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RESEARCH ARTICLE

Calcium content, *in vitro* digestibility, and bioaccessibility in leaves of spinach (*Spinacia oleracea*), sweet potato (*Ipomea batatas*), and drumstick tree (*Moringa oleifera*) [version 1; peer review: 3 approved with reservations]

Jonathan C. Allen¹, Joseph Y. Issa², Weiting Cai³¹Department of Food, Bioprocessing and Nutrition Science, North Carolina State University, Raleigh, NC 27695-7624, USA²Malawi Industrial Research and Technology Development Centre, Blantyre, Malawi, Malawi³Purall (Shanghai) Import & Export Co. Ltd, Shanghai Shi, Shanghai, China

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Abstract

Low calcium intake, poor calcium absorption, excessive calcium losses, or some combination of these factors contribute to calcium deficiency diseases. Calcium insufficiency is associated with osteoporosis, hypertension and colon cancer among other diseases. For individuals who do not have access to milk and dairy products, do not tolerate them, or prefer not to consume them, plants may be alternative sources of calcium. However, calcium bioavailability may be low in plant foods because calcium forms complexes with oxalates, phytate and other competing minerals.

The objective of this study was to compare the calcium content, digestibility and bioaccessibility in the leaves of spinach (*Spinacia oleracea*), sweet potato (*Ipomea batatas*), and drumstick tree (*Moringa oleifera*). Calcium content was analyzed in dry leaf powder by atomic absorption, followed by a two-stage *in vitro* digestion and dialysis against a mock serum solution to determine calcium digestibility and bioaccessibility. *Moringa oleifera* had higher calcium content than spinach and sweet potato leaves: 1.54±0.11% of dry matter for three *Moringa* samples (two African and one from India), and 0.99±0.001 and 1.06±0.001, respectively, in spinach and sweet potato leaves. The mean *in vitro* calcium digestibility was 1.62±0.08% in spinach, 3.4±0.68% in sweet potato leaves and 33.7±9.6% for *Moringa*. A dialysis system was designed to model bioaccessibility of calcium, revealing that bioaccessible calcium in sweet potato leaves was a non-significant 1.4 times higher and in *Moringa* was 9.2 to 19.4 times higher than in spinach. Therefore, the calcium contained in *Moringa* leaves does not appear to be associated with poorly bioavailable complexes such as oxalate. We confirmed previous reports that bioaccessibility of the calcium is low in spinach. These findings imply

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1. **Mark L Failla**, Ohio State University, Columbus, OH, USA
2. **Antonio Cilla**, University of Valencia, Burjassot, Valencia, Spain
3. **Linda Mårtensson**, Swedish University of Agricultural Science, Uppsala, Sweden

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that increased utilization of *Ipomea batatas* and *Moringa oleifera* leaves might increase calcium intake in people in tropical and warm temperate regions where these plants grow, or these plants might become a valuable export crop.



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Corresponding author: Jonathan C. Allen (Jon_allen@ncsu.edu)

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Introduction

Calcium is an essential element responsible for bone strength and regulation of numerous functions in cells and tissues, such as muscle contraction and exocytosis. Approximately 1 to 2% of the human body is calcium. Perhaps 99% of that quantity is immobilized in bones and teeth. The remaining 1% is either ionized calcium or bound in soft tissues in a way that can more readily transfer among tissue compartments. Dietary calcium is absorbed by both active transport and passive diffusion across the intestinal mucosa. Active transport of calcium ions into enterocytes and out on the serosal side depends on the action of 1,25-dihydroxyvitamin D and its intestinal receptors. However, passive diffusion involves the movement of calcium between mucosal cells, is dependent on the luminal:serosal calcium concentration gradient, and may involve low-molecular weight calcium complexes in addition to calcium ions¹.

Calcium deficiency diseases can result from low calcium intake (primary deficiency), poor calcium absorption (secondary deficiency), or excessive calcium losses. Calcium insufficiency in various life stages may be associated with osteoporosis, hypertension, colon cancer or other diseases. Individuals who do not have access, do not tolerate, or prefer not to consume milk and dairy products may prefer or require, plant sources of calcium. However, calcium bioavailability may be reduced in plant foods due to binding to oxalates or phytate, or to minerals competing for absorptive pathways. Benway and Weaver² reported *in vitro* bioavailability for calcium of 0.3, 26 and 76% for spinach, wheat, and kale, respectively. Rapidly growing spinach forms high concentrations of insoluble calcium oxalate when the growth medium is high in available calcium³. High levels of oxalate were also observed in the leaves of sweet potato (*Ipomea batatas*)⁴. The formation of calcium oxalate crystals in numerous plant species may be beneficial to the plant but may be deleterious to human and animal consumers of plant tissues⁵. The leaves of the sweet potato are rarely consumed in Western countries but are a staple food or a delicacy in many African countries, including Zimbabwe, Zambia and Nigeria where the fresh leaves are cooked⁶. Different varieties are sometimes selected for leaf and root consumption. Purple and green sweet potato leaves in Tanzania were shown to contain significant quantities of nutritional and antinutritional compounds⁷. Purple sweet potato leaves that are commonly consumed in Asian countries have been investigated for the bioactivity of their polyphenol content⁸.

Moringa oleifera (drumstick tree) grows in tropical and sub-tropical climates. In the tropical regions of Asia, Africa and South America, leaves from both naturally occurring and cultivated *Moringa* are eaten fresh. The fruits, the flowers and the immature pods of this tree are also edible and they are used to varying extents in traditional diets in many tropical and sub-tropical regions⁹. In addition, *M. oleifera* is used as a medicinal plant¹⁰. In traditional herbal medicine different ethnic groups use parts of the *Moringa* tree differently¹¹. As a traditional food plant in Africa, *M. oleifera* has the potential to improve nutrition, boost food security, foster rural development, and support sustainable land care. In some parts of the world such as Senegal and Haiti, health workers have been treating malnutrition in small children, pregnant and nursing women with *Moringa* leaf powder¹². The leaves of *M. oleifera* are a good source of protein, vitamin A, B and C and minerals such as calcium and iron¹³.

Odoro *et al.*¹⁴ found the calcium content in *M. oleifera* to average 2009 mg per 100 g sample. According to Price¹², *M. oleifera* fresh pods and leaves and dried leaf powder had 30 mg, 440 mg and 2003 mg per 100 g of edible portion, respectively. A study on the nutritional and functional properties of *M. oleifera* leaves found that fresh mature leaves and fresh young shoots had 454 ± 63 mg and 82 ± 31 mg of calcium per 100 g of sample, respectively¹⁵. A previous study on *in vitro* iron bioavailability found that boiling the fresh leaves and dried powder of *M. oleifera* enhanced the *in vitro* iron bioavailability by 3.5 and 3 times, respectively¹⁶. The **USDA Nutrient Database** lists 152 mg of calcium per 100 g of boiled drumstick leaves, 136 mg of calcium per 100 g of boiled spinach, and 33 mg of calcium per 100 g of boiled sweet potato leaves (<http://ndb.nal.usda.gov/ndb/search/list>). Although the literature shows high calcium content in these plant materials, there are fewer data on the calcium bioavailability. The study reported here was therefore designed to investigate the potential calcium bioavailability in spinach (*Spinacia oleracea*), sweet potato (*Ipomea batatas*), and drumstick tree (*Moringa oleifera*) leaves by measuring bioaccessibility using an *in vitro* simulated digestive system. True bioavailability cannot be determined from an *in vitro* system because it does not distinguish between forms of calcium that can be absorbed by regulated active transport processes and passive absorption, nor between retention and rapid renal excretion.

Materials and methods

Sample collection

Green and mature *Moringa* leaf samples were collected from several trees (about 3 trees in each area) in low-lying hot areas of Malawi where it grows naturally and the conditions are favorable for its growth. In these areas, average temperatures are 30°C. The temperature can reach up to 40°C in the dry season. The sample collection areas, Lilongwe and Karonga, are along the lake shore of Lake Malawi and Shire River. These areas are generally hot and flat with sandy loam soils. Another *Moringa oleifera* sample was bought in USA from an Internet distributor (MoringaSource.com). This commercial product was originally imported from India and sold in the United States as powdered *Moringa oleifera* leaf packed in aluminum foil bags. Fresh sweet potato leaves (Covington variety) were harvested at the North Carolina State University Central Crops Research Station, Clayton, NC, washed and stored at -20°C prior to use in this study. Fresh spinach leaves were bought from a local grocery store in Raleigh, NC, USA. All leaf samples were air dried, powdered in a mortar, and packaged in plastic bags. All samples were stored at room temperature and analyzed together at North Carolina State University.

Evaluation of total calcium

Total calcium in the samples was determined by atomic absorption spectrophotometry (AAS). Powdered dry leaf samples were subjected to dry-ashing before the determination of calcium. A 0.1 g sample of each material was weighed and ashed at 525°C for 8 hours. The ash was dissolved with 0.1% HCl and 0.5% lanthanum oxide (La₂O₃) followed by AAS analysis to determine calcium content.

Evaluation of calcium digestibility

In vitro digestion was used to determine calcium digestibility. The leaf powders were subjected to a simulated gastric phase followed by intestinal phase digestion, modified from the method of

Fogleman *et al.*¹⁷. Pepsin enzyme from porcine gastric mucosa (Pcode 1000848645 Sigma-Aldrich, St. Louis, MO, USA) and hydrochloric acid were used in the gastric phase digestion. A 0.1 g sample of leaf powder was combined with 0.25 mL of pepsin solution (40 mg pepsin/mL of 0.1 M HCl), and pH was then adjusted to 2.0 with additional HCl. The mixture was incubated in a shaking water bath at 37°C for 120 strokes per minute for 2 hours, and then placed on ice for 10 minutes. pH was raised to 7 by adding 1 M NaHCO₃. This gastric digestion was followed by pancreatic enzymes (pancreatin from porcine pancreas, P1750, Sigma-Aldrich) and porcine bile extract (B8631, Sigma Aldrich) solution in the intestinal phase digestion. The enzyme stock contained 0.05 g pancreatin and 0.3 g bile extract in 25 mL 0.1 M NaHCO₃. A 1.25 mL portion of the pancreatin-bile extract stock solution was added to each digested leaf sample, with additional incubation in a shaking water bath at 37°C for 120 strokes per minute for 2 hours. The mixtures were placed on ice for 10 minutes and adjusted to pH 7.2 by addition of 0.5 M NaOH. All samples were brought to a common volume with distilled water and filtered through Whatman No.4 filter paper. The solution was diluted appropriately with 0.1 M HCl and 0.5% lanthanum oxide (La₂O₃) followed by AAS analysis to determine calcium content.

Evaluation of calcium bioaccessibility

The bioaccessibility of calcium in the samples was determined by simulated *in vitro* absorption of calcium by using dialysis tubes. This involved the passive trans-membrane diffusion of calcium ions from one medium to another. A 10 mL portion of each sample (previously digested) was loaded inside of the dialysis tubing in a Floatalyzer™ G2 apparatus (Spectrum Laboratories, Inc., Rancho Dominguez, CA) by pipettes. The outside compartment of the dialysis tubing container for each sample was loaded with 25 mL of 0.9% NaCl solution with 0.1% bovine serum albumin (fraction V, Sigma-Aldrich). The samples were then dialyzed at room temperature for 24 hours. The volumes of the solution outside and inside the dialysis tubing were measured and emptied in separate test tubes for calcium analysis. Calcium was analyzed by atomic absorption, and the calcium that was able to pass to the outside of the dialysis tubing corresponded to the bioaccessible calcium.

Calculation of calcium bioaccessibility

Bioaccessible calcium was calculated as the product of the calcium concentration in the external fluid and the combined fluid volume inside and outside the Floatalyzer™. The bioaccessibility of calcium was the amount of dialyzable calcium expressed as a percentage of total calcium in the sample. Calcium digestibility was calculated as the quantity of calcium in the filtrate divided by the calcium content in the original powdered leaf sample.

Data analysis

SAS and JMP software (SAS, Inc., Cary, NC) were used to statistically analyze the data. The Fit X by Y one-way analysis of variance subroutine was used to see the relationship among the two *Moringa oleifera* samples from Malawi, the *Moringa oleifera* sample from India, sweet potato leaves, and spinach in terms of calcium content, digestibility and bioaccessibility. The entire digestion procedure was replicated three times, using duplicate or triplicate samples in each replicate. Two samples of spinach, three samples of sweet potato leaves, and one sample from *Moringa* #4 could not be

included in the data analysis, because the atomic absorption spectrophotometer did not report a meaningful calcium concentration possibly due to consumption of the sample in the analyzer. Tukey's Studentized Range (TSR) test was used to determine whether or not significant differences existed in the mean values of the sample calcium content, digestibility and bioaccessibility at $P \leq 0.05$.

Results

Calcium content

Calcium content and digestibility of *Moringa oleifera*, spinach and sweet potato leaves are provided in Table 1.

Calcium from the samples was expressed as percentage of the dry matter in the three *Moringa oleifera* and the spinach and sweet potato leaves. The results indicated that *Moringa oleifera* leaves had a higher percentage of calcium than spinach and sweet potato leaves. However, among the three *Moringa oleifera* samples, there was a significant difference in the percentage calcium content ($P < 0.05$) using Tukey's Studentized Range Test at $\alpha = 0.05$. The mean percentage calcium \pm SEM was 1.67 ± 0.001 , 1.44 ± 0.018 and 1.55 ± 0.00 for *Moringa oleifera* samples from India (M3), from Karonga, Malawi (M4) and another sample from Lilongwe, Malawi (M5), respectively. The mean percentage calcium in spinach and sweet potato leaves was 0.99 ± 0.0003 and 1.06 ± 0.001 , respectively.

Calcium digestibility

The digestible calcium from *Moringa oleifera* samples and the control samples, spinach and sweet potato leaves are shown in Table 1.

Digestibility of calcium from the leaf powder was expressed as percentage of the calcium in dry matter that was solubilized by the digestion procedure. The digestibility results indicated that the three *Moringa oleifera* samples had higher digestible calcium than spinach and sweet potato leaves. Using Tukey's Studentized Range Test at $\alpha = 0.05$, there was no significant difference in total digestible calcium between the two samples from Malawi, M4 and M5.

Table 1. Total calcium content as percent of dry matter, and digestible calcium as a percent of total Ca (g solubilized Ca/100 g Ca in dried leaf \times 100) from *Moringa oleifera*, spinach and sweet potato leaves (mean \pm standard error of the mean with N = 2–4 replicates per sample for total Ca, N = 3 replicate experiments on duplicate or triplicate samples for digestible Ca).

Sample ^a	Total Ca (%) (N)	Digestible Ca (%) (N)
S	0.99 ± 0.0003^e (2)	1.35 ± 0.21^c (5)
SP	1.06 ± 0.001^d (2)	3.79 ± 0.23^c (4)
M3	1.67 ± 0.001^a (3)	22.77 ± 1.04^b (7)
M4	1.44 ± 0.018^c (4)	46.57 ± 8.34^a (6)
M5	1.55 ± 0.00^b (2)	37.69 ± 0.79^a (7)

^aSamples: M3 = *Moringa* sample from India, M4 = first *Moringa* sample from Karonga, Malawi, M5 = second *Moringa* sample from Lilongwe, Malawi, S = spinach, SP = sweet potato leaves.

^{abc}For the dependent variable within each column, the calcium sources (sample) in rows not connected by the same letter are significantly different from each other as determined by ANOVA and the Tukey-Kramer HSD test with $\alpha = 0.05$.

However, there was a significant difference in percentage of digestible calcium between *Moringa oleifera* samples from India and Malawi ($P < 0.05$). The mean (\pm SEM) percentage of digestible calcium was 22.77 ± 1.04 , 46.57 ± 8.34 and $37.69 \pm 0.76\%$ for *Moringa oleifera* samples from India (M3), from Malawi (M4) and the second sample from Malawi (M5), respectively. The mean percentage digestible calcium in spinach and sweet potato leaves were 1.35 ± 0.21 , and $3.79 \pm 0.21\%$, respectively.

Bioaccessibility of calcium

The bioaccessibility of calcium from *Moringa oleifera* samples and the control samples, spinach and sweet potato leaves, are provided in Table 2.

Bioaccessibility of calcium from the samples was expressed as percentage of the dry matter. Bioaccessible calcium was equivalent to dialyzable calcium. The bioaccessibility results from spinach and sweet potato leaves and the three *Moringa oleifera* samples indicated that *Moringa oleifera* had higher dialyzed calcium than spinach and sweet potato leaves. Using Tukey's Studentized Range Test at $\alpha = 0.05$, there was no significant difference in total bioaccessible calcium between the two *Moringa oleifera* samples from Malawi, but these had more bioaccessible calcium than the *Moringa oleifera* sample from India. Also, the percentage bioaccessible calcium in all *Moringa oleifera* samples was greater than that in spinach and sweet potato ($P < 0.05$).

Table 2 also shows the percentage of the digestible calcium that was dialyzable. Despite the substantial variation in the data, the table shows that a greater amount of the digestible calcium from spinach was dialyzable compared with the *Moringa* samples or sweet potato leaves, suggesting that the calcium oxalate in spinach is largely indigestible and remains in the initial filtrate, whereas the complexes that can be filtered can also pass through the dialysis membranes. The filtration step in the digestibility assay retained particles greater than 20–25 μm and allowed calcium in smaller particles to pass through the fluid that was used in the dialysis procedure. The

dialysis membrane only allows molecular complexes from 500 to 1000 Da to go through and be counted in the "bioaccessible" fraction. The bioaccessible fraction was 45% of the digestible calcium fraction for spinach, but only 22 to 32% for sweet potato and *Moringa* leaves (Table 2).

Data of calcium content, digestibility and bioaccessibility of *Moringa oleifera*, spinach and sweet potato leaves

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.943482>

Discussion

In vitro methods for assessing the bioaccessibility of essential minerals are widely used because they are simple, fast, and inexpensive. Teixeira *et al.*¹⁸ recently measured *in vitro* digestibility of protein fractions in *Moringa oleifera* with different protein extraction pretreatments. The protein digestibility was relatively low, in contrast to the calcium bioaccessibility data we present here. Kamchan *et al.*¹⁹ used an *in vitro* method to study the bioavailability of calcium in vegetables, legumes and seeds; in their study, fresh vegetables were prepared by blanching in boiling deionized water for three minutes followed by homogenization in a food processor whereas seed and pod samples were cooked and homogenized in an electric blender and all the samples were stored in polyethylene bottles at -20°C before analysis. In contrast, in our study *Moringa oleifera* leaf samples were air dried, pounded into powder and stored in polyethylene bags before being subjected to chemical analysis. We assessed calcium content, digestibility and bioaccessibility in *Moringa oleifera* leaf powder from Malawi and India, along with spinach and sweet potato leaf powder. There was a statistically significant difference in the percentage calcium content from the *Moringa oleifera* samples from India and the two samples from Malawi. This may be due to differences in the growing conditions because some soils have higher amounts of calcium. The amount of digestible calcium in the two *Moringa oleifera* samples from Malawi was statistically similar, and the samples from Malawi differed in this respect from the sample from India. This may be due to similar growing conditions for the samples from Malawi, which may differ from the growing conditions in India, or it may be due to genetic drift in the *Moringa* genome²⁰. Dialyzed calcium was significantly different between the two samples from Malawi and one sample from India when a Tukey's Studentized Range Test with $\alpha = 0.05$ was used. The dialyzed calcium from all the *Moringa oleifera* samples was significantly higher than spinach and sweet potato leaves. The bioaccessible calcium in spinach in this study was similar to the calcium bioavailability of spinach reported by Benway and Weaver². *In vitro* methods used to study calcium dialyzability from different vegetables revealed percentage dialyzable calcium in kale, celery and Chinese cabbage to be 38.9 ± 2.1 , 36.2 ± 4.1 and 32.2 ± 4.6 , respectively¹⁹. This may be due to different chemical and biological compositions of these plants that inhibit absorption of calcium, such as phytate, oxalate, and dietary fiber, or due to different calculation methods. Ezeike *et al.*²¹ were able to extract 0.28 mg of oxalate per

Table 2. Calcium bioaccessibility from *Moringa oleifera*, spinach and sweet potato leaves. Values represent means \pm SEM of the number of replicates shown in parentheses.

Sample ¹	Bioaccessible Ca (% of total Ca) (N)	Dialyzable Ca as % of digestible Ca (N)
S	0.62 ± 0.17^c (5)	45.1 ± 8.9^a (5)
SP	0.85 ± 0.08^c (4)	22.4 ± 2.0^b (4)
M3	5.72 ± 0.85^b (7)	24.8 ± 3.3^b (7)
M4	10.03 ± 1.36^a (6)	24.1 ± 4.6^b (6)
M5	12.01 ± 1.14^a (7)	31.8 ± 2.7^{ab} (7)

¹Samples: M3 = *Moringa* sample from India, M4 = first *Moringa* sample from Karonga, Malawi, M5 = second *Moringa* sample from Lilongwe, Malawi, S = spinach, SP = sweet potato leaves.

^{abc}For the dependent variable within each column, the calcium sources (sample) in rows not connected by the same letter are significantly different from each other as determined by ANOVA and the Tukey-Kramer HSD test with $\alpha = 0.05$.

gram of dry matter from *Moringa oleifera* leaves, and cited data to calculate that spinach contains 100 mg oxalate per gram of dry matter. Oxalic acid in fresh sweet potato leaves was 3–5 mg per gram fresh weight, or about 16–27 mg per gram of dry matter²². The results from the study presented here are different from those found by Kamchan *et al.*¹⁹ probably because different species of plants and different sample preparatory methods were used. An *in vitro* iron bioavailability study¹⁶ found that boiling the fresh leaves and dried powder of *Moringa oleifera* enhanced the *in vitro* iron bioavailability by 3.5 and 3 times, respectively. Whether a similar preparatory method would enhance the calcium bioaccessibility requires further study.

Price¹² found calcium content in *Moringa oleifera* pods, leaves and leaf powder to be 30 mg, 440 mg and 2003 mg per 100 g of edible portion, respectively. We found in our study that the percentage calcium content in powdered *Moringa oleifera* leaves is 1.67 ± 0.001 for samples from India (M3), 1.44 ± 0.018 for samples from Karonga, Malawi (M4), and 1.55 ± 0.00 , for samples from Lilongwe, Malawi (M5). If we translate these results per 100 g edible sample, it yields 1.67 g (1670 mg), 1.44 g (1440 mg) and 1.55 g (1550 mg) for M3, M4, and M5, respectively, averaging 1553 mg/100 g. These results are not very different from that of Price¹² for the leaf powder, which was 2003 mg per 100 g sample. The current calcium RDA for pregnant and lactating women is 1300 mg/day for women aged 14–18 years and 1000 mg/day for women aged 19–50 years²³. A dietary intake study undertaken in Malawi, Africa, showed that calcium intake from women was lower than the recommended intake with average intake of 620 mg/day for non-lactating women and 622 mg/day for lactating women²⁴. Another nutrient intake study conducted in South Africa in three ethnic groups (black, white and mixed ancestry subjects) found that mean dietary calcium intake was higher in white subjects than in black and mixed ancestry subjects; however, calcium intake was low in all the groups with about half of the RDA of 1000 mg/day²⁵. If the dietary intake of *Moringa oleifera* is increased, calcium intake could also be tremendously increased, especially in developing countries such as Malawi, where growing these trees requires little care. This can be achieved by promoting plantation of these trees in areas where they grow easily.

According to our study, only 64 g of dried *Moringa* leaf powder would supply the 1000 mg/day RDA. From this intake, 357 mg of calcium are estimated to be digestible, and 93 mg of calcium are estimated to be bioaccessible, or available for absorption, based on the average of the *Moringa oleifera* plant samples tested in our study. This study also shows sweet potato leaves to be a good source of calcium but more similar to spinach than to *Moringa oleifera* leaves.

Conclusion

The percentage dialyzed or bioaccessible calcium is higher in *Moringa oleifera* leaves than other vegetable sources such as spinach and sweet potato leaves. Percentage dialyzed calcium of *Moringa oleifera* grown in Malawi was slightly higher than the sample grown in India. Because there is evidence from related research that percentage calcium dialyzability differs in different foods, especially vegetable foods because different inhibitory components may be present, additional research can be conducted to find out the inhibitory components in *Moringa oleifera* leaves. Such inhibitory components may be the contributing factors for the differences in percentage bioaccessible calcium in different plant leaf samples.

Data availability

figshare: Data of calcium content, digestibility and bioaccessibility of *Moringa oleifera*, spinach and sweet potato leaves <http://dx.doi.org/10.6084/m9.figshare.943482>²⁶

Author contributions

JCA was responsible for editing the manuscript, experimental design and final data analysis. JYI proposed the research area, developed methods and wrote the first draft of the manuscript. YC conducted the laboratory analyses and prepared the data for analysis.

Competing interests

The authors have no competing interests in the publication of this paper.

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Version 1

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Linda Mårtensson

Department of Food Science, Swedish University of Agricultural Science, Uppsala, Sweden

The article "*Calcium content, in vitro digestibility, and bioaccessibility in leaves of spinach (*Spinacia oleracea*), sweet potato (*Ipomea batatas*), and drumstick tree (*Moringa oleifera*)*" by Allen *et al.* concerns important issues of Calcium intake when dairy products are replaced, irrespective of the cause behind the need or desire to the substitution. The findings are valuable for global nutritional security as well as for further research. However, I find numerous ways to improve the publication in its current format.

Overall suggestions for improvement:

1. The use of Latin name and common name should be done in a more consistent style throughout the entire article. Latin should preferably be used as the standard throughout the article and for all species/crops in the study.
2. The number of significant digits should also be consistent throughout the article: *e.g.* 0.99 has two but 1.54 has three.
3. Use of abbreviations: the first time a term is used it should be spelled out and then can be written as an abbreviation in brackets after. In the continued text use the abbreviation only, *e.g.* *HCl* is used before the spelled out *hydrochloric acid*.

Specific suggestions for improvement:

Abstract

1. The sentence "... revealing that bioaccessible calcium in sweet potato leaves was a non-significant 1.4 times higher and in *Moringa* was 9.2 to 19.4 times higher than in spinach." must be clarified. The results provide more information than this and can be summarized in the abstract.

2. Add % after results in second paragraph.
3. Second paragraph: "...1.4 times higher than..." – what are you comparing to?

Introduction – no comments

Materials and methods

1. Page 4, line 18: "*common volume*" - The authors are advised to give the actual volume here.
2. Title "*Calculation of calcium bioaccessibility*" should include digestibility.
3. Keep the order in presentation - e.g. digestibility should be presented first in "*Calculation of calcium bioaccessibility*" to follow earlier flow.
4. Page 4, second column: "*because the atomic absorption spectrophotometer...*" - Clarify this.
5. P value and alfa are mixed throughout the results section, which needs to be either clarified or changed.

Results

1. Both starting sentences in the "*Results*" section are misplaced. They would perhaps be better off in the materials and methods section?
2. Table 1: hyperlinks (and explanations?) are missing for indications d and e.
3. Results need not be repeated in result text if already presented in tables.
4. Results need not to be repeated in the discussion.

Discussion

1. Soil chemistry is reflected upon but information/data is lacking – Is it possible to supplement?
2. Results should not be repeated here.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 06 June 2014

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Antonio Cilla

Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Burjassot, Valencia,

Spain

The manuscript of Prof. Allen and colleagues highlights the potential use of leaves of spinach, sweet potato and drumstick tree as an alternative to other calcium dietary sources such as milk and dairy products. In addition they measure the bioaccessibility, as a prior step of bioavailability, to check if these leaves can be a potential source of available calcium in the geographic areas in which they are grown or cultivated easily and are a cost-effective approach for people in these zones. Research in this field is very important in food science and nutrition since results of this kind provide a scientific rationale, as mentioned by the authors, to improve nutrition, boost food security, foster rural development and support sustainable land care. However, I suggest some modifications to improve the study.

1. Bioaccessibility (defined as a fraction of a compound that is released from its food matrix within the gastrointestinal tract and that becomes available for absorption) is a term which comprises both solubility and dialyzability (Fernández-García *et al. Nutr. Res.* 2009, 29, 751-760). The results, as demonstrated in many other publications, should be expressed as % bioaccessibility (understood as % dialysis or % solubility). See examples (Perales *et al. J. Agric. Food Chem.* 2005, 53, 3721-3726 or Galán *et al. J. Agric. Food Sci.* 2014, 94, 515-521). This should be applied to the entire text.

Abstract:

2. Second Paragraph - *"revealing that bioaccessible calcium in sweet potato leaves was a non-significant 1.4 times higher ..."* – what is this in comparison to?

Introduction:

3. Polyphenols can also bind calcium and other divalent minerals and thus reduce their bioavailability. – This should be included in the introduction.

4. You need to specify that there is fewer data on calcium bioavailability and bioaccessibility.

Materials and methods:

5. Evaluation of calcium bioaccessibility: the authors should indicate in the last sentence that this "...corresponded to the bioaccessible (dialyzable) calcium."

Results:

6. As reported by the previous reviewer (Dr. Failla) please do not duplicate table results in the text.

7. In table 2 the nomenclature in the 2nd column needs to be changed to Ca bioaccessibility (% of total Ca).

8. How are the authors sure that the complexes formed are with oxalate and not with phytate and/or polyphenols?

Discussion:

9. I do not understand why authors compare bioaccessibility of calcium with that of proteins from the study of Teixeira *et al.*

10. The authors introduce the study of Kamchan *et al.* but do not include anything about this study's findings or compare them with their own results.

11. Sometimes the terms bioavailability and bioaccessibility are used indistinctly, but they are not the same. The authors are comparing bioaccessibility with bioaccessibility (solubility) and not bioavailability in the work of Benway and Weaver. This must be modified.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 14 March 2014

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Mark L Failla

Department of Human Nutrition, Ohio State University, Columbus, OH, USA

This article by Prof. Allen and associates addresses the important issue of inadequate intake of calcium by those who do not have access to, or the ability/willingness to consume milk and dairy products. The possibility that leaves from three common plant foods in rural areas or developing countries can serve as an alternative source of calcium merits investigation. The results provide novel information regarding calcium content and dialyzability in dried powders of leaves from spinach, sweet potato and drumstick. These results will be of interest to nutrition and food scientists, horticulturists and food processors.

I have several specific suggestions for enhancing the presentation:

1. **Abstract:** Use of the term "*mock serum solution*" is a stretch for the presence of BSA outside the dialysis bag. Plasma contains numerous proteins and low molecular weight compounds that can bind a portion of plasma calcium. The rationale for use of BSA should be added to the manuscript e.g., serves as a "sink" for calcium diffusing into the outer solution, some calcium in plasma and some of the divalent metal in plasma complexes with albumin. I suggest that albumin be substituted for mock serum solution.
2. **Abstract:** middle of paragraph 2. "*Moringa oleifera had higher calcium content.....*". Add % after 0.99 and 1.06.
3. **Abstract:** paragraph 2. "*A dialysis system was designed...*" "*1.4 times higher*" - than what?
4. The term bioaccessibility generally refers to availability for uptake by, or paracellular transfer across the epithelial cell barrier. Passive diffusion across the barrier represents a portion of calcium absorption; particularly when the lumen concentration of soluble calcium is relatively high. As the investigators determined the amount of dialyzable calcium I suggest that it is not appropriate to use the term bioaccessibility in the title and throughout the manuscript. The great majority of papers reporting results for electrolytes and low molecular weight organic compounds obtained using in vitro digestion coupled with dialysis

refer to dialyzability; not bioaccessibility.

5. **Results:** The text and tabular data are quite redundant with means and SD from the table stated in text. The text can be less specific, i.e., delete the numerical details.
6. **Discussion:** The translation of the *in vitro* results to the amount of calcium provided by powdered leaves is excellent. However, individuals in poor rural areas of developing countries generally consume boiled leaves from the three plants. How much calcium would 1 serving of leaves from each plant provide? Might a distinction of use of these leaves in a dried form as a supplement vs as an actual food, provide a more balanced viewpoint?

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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