

## Negative Effect of Camu-Camu (*Myrciaria dubia*) Despite High Vitamin C Content on Iron Bioavailability, Using a Caco-2 Cell Model

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It is well known that vitamin C is an important enhancer of nonheme iron bioavailability due to its high reducing capacity. Camu-camu, a fruit that grows in the jungle of Peru, contains high amount of vitamin C (2,780 mg per 100 g). In this study, we investigated the effect of camu-camu on nonheme iron bioavailability from two different meals (rice with lentils and wheat flour porridge) using an *in vitro* Caco-2 cell model. These two meals were treated with three different camu-camu juice concentrations ( $C_0 = 0$  g,  $C_1 =$  typical consumption, and  $C_2 = 3X$  typical consumption). The results showed that camu-camu reduced rather than enhanced nonheme iron bioavailability. The inhibiting trend was significant ( $p < 0.0001$ ) in the wheat flour porridge (from 124 to 91 and 35  $\mu$ g ferritin/ $\mu$ g protein, for  $C_0$ ,  $C_1$  and  $C_2$ , respectively). With the rice with lentils, there was no significant effect of camu-camu due to the high polyphenols and phytate contents of the meal. Relative bioavailability values obtained showed significant decrease with increasing camu-camu juice concentration for both meals. As expected, the ascorbic acid added to the meals at a concentration equivalent to that present in  $C_2$ , had no effect on bioavailability with rice meal but increased significantly with wheat flour meal. The findings of this study suggest that camu-camu, in the traditional way of preparation, may significantly reduce nonheme iron bioavailability because of its high polyphenol content which overrides the beneficial effect of its high ascorbic acid content.

### INTRODUCTION

Iron deficiency anemia is a major public health problem, especially in developing countries where iron content in the diet is poor and is mostly in the form of poorly available nonheme iron [UNICEF, UNU, WHO, 2001]. In a national survey conducted in Peru in 2009 [Endes Continua, 2009], it was found that twenty percent of women of reproductive age, and thirty-seven percent of children under the age of five, suffer from iron deficiency anemia.

Peruvians often drink fruit juice or soda along with their meals. Camu-camu (*Myrciaria dubia*), is a fruit found in the jungle of Peru with high vitamin C content (2,780 mg per 100 g of fruit) and juice is often made from this fruit [Collazos, 1996]. It is well known that vitamin C is an enhancer of nonheme iron absorption due to its high reducing capacity [Lynch & Cook, 1980]. It causes ferric reduction to ferrous and makes iron more soluble. Ferric iron reduction is important because it is mostly insoluble and poorly absorbed [Proulx & Reddy, 2007]. This study was therefore conducted to investigate whether camu-camu, as a good source of vitamin C can improve nonheme iron bioavailability from se-

lected meals commonly consumed in Peru. We used *in vitro* digestion coupled with Caco-2 cell iron uptake to assess iron bioavailability. Caco-2 cells are human adenocarcinoma cell line that exhibit enterocyte-like biochemical and morphological characteristics and have been used widely for studies of bioavailability of heme and nonheme iron due to their high correlation with iron absorption studies in humans [Au & Reddy, 2000; Proulx & Reddy, 2006, 2007; Yun *et al.*, 2004].

### MATERIALS AND METHODS

#### Camu-camu juice

Camu-camu fruits were obtained from Iquitos, Loreto, Peru and were sent to Lima from the National Institute for Agrarian Innovation (INIA). They were processed manually as follows. The fruits were crushed and seed and peel were separated from the pulp. The pure camu-camu juice obtained was then placed in 50 mL vials and frozen at  $-80^{\circ}\text{C}$ , before shipping to the United States.

#### Vitamin C determination

Ascorbic acid in camu-camu juice was measured using the method that is based on the reduction of  $\text{Fe}^{+3}$  to  $\text{Fe}^{+2}$  by ascorbic acid, followed by chelation of  $\text{Fe}^{+2}$  by ferrozine as a chromogen [McGown *et al.*, 1982].

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### Nonheme iron content determination

Nonheme iron content of each meal was determined by the modified method of Torrance & Bothwell [1968] using ferrozine instead of bathophenanthroline disulfonic acid as a chromogen in a microplate assay [Chidambaram *et al.*, 1989].

### Meals

Two meals, namely rice with lentils and wheat flour porridge which are common dishes in Peru were prepared as a regular adult portion sizes and used in the experiments. The rice and lentil meal was prepared from 110 g of rice and 70 g of lentils. These ingredients were cooked and homogenized with iron-free water (260 mL). In preparing the wheat flour porridge, wheat flour (28 g) was dissolved in water, heated to boil and it was further cooked after adding sugar (11 g) and powdered milk (17 g). Both meals were weighed and saved as small batches at  $-20^{\circ}\text{C}$ . Camu-camu juice was prepared by mixing the pulp with water 1:5 as it is usually consumed as a refreshment drink in 1:5 water dilution (240 mL per serving). Three different treatments in triplicates were prepared with each meal as follows. Water ( $C_0$ ) was added to the first aliquot; Two different amounts of Camu-camu juice were added to the second ( $C_1$ ) and the third ( $C_2$ ) sample.  $C_1$  represents typical intake level in Peru and  $C_2$  contains three times the vitamin C concentration in  $C_1$ . The amount of juice added ( $C_1$ ) was proportional to the fraction of one serving of meal. To further understand the effect of camu-camu on iron bioavailability, 918 mg ascorbic acid was added to each of the two meals. This was equal to the amount of ascorbic acid in camu-camu juice that was used in  $C_2$  sample. The food ingredients, required for preparing the meals, were obtained from local supermarket in Ames, Iowa. Deionized water and iron-free water were used throughout the study.

### In vitro digestion

*In vitro* digestion of samples and iron bioavailability measurements were done as described by Proulx & Reddy [2006]. Brief description of the protocol is provided below. Aliquots of both meals (with camu-camu or with vitamin C) were subjected to *in vitro* digestion followed by Caco-2 cell uptake. For simulating gastric and duodenal digestion, pepsin and pancreatin/bile extract were added to the samples. Pepsin was prepared by solubilizing 0.2 g of porcine pepsin A (1:60000) in 5 mL of 0.1 mol/L HCl. Pancreatin and bile solutions were prepared by dissolving 0.05 g of porcine pancreatin (4 x USP) and 0.3 g bile extract in 25 mL of 0.1 mol/L sodium bicarbonate ( $\text{NaHCO}_3$  0.1 mol/L). Trace minerals were removed from the mixture of pepsin and pancreatin by treatment with Chelex-100 (BioRad), as described by Glahn *et al.* [1998]. Samples were weighed to deliver 200  $\mu\text{g}$  of total iron, mixed with water, and adjusted to pH 2 using 0.1 mol/L HCl. Pepsin solution was added (1 mL) and incubated at  $37^{\circ}\text{C}$  with shaking at 800 rpm for 1 h. Following the pH adjustment to 6.0 with 1 mol/L  $\text{NaHCO}_3$  solution, pancreatin and bile solution were added (5 mL), and incubation was continued for 15 min. Samples were heat-treated (4 min at  $100^{\circ}\text{C}$ ) to inactivate proteolytic enzyme activity [Jovani *et al.*, 2001] and centrifuged, and the supernatant was used for iron bioavailability experiments.

### Iron bioavailability determination using the Caco-2 cell model

Bioavailability was determined using ferritin synthesis as an index of bioavailable iron in response to iron uptake by the Caco-2 cells which is common method used by other researchers [Glahn *et al.*, 1998; Proulx & Reddy, 2006; Yun *et al.*, 2004]. Ferritin values were normalized to cell protein concentration. All reagents for cell culture work were from Sigma Aldrich or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD) and the following experiments were conducted during passages 34–37. Cells were grown in a culture flask with Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% v/v nonessential amino acids, and 1% v/v antibiotic-antimycotic solution and maintained at  $37^{\circ}\text{C}$  in an incubator with 5%  $\text{CO}_2$ . Media was changed 3 times weekly. At day 7, the cells were rinsed with Earle's Balanced Salt Solution (EBSS), trypsinised to dissociate the cells, and centrifuged for 5 min. The cells were seeded on collagenized (Type 1 Rat tail collagen) 12-well cell culture plates (Corning Costar) at a density of  $5 \times 10^4$  cells/cm<sup>2</sup>, for iron bioavailability experiments. The cell culture plates were maintained in the incubator with conditions similar to those of the cell culture flask. Iron bioavailability experiments were conducted 15 d post seeding. Serum-free media without FBS (0.5 mL) as suggested by earlier studies [Glahn, 1998], prepared with DMEM with 1% v/v nonessential amino acids, 1% v/v antibiotic-antimycotic solution, 10 mmol/L piperazine- $\text{N,N}'$ -bis-[2-ethanesulfonic acid] (PIPES), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5  $\mu\text{g}/\text{L}$ ), triiodothyronine (34  $\mu\text{g}/\text{L}$ ), and epidermal growth factor (20  $\mu\text{g}/\text{L}$ ), was mixed with equal volume of sample supernatants and was added to cell monolayer after rinsing the cells with Earl Balanced Salt Solution (EBSS) and then incubated for 2 h. An additional 0.5 mL of serum-free media was added followed by a further incubation for 22 h. After 24 h total incubation, cells uptake solution was removed and rinsed with EBSS. Then, cells were lysed by adding 0.5 mL of deionized water to each well and sonicated with a probe-type sonic dismembrator at lowest setting ( $<1$  W output) for 15 s. Total cellular protein was determined in the lysates by the Bradford Coomassie Assay (Pierce Laboratories, Rockford, IL). Ferritin in the lysates was determined by radioimmunoassay (Fer-Iron II, Ramco Laboratories, Stafford, TX). Iron bioavailability was expressed as  $\mu\text{g}$  ferritin/ $\mu\text{g}$  cell protein. The relative bioavailability (RBA) of  $C_1$  and  $C_2$  was expressed as percentage of  $C_0$  bioavailability. ANOVA with Tukey's multiple comparison test was used to determine the differences among three treatments for each meal and unpaired t-test was used to compare RBA of  $C_1$  and  $C_2$  for each meal. The differences were considered significant at  $p \leq 0.05$ .

## RESULTS AND DISCUSSION

Proximate analysis indicated that the vitamin C content of camu-camu juice was 638 mg/100 g juice. The vitamin C contents of the different concentrations of camu-camu used in this study were 306 mg for  $C_1$ , and 918 mg for  $C_2$ .

TABLE 1. Nutrient composition of meals.

Meal	Portion description	Energy (Kcal)	Protein (g)	Fat (g)	Carbohydrates (g)	Iron (mg)	Ascorbic acid (mg)
Rice with lentils	Per serving	638.7	25.39	0.742	127.6	8.8	3.08
	Per 100 g raw mix	354.83	14.1	0.41	70.89	4.89	1.71
Wheat flour porridge	Per serving	197.31	6.63	0	44.67	1.05	0.86
	Per 100 g raw mix	352.34	11.83	0	79.76	1.87	1.54

TABLE 2. Effect of different concentrations of camu-camu on iron bioavailability in two meals.

Result description	Diet	C <sub>0</sub>	C <sub>1</sub>	C <sub>2</sub>	P
Iron bioavailability ( $\mu\text{g}$ ferritin/ $\mu\text{g}$ protein)	Rice with lentils	28 $\pm$ 2 <sup>a</sup>	31 $\pm$ 2 <sup>a</sup>	25 $\pm$ 1 <sup>a</sup>	0.0853
	Wheat flour porridge	124 $\pm$ 4 <sup>a</sup>	91 $\pm$ 6 <sup>b</sup>	35 $\pm$ 2 <sup>c</sup>	<0.0001
Relative bioavailability (% of control)	Rice with lentils	-----	111.7 $\pm$ 6.7 <sup>a</sup>	90.7 $\pm$ 5.3 <sup>b</sup>	0.0279
	Wheat flour porridge	-----	73.7 $\pm$ 4.4 <sup>a</sup>	28.1 $\pm$ 1.5 <sup>b</sup>	< 0.0001

Different superscripts in the same row are significantly different. Comparisons were made within the same meal.

The control treatment (C<sub>0</sub>) had no camu-camu juice. Non-heme iron contents of the food samples were 14.7 mg and 3.3 mg per serving for the rice with lentils and wheat flour porridge, respectively. These values were higher than those from the USDA Nutrient Database (Table 1).

Results on the effect of camu-camu on iron bioavailability are shown in Table 2. In the rice with lentils meal, bioavailability of iron was not significantly ( $p>0.05$ ) different among the three different levels of camu-camu. On the contrary, a clear trend was observed for the wheat flour porridge, in which the bioavailability was reduced as concentration of camu-camu increased. The bioavailability values for the three different levels of camu-camu concentration in the wheat flour porridge were significantly different from each other ( $p<0.0001$ ). Iron bioavailability of C<sub>0</sub> without camu-camu was 3.5 times higher than C<sub>2</sub> and 1.4 times higher than C<sub>1</sub>.

The inverse relationship between camu-camu concentration and iron bioavailability was made clearer by the RBA values (Table 2). For both meals, RBA values significantly decreased with increasing concentration of camu-camu. This is contrary to expectation considering the high vitamin C content of camu-camu (306 mg in C<sub>1</sub> treatments and 918 mg in C<sub>2</sub> treatments). Cook & Monsen [1977], reported a proportional increase in iron absorption by adding 25 to 1,000 mg of ascorbic acid to a semisynthetic meal containing 4.1 mg iron; and Diaz *et al.* [2003] showed that intake of 25 mg of ascorbic acid, in lemonade, twice a day for 2 weeks, doubled the absorption of iron from typical Mexican foods containing between 6.2 and 8.6 mg of iron. This unexpected observation of the inhibitory effect of camu-camu juice on iron absorption may be explained by the high polyphenols content of camu-camu. Apart from having a high content of vitamin C, camu-camu is also rich in polyphenols containing about 2.4% [Munoz *et al.*, 2007]. Polyphenols are potent inhibitors of iron absorption because they can bind iron forming insoluble complexes [Siegenberg *et al.*, 1991].

Comparing results for the two meals, bioavailability values were generally higher in the wheat flour porridge meal than the rice with lentils. The lower bioavailability in the rice

with lentils meal (28  $\mu\text{g}$  ferritin/ $\mu\text{g}$  protein) compared with the wheat flour (124  $\mu\text{g}$  ferritin/ $\mu\text{g}$  protein) may be due to the combined effect of phytic acid and polyphenols associated with lentils. Since bioavailability of rice with lentils

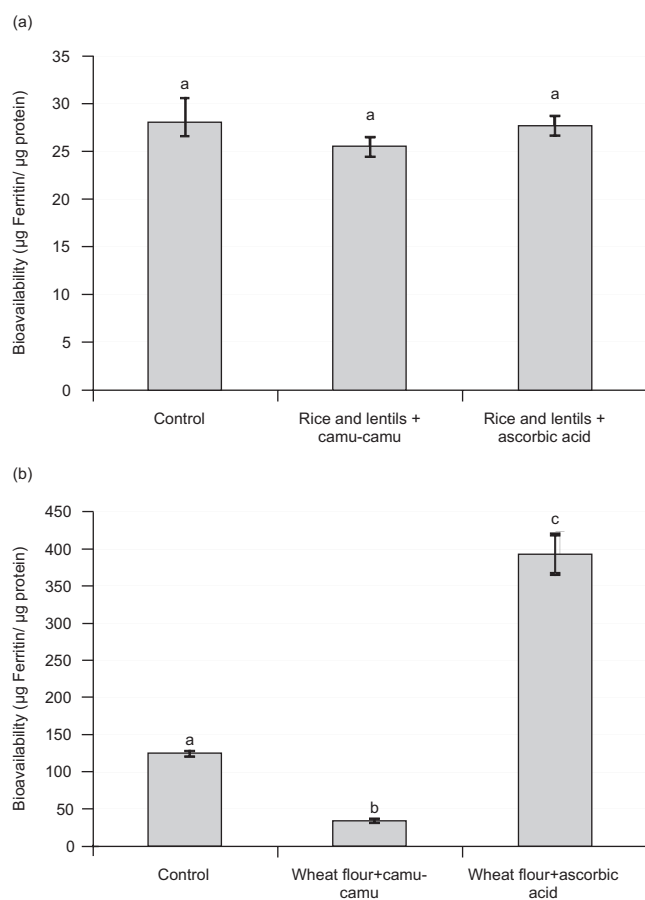


FIGURE 1. Effect of ascorbic acid and camu-camu on iron bioavailability in rice with lentils (a) and wheat flour porridge (b) ( $n=2-3$ ). Treatments for each meal: control: meal alone; camu-camu: meal with added camu-camu juice containing 918 mg ascorbic acid; ascorbic acid: meal with added 918 mg ascorbic acid. ANOVA with Tukey's multiple comparison test was used to detect the differences among three treatments. Bars with different letters are significantly different.

was already low, the camu-camu juice did not show further significant reduction. On the other hand, in the wheat flour meal, iron bioavailability was reasonably higher in the control ( $C_0$ ) meal, and the inhibitory effect of camu-camu juice was more pronounced due to its high polyphenol content. However, when the RBA was compared between  $C_1$  and  $C_2$ , they were significantly different for both rice with lentil ( $p < 0.03$ ) and wheat porridge ( $p < 0.0001$ ) (Table 2).

Adding ascorbic acid similar to the concentration present in  $C_2$ , provided us some understanding of camu-camu's inhibiting effect. Ascorbic acid had no effect which is similar to adding camu-camu with rice/lentil meal (Figure 1A), again suggesting phytic acid and polyphenols inhibition is too strong to overcome by ascorbic acid. Whereas, adding ascorbic significantly increased bioavailability by 3-fold with wheat porridge in contrast to a significant decrease with the addition of camu-camu (Figure 1B). This observation suggests that the polyphenol content of camu-camu overrides the enhancing effect of ascorbic acid in camu-camu leading to a reduction in iron bioavailability from the meals, particularly from the wheat flour porridge.

## CONCLUSION

This study suggests that camu-camu juice may reduce iron bioavailability in the meal due to its extremely high polyphenols content which inhibits iron bioavailability, counteracting the enhancing effect of vitamin C. This might also imply that, though the fruit may not be useful in improving nonheme iron absorption it may be useful in protecting from chronic diseases by providing antioxidant effect due to its high polyphenol content.

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