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Analytical Methods

Bioavailability of calcium and its absorption inhibitors in raw and cooked green leafy vegetables commonly consumed in India – An in vitro study

Augustine Amalraj [1], Anitha Pius *

Department of Chemistry, The Gandhigram Rural Institute – Deemed University, Gandhigram, Dindigul 624 302, Tamil Nadu, India

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ABSTRACT

The objectives of this research were to assess the bioavailability of calcium using equilibrium dialysis after simulated gastric digestion method in 20 commonly consumed green leafy vegetables (GLVs) from the typical Indian diet, provide data on the content of calcium absorption inhibitors, like oxalate, phytate, tannin and dietary fibres, and evaluate the inhibitory effect of these compounds on calcium bioavailability in raw and cooked GLVs. Cooking did not affect significantly calcium bioavailability in any GLVs. *Sesbania grandiflora* had a very high content of total oxalates, tannins and dietary fibers, which reduced calcium bioavailability. Calcium content was determined by atomic absorption spectroscopy, oxalate by titrimetry, phytate and tannin by colorimetric and dietary fibres by an enzymatic gravimetric method. *Chenopodium album*, *Alternanthera philoxeroides* and *Centella asiatica*, with lower total calcium content, had nearly twice as much bioavailable calcium than other GLVs, because of low fibres, oxalate, phytate and tannin content.

1. Introduction

Calcium, the most abundant mineral in the body, is found in some foods, added to others, available as a nutritional supplement, and present in some medicines. The body's calcium supply is stored in the bones and teeth where it supports their structure and function. Bone undergoes continuous remodelling, with constant resorption and deposition of calcium into new bone. The balance between bone resorption and deposition changes with age. Bone formation exceeds resorption in periods of growth in children and adolescents, whereas in early and middle adulthood both processes are relatively equal. In ageing adults, particularly among postmenopausal women, bone breakdown exceeds formation, resulting in bone loss that increases the risk of osteoporosis over time (IOM, 2010). Calcium (Ca) is an essential nutrient for plants and animals, with key structural and signalling roles, and its deficiency in plants can result in poor biotic and abiotic stress tolerance, reduced crop quality and yield. Likewise, inadequate calcium (Ca) intake and poor absorption of Ca in human are among several risk factors for osteoporosis and some other diseases (e.g. rickets, hypertension and colorectal cancer) (Centeno, de Barboza, Marchionatti, Rodriguez, & de Talamoni, 2009).

Osteoporosis is a condition that mainly affects older people and is linked to enhanced decalcification and demineralization of bones. Most, but not all, studies show that increasing Ca intake in later life decreases the occurrence of osteoporotic fractures and it is universally accepted that sustaining the recommended dietary intake (RDI) of Ca throughout life is beneficial for health later in years (Michaelsson, 2009).

In most developing countries, vegetables are the most reliable and affordable source of minerals and vitamins for families (Mosha, Gaga, Pace, Laswai, & Mtebe, 1995). In India, most of the people adopt a vegetarian lifestyle. Plant-food-based diets are rich in bioactive compounds, which are believed to be advantageous for the prevention of non-communicable chronic diseases, such as cancer, diabetes mellitus, etc. Green leafy vegetables occupy an important place among the food crops as these provide adequate amounts of many vitamins and minerals for humans. They are rich source of carotene, ascorbic acid, riboflavin, folic acid and minerals like calcium, iron and phosphorous. The contribution of minerals and vitamins from vegetable in human nutrition is, however, limited due to the presence of anti-nutritional factors, which render some of the nutrients unavailable for absorption. The most common anti-nutritional factors present in GLVs that decrease the

* Corresponding author. Tel.: +91 451 2452371; fax: +91 451 2454466.
E-mail address: dranithapius@gmail.com (A. Pius).
[1] Tel.: +91 451 2452371; fax: +91 451 2454466.

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bioavailability of some minerals, especially calcium, are oxalate, phytate, tannin and dietary fibres (DF) (Mosha et al., 1995).

The bioavailability of minerals has generated increasing interest in the field of nutrition. Bioavailability should be determined by in vivo measurements, ideally in humans. However, such studies are difficult, expensive, and provide limited data (Camara, Amaro, Barbera, & Clemente, 2005). While animal assays are less expensive, they are somewhat limited by uncertainties with regard to differences in metabolism between animals and humans. As an alternative to human and animal studies in vivo are measures made using simple, rapid and inexpensive methods in vitro (Luten et al., 1996).

In vitro estimation of the bioavailability of minerals from foods involves the simulation of gastrointestinal digestion and measurement of the mineral soluble fraction or the mineral fraction that dialyses across a semi-permeable membrane of a certain pore size. These methods are widely used because of their good correlation with in vivo studies. In vitro methods have been used to estimate the bioavailability of minerals from different foods and also from dishes and composite diets (Camara et al., 2005).

In the present study, 20 GLVs commonly consumed in India were selected for determining their calcium content and bioavailability in vitro. Various factors that may influence bioavailability of calcium, namely oxalate, phytate, tannin and dietary fibre were also quantified. Bioavailability of calcium was correlated with the concentration of these inhibitory factors since there is very little information in the literature about the content of oxalate, phytate, tannin and dietary fibres in raw and cooked GLVs. Therefore, one of the objectives of this study was to analyse the effects of cooking on the content of oxalate, phytate, tannin and dietary fibre. These data form a basis for better food selection and, consequently, improved nutritional advice for local populations in India as well as potentially preventing the onset of fluorosis.

2. Materials and methods

2.1. Materials

Locally grown and commonly consumed fresh GLVs were purchased from different markets in Dindigul District, Tamil Nadu (India) and healthy, disease-free, edible parts selected for this study. All chemicals used in the experiments were of analytical (AR) grade and were obtained from Sigma Aldrich India Ltd., Mumbai, India. Millipore – MilliQ distilled water was employed during the complete study.

2.2. Preparation of the samples

Twenty samples of GLVs collected were *Acalypha indica*, *Allmania nodiflora*, *Alternanthera dentate*, *Alternanthera lehmannii*, *Alternanthera philoxeroides*, *Alternanthera sessilis*, *Amaranthus blitum*, *Amaranthus dubius*, *Amaranthus polygonoides*, *Amaranthus spinosus*, *Basella alba*, *Centella asiatica*, *Chenopodium album*, *Hibiscus sabdariffa (Linn)*, *Marsilea villosa*, *Moringa oleifera*, *Pisonia alba*, *Sesbania grandiflora*, *Solanum nigrum* and *Trigonella foenum-graecum* were selected. They were cleaned and washed with Millipore water after separating the non-edible portion. The thoroughly drained greens were cut into 1 cm pieces and they were divided into two parts. One part was cooked using Millipore – MilliQ distilled water in microwave oven until the water was evaporated and marked. The cooked and fresh samples were dried in glass dishes in a hot air oven at 50 ± 5 °C. The dried samples were ground to fine powder and stored in airtight containers. The dried samples were used for the estimation of total, soluble and bioavailable calcium as well as oxalate, phytate, tannin and dietary fibres.

2.3. Determination of total calcium content

Finely ground GLV samples were ashed in a muffle furnace at 550 °C for 10 h and the ash was dissolved in conc. HCl. Calcium content was determined by atomic absorption spectrometer (Perkin – Elmer A Analyst 100). Instrumental conditions: wavelength = 422.7 nm, slit = 0.7 nm, recommended flame = air–acetylene, oxidising (lean, blue) and nebulizer = spoiler. Lanthanum chloride (1%) was added to the mineral solution to avoid interference from phosphate. Calibration of the instrument was performed using commercial standards. All measurements were carried out using standard flame operating conditions, as recommended by the manufacturer.

2.4. Evaluation of calcium bioavailability by in vitro simulated gastrointestinal method

Bioavailability of calcium from selected GLV samples were determined using an in vitro method described by Luten et al. (1996), involving simulated gastrointestinal digestion (Fig. S1; supporting information) with suitable modifications. All finely ground GLVs were subjected to simulated gastric digestion by incubation with pepsin (pH 2.0) at 37 °C for 2 h. Titratable acidity was measured in an aliquot of the gastric digest, by adjusting the pH to 7.5 with 0.1 mol L^{-1} NaOH in the presence of pancreatin–bile extract mixture (1 L of 0.1 mol L^{-1} sodium bicarbonate containing 4 g pancreatin + 25 g bile extract). Titratable acidity was defined as the amount of 0.1 mol L^{-1} NaOH required to attain a pH of 7.5. To simulate intestinal digestion, segments of dialysis tubing (Molecular mass cut off between 12,000 and 14,000 Da) containing 25 ml aliquots of sodium bicarbonate solution, being equivalent in moles to the NaOH needed to neutralize the gastric digest (titratable acidity) determined as above, were placed in Erlenmeyer flasks containing the gastric digest and were incubated at 37 °C with shaking for 30 min or longer until the pH of the digest reached 5.0. Five millilitres of the pancreatin–bile extract mixture was then added and incubation was continued for 2 h or longer until the pH of the digest reached 7.0. At the end of simulated gastro-intestinal digestion, calcium present in the dialyzate was analysed by atomic absorption spectrometry. The dialyzable portion of the total calcium present in the sample (expressed as percent) represented the bioavailable calcium.

Bioavailability (%) was calculated as follows: bioavailability (%) = $100 \times D/T$, where, D is the calcium content in the dialyzable portion for the bioavailable fraction (mg calcium/100 g GLVs), and T is the total calcium content (mg calcium/100 g GLVs).

2.5. Determination of inhibitory factors

2.5.1. Determination of oxalate

The GLVs were analysed for total oxalates and soluble oxalates by precipitation with calcium oxalate from deproteinized extract and subsequent titration with potassium permanganate (Association of Official Analytical Chemists – AOAC, 2000). For total oxalate determination, 5 g sample and 200 ml distilled water are placed in a 600-ml Berzelius beaker, and this mixture was stirred for 15 min. Then 100 ml distilled water and 55 ml of 6 mol L^{-1} HCl were added and boiled with reflux for another 15 min; after this time, the mixture was allowed to cool. Then it was adjusted to 500 ml with distilled water and left overnight. Then the mixture was filtered. The determination of oxalate was carried out by precipitation as calcium oxalate (AOAC, 2000). To 25 ml of filtrate, 5 ml tungstophosphoric acid solution (prepared by mixing 2.5 g $Na_2WO_4 \cdot H_2O$ to 4 ml H_3PO_4 with concentration 1.2 mol L^{-1}, and diluting to 100 ml) was added and left to stand for at least 5 h. After this period of time, the mixture was filtered, an aliquot of 20 ml was taken