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Micellarization and Intestinal Cell Uptake of β-Carotene and Lutein from Drumstick (*Moringa oleifera*) Leaves

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ABSTRACT The leaves and pods of the drumstick tree are used as food and medicine in some Asian and African countries. Although relatively high concentrations of β-carotene and lutein have been reported in the leaves, the bioavailability of these carotenoids from this source is unknown. We have analyzed the digestive stability and bioaccessibility of carotenoids in fresh and lyophilized drumstick leaves using the coupled *in vitro* digestion/Caco-2 cell model. β-Carotene and lutein were stable during simulated gastric and small intestinal digestion. The efficiency of micellarization of lutein during the small intestinal phase of digestion exceeded that of β-carotene. Addition of peanut oil (5% vol/wt) to the test food increased micellarization of both carotenoids, and particularly β-carotene. Caco-2 cells accumulated β-carotene and lutein from micelles generated during digestion of drumstick leaves in a time- and concentration-dependent manner. The relatively high bioaccessibility of β-carotene and lutein from drumstick leaves ingested with oil supports the potential use of this plant food for improving vitamin A nutrition and perhaps delaying the onset of some degenerative diseases such as cataracts.

KEY WORDS: • ***bioavailability*** • ***Caco-2 cells*** • ***β-carotene*** • ***drumstick leaves*** • ***lutein*** • **Moringa oleifera**

INTRODUCTION

VITAMIN A DEFICIENCY continues to represent a significant public health problem in developing countries, particularly for children.[1,2] For example, it was recently reported that 55% of apparently healthy school-aged children (6–16 years) in a southern city of India had subclinical vitamin A deficiency based on serum retinol values.[3] Although supplementation with a massive dose of vitamin A has been used to treat vitamin A-deficient children, the sustainability of diet-based approaches is preferable. Thus, identification and popularization of carotenoid-rich foods, food fortification, biofortification, and genetically modified varieties of staple foods enriched in provitamin A and vitamin A represent potential strategies for the prevention of vitamin A deficiency.[4,5]

Moringa oleifera, commonly known as the "drumstick" or "horseradish" tree, is used in traditional Indian medicine for treating various illnesses. Extracts of drumstick leaves have been reported to exhibit anticancer, antioxidant, and anti-inflammatory activities.[6–8] The pods and leaves of the tree are also used in recipes in Southern India. The leaves are a rich source of essential amino acids, vitamins, and minerals.[9] Because drumstick leaves also contain relatively high concentrations of β-carotene, supplementation of drumstick leaf powder is being considered as a potential source to increase the vitamin A status of children in India.[10] However, the bioavailability of provitamin A carotenoids and xanthophylls in drumstick leaves is not known. Carotenoid bioavailability from plant foods is affected by numerous factors, including the physicochemical properties of the carotenoids, food matrix, post-harvest processing, style of preparation, and composition of the meal, as well as the phenotype (nutritional and physiological status) and genotype of individuals.[11] Carotenoid bioavailability in human subjects is generally estimated by monitoring the quantity of ingested carotenoids appearing in the triacylglycerol-rich fraction of plasma after feeding test meals.[12,13] A more accurate method for determining the bioavailability of carotenoids from a meal requires feeding plant foods intrinsically labeled with stable isotopes.[14,15] However, expenses associated with instrumentation and technical support, as well as the labor intensity associated with both methods, limit their use as tools for screening the impact of diverse styles of cooking and other components in a meal on the bioavailability of carotenoids.

Here, we investigated the digestive stability, efficiency of micellarization, and cellular uptake of β-carotene and lutein in drumstick leaves using the coupled simulated *in vitro* digestion/Caco-2 cell model. Further, the effect of oil on the

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micellarization of β-carotene and lutein was also studied. This coupled system has been shown to be a cost-effective *in vitro* approach to examine the bioaccessibility of carotenoids and xanthophylls from foods and meals.[16–19] The model consists of two steps. First, test foods are subjected to simulated gastric and small intestinal phases of digestion. Then the fraction containing carotenoids incorporated into micelles generated during simulated digestion is added to cultures of enterocyte-like Caco-2 human intestinal cells. Previous studies have shown that Caco-2 cells are capable of transporting carotenoids across the apical membrane, converting β-carotene to retinol, and secreting carotenoids and retinyl esters in chylomicrons.[17–21]

MATERIALS AND METHODS

Chemicals and drumstick leaves

Drumstick leaves were either packed into zip-lock bags under nitrogen or lyophilized and powdered using a kitchen blender. Fresh leaves and dried powder contained 76% and 0.9% water by weight, respectively. For preparation of the puree, fresh drumstick leaves (62 g) were homogenized in water (150 mL) with a kitchen blender for 7 minutes. Lyophilized leaf powder and pureed drumstick leaves were stored at −20°C under a blanket of nitrogen for a maximum of 2 months.

In vitro *digestion*

Details of the procedure for simulated gastric and small intestinal phases of digestion were described previously.[16] Digestion reactions (50 mL total volume) contained either 2.0 g of pureed drumstick leaves or 0.5 g of lyophilized drumstick leaf powder. Either 0 or 100 μL of peanut oil was added to the drumstick samples before initiation of the gastric phase of digestion. Peanut oil was selected as the lipid source since it is routinely used for cooking in Southern India. At the end of the simulated digestion, an aliquot of the product (*i.e.*, digesta) was centrifuged (model L7–65, Beckman, Fullerton, CA) at 167,000 *g* at 4°C for 30 minutes to separate the aqueous fraction containing mixed micelles from nondigested material. The aqueous fraction was filtered (0.22 μm pore size) to remove microcrystalline carotenoid aggregates and microbial contamination. The filtrate was referred to as the micellar fraction. Aliquots of digesta and micellar fraction were stored at −20°C under nitrogen and analyzed within 1 week. To study the uptake of micellarized carotenoids, aliquots of aqueous fraction were immediately diluted (1:4) in Dulbecco's modified Eagle's basal medium containing 1% nonessential amino acids and 4 m*M* L-glutamine.

Uptake of micellarized carotenoids by Caco-2 cells

Stock and test cultures of Caco-2 human intestinal cells (HTB37, American Type Culture Collection, Baltimore, MD) (passages 28–32) were maintained as previously described.[16,17] Uptake of β-carotene and lutein from the micellar fraction generated during *in vitro* digestion of drumstick plus peanut oil was studied using monolayers of Caco-2 cultures at 11–12 days post-confluency. Monolayers were washed twice with basal medium at 37°C before addition of 2 mL of Dulbecco's modified Eagle's medium containing 25% micellar fraction. At indicated times, the medium was removed, and monolayers were washed once with ice-cold phosphate-buffered saline, pH 6.8, containing 2 g/L albumin to remove residual carotenoids adhering to the cell surface and twice with cold phosphate-buffered saline only. Cells were collected, pelleted by centrifugation, and stored under nitrogen at −80°C for a maximum of 2 weeks before analysis. To determine stability of micellar carotenoids in the cell culture incubator, medium containing 25% micellar fraction was added to culture dishes without cell monolayers and incubated for 4 hours. Samples of medium at 0 and 4 hours were stored under nitrogen at −80°C for a maximum of 2 weeks before analysis.

Extraction and analysis of carotenoids

Either 2 g of pureed fresh leaves or 0.5 g of lyophilized leaf powder, 0.4 g of calcium carbonate, and 0.2 g of Celite® (World Minerals Corp., Santa Barbara, CA; obtained from Fisher Scientific Co., Fairlawn, NJ) were added to 50 mL of methanol for analysis of total carotenoids. The mixture was homogenized for 1 minute, and the methanol-soluble fraction was separated from insoluble materials by filtration through Whatman #1 filter paper. The extraction was repeated until material on the filter paper was colorless. The crude methanol extract (200 mL) was saponified by adding 75 mL of 30% KOH in methanol and incubating at room temperature in the dark with stirring for 45 minutes. Crude extract was transferred to a separatory funnel, and the carotenoids were extracted into petroleum ether:acetone (2:1 vol/vol) until no color was observed in the upper organic phase. The volume of the organic phase was adjusted to 250 mL with hexane in a volumetric flask, and 5-mL aliquots of the extract were transferred to glass tubes, dried under nitrogen, and stored at −20°C.

Frozen samples (1–2 mL) of digesta, micellar fraction, and medium added to wells without cells were thawed. Samples were extracted by adding 3.0 mL of petroleum ether:acetone (2:1 vol/vol) containing 4.5 mmol/L butylated hydroxytoluene, vortex-mixed for 1 minute, and centrifuged (2,000 *g* for 5 minutes) to separate the organic and aqueous phases. The extraction procedure was repeated a total of three times, and the petroleum ether fractions were combined and dried at 37°C under nitrogen. The film was resolubilized in ethyl acetate:methanol (1:1 vol/vol) and analyzed immediately. Using apo-8 carotenol as a recovery standard, extraction efficiency was 90–105%.

Cell pellets were thawed on ice before addition of 1 mL of phosphate-buffered saline with 10% ethanol containing 4.5 mmol/L butylated hydroxytoluene and sonicated on ice for 10–15 seconds. After addition of 1 mL of petroleum

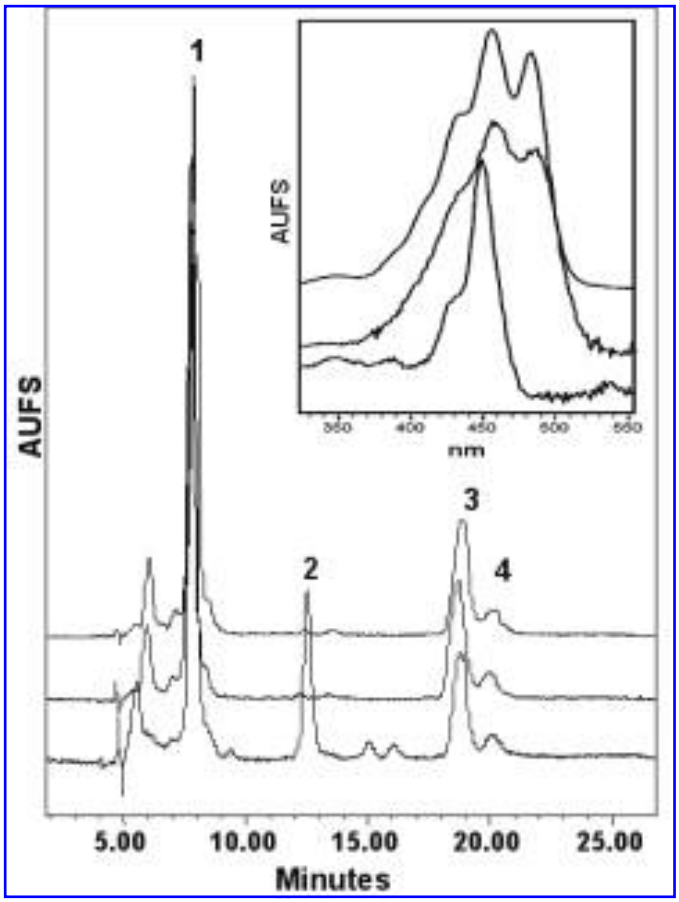

FIG. 1. Reversed-phase high performance liquid chromatography analysis of carotenoids in drumstick fresh leaves, powdered leaves, and digested leaf powder. Carotenoids from fresh, powdered leaves and digesta were extracted and analyzed as described in Materials and Methods. Peak identification for representative chromatograms is as follows: 1, all-*trans*-lutein; 2, apo-8 carotenol (recovery standard); 3, all-*trans*-β-carotene; and 4, unknown. (**Inset**) Ultraviolet-visible absorption spectra of lutein (peak 1), β-carotene (peak 3), and peak 4. AUFS, absorbance units full scale.

ether:acetone (2:1 vol/vol), carotenoids were extracted as above.

Carotenoids were separated by high performance liquid chromatography using a Waters (Milford, MA) model 2695 system equipped with an autosampler injector and a Waters model 2996 ultraviolet-visible photodiode array detector controlled by an Empower workstation (Waters). Separation was carried out using a Vydac 5-μm (particle size) analytical-scale C_{18} reversed-phase column (250 mm $\times$ 4.6 mm inside diameter) protected by a 4-μm (particle size) C_{18} Nova-Pak C18 guard column (20 mm $\times$ 3.9 mm, Waters) at ambient temperature and a flow rate of 1.0 mL/minute. Carotenoids were eluted from the column using a gradient with 100% solvent A (98% methanol:2% 1 mol/L ammonium acetate) for 5 minutes, 80% A:20% solvent B (100% ethyl acetate) from 5 to 25 minutes, and 100% A from 25 to 30 minutes. Carotenoids were identified and quantified by comparing retention times, spectral profiles, and peak areas with pure standards.

Protein assay

The protein content of cell samples was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard.

Statistical analysis of data

Data were analyzed using SPSS version 7.0 (SPSS, Chicago, IL). Descriptive statistics including mean and SD were calculated for the stability and efficiency of micellarization of carotenoids from digested foods, the stability of micellarized carotenoids in cell culture medium, and the uptake of carotenoids by Caco-2 cells. Means were compared using one-way analysis of variance followed by least significant differences test. The time-dependent accumulation of carotenoids was analyzed by paired *t* tests. Differences were considered significant at $P < .05$. All experiments were conducted using three independent samples, and each experiment was repeated at least once to provide a minimum of six independent observations.

RESULTS

Carotenoid content of drumstick leaves

Carotenoid analysis of pureed drumstick leaves using high performance liquid chromatography revealed two major peaks identified as all-*trans*-lutein (peak 1) and all-*trans*-β-carotene (peak 3) by ultraviolet-visible absorption spectra (Fig. 1). A third peak (peak 4) had a slightly longer

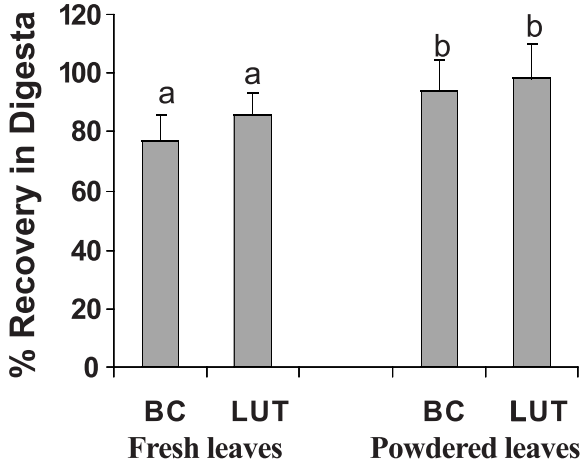

FIG. 2. Recovery of β-carotene (BC) and lutein (LUT) from drumstick leaves and powder after simulated digestion. Digestive stability represents the percentage of carotenoids recovered in digesta compared to that in pureed drumstick leaves. Data are mean $\pm$ SD values for six independent samples. Means not sharing a common letter above the columns differ significantly ($P < .001$).

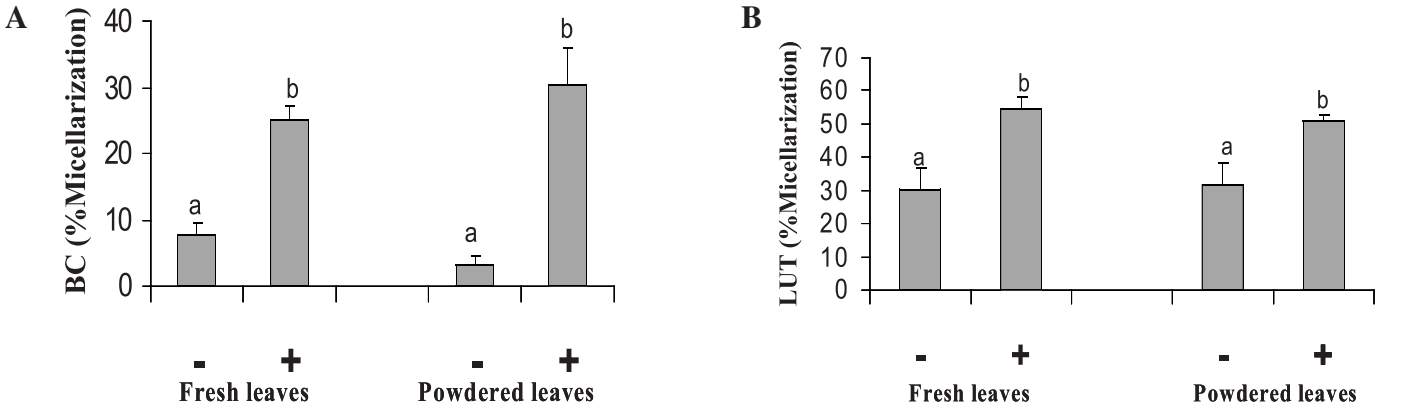

FIG. 3. Peanut oil increases the micellarization of (**A**) β-carotene (BC) and (**B**) lutein (LUT) during simulated digestion of drumstick fresh leaves and powder. Drumstick fresh leaves or powder was digested *in vitro* in the absence (−) and presence (+) of 5% (vol:wt) peanut oil. The efficiency of micellarization represents the percentage of the carotenoid in the test food transferred to the filtered aqueous fraction during simulated digestion. Data are mean ± SD values for six samples independently digested. Means not sharing a common letter above the columns differ significantly ($P < .001$).

retention time than β-carotene. However, it did not exhibit a typical carotenoid absorption spectrum and was not investigated further (Fig. 1, inset). The carotenoid profile of lyophilized leaf powder was similar to that of fresh leaves with lutein and all-*trans*-β-carotene as the predominant peaks (Fig. 1). The concentrations of lutein and β-carotene in fresh leaves were 418 and 272 mg/kg of dry weight, respectively, and 472 and 166 mg/kg of dry weight in powdered leaves, respectively. The ratio of lutein to β-carotene (1.7) was lower in fresh leaves than in lyophilized powder (2.5). Although *cis* isomers of β-carotene and lutein can form during storage and processing,[22] they were not detected in extracts of leaf powder or digested leaf powder (Fig. 1). These data suggest that some β-carotene was degraded during preparation of the lyophilized powder.

Digestive stability and micellarization of carotenoids from drumstick leaves

Digestive stability refers to the recovery of carotenoids present in the starting material after simulated gastric and small intestinal phases of digestion. Micellarization represents the amount of carotenoids present in the filtered aqueous (*i.e.*, micellar) fraction. Recovery of β-carotene and lutein in digesta ranged from 80% to 95% (Fig. 2), with recovery of these carotenoids from digested powdered leaves being greater ($P < .001$) than from digested fresh leaves. Efficiency of micellarization of β-carotene was 7% and 3.6% for digested fresh and powdered leaves without added oil, respectively (Fig. 3A). Partitioning of lutein into micelles during digestion of drumstick leaves without oil was more efficient, *i.e.*, approximately 30% (Fig. 3B). When peanut oil (5% vol/wt) was added to drumstick leaves, micellarization of β-carotene and lutein significantly ($P < .001$) increased to 25.2–30.3% and 51.0–54.7%, respectively (Fig. 3).

Uptake of micellarized carotenoids by Caco-2 cells

Cellular accumulation of both β-carotene and lutein was proportional to time of incubation (Fig. 4) and the concentration of micellarized carotenoids in medium. The quantities of β-carotene and lutein accumulated in cells were significantly ($P < .001$) greater after 4 hours than after 2 hours of incubation. The percentages of β-carotene (34.3 ± 3.6%) and lutein (37.1 ± 1.3%) in medium that were accumulated by the cells during the 4-hour incubation were not significantly different ($P > .05$). Thus, the greater amount of lutein compared to β-carotene in cells after 2 and 4 hours of incubation reflects the higher concentration of lutein in micelles generated during simulated digestion.

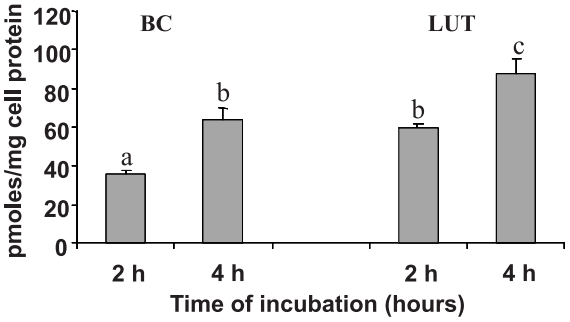

FIG. 4. Uptake of micellarized β-carotene (BC) and lutein (LUT) from digested powdered drumstick leaves with peanut oil by Caco-2 cells. Differentiated monolayers of Caco-2 cells were incubated with 2 mL of medium containing Dulbecco's modified Eagle's medium diluted (1:4) with micellar fraction generated during simulated digestion of drumstick leaf powder and not leaves for 2 or 4 hours. Data are mean ± SD values for six independent observations. Means not sharing a common letter above the columns differ significantly ($P < .001$.)

Stability of micellar carotenoids

β-Carotene and lutein in micelles generated during digestion of powdered drumstick leaves were stable in the cell culture environment with 99% and 89%, respectively, of the initial quantities recovered after 4 hours of incubation of medium in cell-free dishes.

DISCUSSION

Our results demonstrate that β-carotene and lutein in fresh and powdered drumstick leaves are stable during simulated gastric and small intestinal digestion and that the efficiency of micellarization of these carotenoids is promoted by the addition of oil (5%) to the plant food. Furthermore, we confirmed that the carotenoids transferred to micelles are bioaccessible since they were accumulated by Caco-2 cells in a time- and concentration-dependent manner.

The lutein and β-carotene content of drumstick leaves analyzed in the present study was similar to that of reported by Laksminarayana *et al*.[23] In contrast, Nambiar and Seshadri[24] reported almost a four times greater quantity of β-carotene (930 mg/kg of dry weight) in drumstick leaves. Differences in the β-carotene concentrations of drumstick leaves between the groups likely reflect variations in location where leaves were harvested, season, and post-harvest processing of the leaves.

Micellarization of carotenoids during digestion is required for transfer of carotenoids to the brush border membrane of enterocytes. The extent of transfer of carotenoids from the food matrix to micelles during simulated digestion is determined by the concentration of the pigments in the filtered aqueous fraction of chyme.[16–19] Sunflower oil and red palm oil have been reported to enhance micellarization of carotenoids during simulated digestion of green leaves from a number of plant foods, including amaranth, sweet potato, pumpkin, and cassava.[25] These results suggest that enhanced micellarization of carotenoids during small intestinal digestion contributed to the fat-mediated stimulation of carotenoid bioavailability in human subjects.[26–28]

Increases in serum and hepatic vitamin A have been reported for vitamin A-deficient rats fed diets containing either fresh or dehydrated drumstick leaves and peanut oil.[24] Our observation that micellarization of β-carotene during digestion of drumstick leaves is efficient in the presence of peanut oil provides a likely explanation for increased vitamin A status in these animals. Moreover, the body weights of the animals fed with drumstick leaves were higher than that of rats fed diet with retinyl acetate, suggesting that drumstick leaves may improve general nutritional status in addition to vitamin A status.[24]

Micellarization of lutein was more efficient than β-carotene during simulated digestion of drumstick leaves. This also has been observed during *in vitro* digestion of other plant foods and meals containing these two carotenoids.[16–18,29] Lutein is located at the surface of oil droplets because of the presence of a polar hydroxyl group on each of the ionone rings, whereas apolar carotenoids such as β-carotene reside in the core of the lipid droplets.[30] This differential localization of xanthophyll and carotenoids in oil droplets favors the transfer of lutein to mixed micelles. Also, lutein in disrupted chloroplasts has the potential to be transferred directly to micelles without the need for oil droplets serving as an intermediate reservoir.[31] It has been reported that lutein interferes with the transfer of β-carotene from oil emulsions to mixed micelles *in vitro*[32] and that xanthophylls competitively inhibit the activity of β-carotene 15,15′-oxygenase.[33] It is unknown if the ratio of lutein to β-carotene in drumstick leaves is sufficiently high for such interactions *in vivo*.

The absorption of carotenoids and their metabolites such as retinyl esters requires that they be taken up from micelles by intestinal epithelial cells, possibly converted to retinyl esters, and secreted in chylomicrons for delivery to peripheral tissues. The bioaccessibility of lutein and β-carotene in the micellar fraction from digested drumstick leaves was confirmed by the time- and concentration-dependent accumulation of these carotenoids by Caco-2 cells. Examination of the bioavailability of β-carotene and lutein in drumstick leaves in vitamin A-deficient human subjects is merited.

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