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Long-term dose-response condensed tannin supplementation does not affect iron status or bioavailability

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Abbreviations:

iAUC: incremental area under the curve

aPRP: acidic salivary proline rich protein

ASA24: automated system for 24-hour dietary recalls

BMI: body mass index

bPRP: basic proline rich proteins

CI: Confidence interval

CRP: C-reactive protein

EDTA: ethylene diaminetetraacetic acid

gPRP: glycosylated salivary proline rich protein

Hb: Hemoglobin

HPLC: High performance liquid chromatography

KSU: Kanas State University

PRP: salivary proline rich proteins

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RDA: Recommended Dietary Allowance

TFA: Trifluoroacetic acid

USDA: United States Department of Agriculture

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1 **Abstract**

2 **Background:** Repeated phytic acid consumption leads to iron absorption adaptation, but the
3 impact of repeated tannin consumption has not been established. Salivary proline-rich proteins
4 (PRPs) may improve iron absorption by precipitating tannins.

5 **Objective**: To determine the effect of long-term, dose-response, condensed-tannin
6 supplementation on iron bioavailability, and status as well as determine the effect of salivary
7 proteins on iron bioavailability during prolonged condensed tannin consumption. A secondary
8 objective was to assess astringency as a potential marker for adaptation to tannins and iron
9 bioavailability.

10 **Methods:** Non-anemic women were enrolled in a double-blind three dose crossover trial (n =
11 11). Three (1.5, 0.25, or 0.03 g) condensed tannin supplements were consumed three times daily
12 for four weeks in random order, with two-week washouts in-between. Before and after
13 supplementation, meal challenges were employed to assess iron bioavailability, iron status,
14 salivary PRP changes, and astringency.

15 **Results:** Tannin supplementation did not change iron bioavailability at any dose (ps > 0.82) from
16 Week 0 to 4 in any dose. Hemoglobin ($p = 0.126$) and serum ferritin ($p = 0.83$) were unchanged
17 by tannin dose from Week 0 to Week 4. There were significant correlations between iron
18 bioavailability, basic PRPs (r = 0.366, $p = 0.003$), and cystatin production (r = 0.27, $p = 0.03$)
19 with tannin supplementation. Astringency ratings did not change significantly within, or between
20 tannin doses (ps > 0.126), but there were negative relationships between bPRP (rs < -0.32, ps <
21 0.21), cystatin (rs < -0.2, ps < 0.28) production, and astringency ratings.

22 **Conclusions:** Condensed-tannin consumption did not affect iron bioavailability or status
23 regardless of supplementation period in premenopausal, non-anemic women. Correlation

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24 analyses suggest that basic PRPs, and cystatins are associated with improved iron bioavailability,
25 and that lower ratings of astringency may predict improved iron absorption with repeated tannin
26 consumption.
27

28 Keywords: tannins, iron bioavailability, salivary proline rich proteins, adaptation, antinutritional
29 factors, proanthocyanidins, iron deficiency anemia
30

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31 **Background**

32 An estimated one billion people suffer from iron deficiency anemia worldwide (1). Most
33 commonly, iron deficiency is found in women, children, vegetarians, and in people who have
34 insufficient iron intake (1). Despite multiple initiatives aimed at reducing iron deficiency anemia
35 in the past 20 years, an estimated 29% of non-pregnant women were anemic in 2011, a 4%
36 reduction since 1995 (2).

37 Tannins have been found to negatively impact iron bioavailability (3-8) by formation of
38 insoluble antinutritional-mineral complexes (9), and this has deterred tannin-rich foods, such as
39 sorghum, from being used within food-aid for regions that are largely undernourished (10).
40 Previous research suggests that long-term tannin consumption may not inhibit iron
41 bioavailability as much as single meal studies predict (11, 12). For example, long-term
42 antinutritional factor consumption in animals (13-15) and humans (16, 17) has resulted in
43 improved non-heme iron bioavailability compared to single-meal studies. In these studies, the
44 negative effects (reduced iron status or bioavailability) of antinutritional factor intake over time
45 have not been sustained, and these findings have been proposed to be due to adaptation to
46 antinutritional factors over time. In studies that have found reductions in iron bioavailability with
47 tannin consumption, individual iron absorption has been highly variable (18, 19) and many
48 individuals consuming diets with large concentrations of tannins maintain normal iron stores
49 (20).

50 Many studies finding reduced iron bioavailability with tannin consumption have used
51 hydrolyzable tannic acid, which is not commonly consumed, or tea tannins, which may be
52 metabolized differently than condensed tannins, which are commonly found in food (11). To the
53 best of our knowledge, no studies have determined the long-term effects of condensed tannin

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54 consumption apart from other antinutritional factors such as fiber or phytates on iron
55 bioavailability or status. In addition, it has not been determined whether long-term condensed
56 tannin consumption results in adaptation, or what mechanisms underlie adaptation if it does
57 occur.

58 Mechanistically, adaptation to tannins may start in the mouth (21). Saliva contains six
59 main classes of salivary proteins: histatins, cystatins, statherins, acidic proline-rich proteins
60 (aPRP), basic proline-rich proteins (bPRP), and glycosylated proline-rich proteins (gPRP) that
61 may exert independent effects on tannins (22). The binding of PRPs to condensed tannins also
62 may prevent condensed tannins from chelating iron thereby improving iron bioavailability (21,
63 22). Tannin-PRP complexes are insoluble within the GI tract (23, 24), preventing tannin-iron
64 chelation throughout digestion. There are a variety of PRP subtypes that make up different
65 salivary profiles, which may be largely genetically determined (25, 26). Genetic determination of
66 salivary profiles favoring effective tannin precipitation may explain why some individuals have
67 greater capacity to consume tannins than others without negative impacts on iron status.
68 Upregulation of PRP secretion when consuming tannins has been shown to improve protein (27)
69 and iron bioavailability in animal studies (12, 14), and animals that do not upregulate PRP
70 synthesis in response to tannin consumption have poor growth outcomes (28).

71 Previously, PRP-tannin binding has been identified in sensory studies because it causes
72 an oral astringency sensation (22). Theoretically, identification of PRP “adapters” may then be
73 possible through simple, inexpensive, astringency testing (22). Further, changes in astringency
74 sensation may indicate an upregulation of PRP production over time with repeated tannin
75 consumption (29).

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76 The primary objectives of the current tannin dose-response crossover trial were to
77 determine the effect of 4-week, multi-meal dose-response condensed-tannin supplementation on
78 iron bioavailability, and status, and to understand the effect of salivary proteins on iron
79 bioavailability during prolonged condensed tannin consumption. A secondary objective was to
80 assess astringency as a potential marker of adaptation to tannins and iron bioavailability.
81 In this study, we hypothesized that 1) condensed-tannin supplementation would not
82 change iron bioavailability (determined by iron absorption) or status (determined by hemoglobin
83 and ferritin) regardless of dose over four weeks, 2) salivary proline-rich protein production
84 would be induced by tannin consumption over time and by higher versus lower tannin doses, and
85 3) proline-rich protein production would be positively associated with improved iron
86 bioavailability after tannin consumption. Secondary hypotheses were that 1) astringency
87 perception would be changed with tannin consumption over time, 2) salivary proline-rich protein
88 production would predict astringency with tannin consumption, and 3) astringency could be used
89 as a surrogate marker for iron bioavailability with tannin consumption.
90

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91 **Methods**

92 ***Inclusion/exclusion***

93 The study protocol was approved by the Institutional Review Board at Kansas State
94 University (KSU; IRB #8121). Due to the length of the study, enrollment was rolling, and is
95 outlined (**Figure 1**). An announcement requesting participants was sent to faculty and students
96 through a university email digest, as well as disseminated though departmental social media
97 channels. In total, 48 women responded, and potential participants were screened in person or via
98 phone (Figure 1). Before screening, participants were required to read and sign an informed
99 consent document, and all procedures, risks and benefits of the study were reviewed verbally.
100 During screening, participants were asked to complete a medical history questionnaire.
101 Premenopausal women, aged 18-35, who were non-obese (body mass index, BMI $\leq$ 30.0 kg/m^2),
102 had no history of oral or gastrointestinal disease, were moderate ($\leq$ 1 drink per day) or non-
103 alcohol consumers, and non-tobacco users were eligible for participation. Non-anemic women
104 were included who had both normal (n = 2, ferritin range 88-100 ng/mL) and marginal (n = 9,
105 ferritin range 7-30 ng/mL) iron stores to minimize losses due to potential exacerbated anemia
106 during the study. Iron absorption has been significantly changed after antinutritional
107 supplementation in non-anemic, iron-replete individuals previously (6, 30). Further exclusion
108 criteria included blood disorders affecting iron status or absorption, current supplementation or
109 medication that would impair iron status, food allergies to supplements, pregnancy or
110 breastfeeding. No participants consumed iron supplementation prior to, or during, the study
111 period (see Supplementary Material for screening questionnaire and exclusion criteria).
112 Participants were compensated for completing study activities.

113 ***Study Design***

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114 *Blinding and randomization*

115 Participants were assigned ID numbers, and a researcher not involved in data collection
116 randomized each participant number to a tannin supplement order (SAS, Carey, NC).
117 Participants, the principal investigator, and the project coordinator were blinded to dose order.
118

119 *Supplementation periods*

120 Supplementation periods consisted of Week 0 and Week 4 meal challenges, with four
121 weeks of tannin supplementation in between (**Figure 2**). Four-week supplementation periods
122 were chosen as completed previously (31) to assess for iron status changes with inhibited iron
123 absorption at each meal, and to allow for time to adapt to tannins. Each participant consumed a
124 powdered, condensed-tannin (Nusci grape seed extract, 95% condensed proanthocyanidins)
125 supplement mixed in an opaque bottle with water and a non-caloric flavor enhancer and
126 sweetener (Mio Original) to improve its palatability, three times daily for four weeks.
127 Supplements were prepared weekly by an outside researcher; participants returned weekly to
128 pick up supplements, and were questioned about supplement adherence. In addition, supplement
129 bottles were checked for total supplement consumption, and adherence issues were noted High
130 (1.5 g), medium (0.25 g), or low (0.03 g) condensed-tannin doses were provided for consumption
131 with three daily meals. These doses represented the amount of condensed tannins from 100g of
132 high tannin red sorghum (32-34), 1 cup of tea (6-8), or the lowest inhibitory vegetable meals
133 cited previously (3, 35). Each Week 4 meal challenge was followed with a two-week washout
134 period to allow normalization of iron absorption and potentially PRPs to the participant's usual
135 diet (36). Two participants had a single washout period of three, instead of two weeks due to
136 participant availability for meal challenges.

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137

138 *Tannin meal challenges*

139 At Week 0 and Week 4 of each supplementation period, participants completed meal
140 challenges at the KSU Physical Activity and Nutrition Clinical Research Consortium.
141 Participants were asked to come in fasted (at least 8 hours) at 7:00 am, having abstained from
142 teeth cleaning (2 hours) and exercise (24 hours) to minimize diurnal variations or other
143 confounding factors in salivary production (37) and iron uptake (38, Figure 2b). Pre-meal saliva
144 was collected by passive drool (2 ml total) in cryovials, and samples were immediately stored in
145 a -80^{0} C freezer. A 20-gauge indwelling peripheral IV catheter was placed either in the median
146 cubital, cephalic, or basilic vein for multiple blood samples, which was flushed and saline locked
147 (no intravenous fluids were provided during the meal challenges except for flushes) with 10 ml
148 of 0.9% isotonic saline between blood collections. From the fasting blood draw, two separate
149 samples were collected in 5 mL serum separator and 3 mL ethylenediaminetetraacetic acid
150 (EDTA) vacutainer tubes to measure serum iron (by spectrophotometry), C-reactive protein
151 (CRP, by nephelometry, sensitivity 0.2 mg/dL), ferritin (by immunoassay, sensitivity 0.1 ng/ml),
152 and whole blood hemoglobin concentrations (by electronic cell cytometry). After fasted blood
153 collection, a challenge meal including a 95 g bagel with 12 g sugar-free strawberry jam, half
154 sprinkled with 15 mg anhydrous ferrous sulfate (39) and the other half with 75 mg ascorbic acid
155 (16, 40), and a 90 g banana, was consumed simultaneously with the participant's assigned
156 supplement dose (1.5 g, 0.25 g, or 0.03 g in 8 fluid ounces). Ferrous sulfate and ascorbic acid
157 weighed with a scale to the nanogram, and weights were recorded for %max iron calculations.
158 Salivary samples were collected 15 minutes following the final bite of the meal to determine
159 salivary protein stimulation after tannin consumption (41). Subsequent blood samples were

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160 collected in tubes and analyzed for serum iron at 180 and 240 minutes. After collection, serum
161 samples were centrifuged at 2500 RPM for 15 minutes after clotting for 20 minutes, and kept at
162 room temperature for analysis. All blood samples were analyzed by a certified laboratory (Quest
163 Diagnostics, Lenexa, KS) within 24 hours.

164 *Regression analysis of three-point time draw for % maximum iron absorption*

165 To minimize blood loss, validity of a three-time blood draw systemto determine iron
166 bioavailability by serum iron was determined. In the first supplementation period, four
167 participants had blood drawn every thirty minutes for four hours to establish correlation with a
168 three-time point draw (0, 180, and 210 minutes) system previously proposed (40). Regression of
169 polynomial lines from data points were calculated on a computer data system (Microsoft Excel,
170 2013), and R^2 values were calculated for goodness-of-fit. From these data, it was verified that
171 time points proposed were representative of the full models previously used (16, 40).
172 Correlations of 0.99+ were seen, thus blood samples were also drawn for serum iron at these
173 three time points to determine % max iron absorption and incremental serum iron area under the
174 curve (iAUC) (40).

175

176 *Incremental AUC and maximum percentage of iron absorption calculation*

177 Serum iron data were used to calculate percentage of maximum iron recovery and iAUC for iron
178 bioavailability analysis.

179 Percentage Iron Recovery: Percentage iron recovery was calculated as below (40).

180

181

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$$\%\ iron\ recovery\ \max = (\frac{serum\ iron\ \max \times\ plasma\ volume)}{total\ iron\ ingested} \times 100$$

182 Where: $iron\ \max = \frac{\mu mol}{L}, plasma\ volume = L, and\ amount\ of\ iron\ is\ in\ \mu mol$

$$plasma\ volume\ (L) = \frac{Blood\ volume\ (ml) \times \left(1 - Packed\ cell\ volume\ (decimal)\right)}{1000}$$

183 And $blood\ volume = 69.6\frac{ml}{kg} body\ weight$

184

185 iAUC by trapezoidal integration

$$\int AUC = \frac{\Delta\ time}{2} \times [\Delta Serum\ iron\ _{time\ 0-180}\ +\ (2 \times \Delta Serum\ iron_{time\ 0-210})]$$

186

187 *Astringency testing*

188 After the peripheral indwelling IV catheter was removed following each meal challenge,
189 participants were asked to complete an astringency test (42, 43). Each participant was given four
190 different concentrations of alum powder in 10 ml distilled water (0.20, 0.15, 0.07, and 0.03%) in
191 random order to sip. They were first given a verbal description of the sensation of astringency
192 and were asked to rate each solution based on their perception of astringency on a 5 point Likert
193 scale (1 = not astringent, 5 = extremely astringent). Participants waited for 30 seconds before
194 testing the next sample.

195

196 *Dietary analysis in supplementation periods*

197 Within each supplementation period, 24-hour dietary recalls were collected on three
198 different days (two weekdays and one weekend day, 44). At the beginning of week two of each
199 four-week supplementation period, participants were emailed a unique username and password

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200 to complete 24-hour dietary recalls for 2 weekdays and 1 weekend day on the automated Self-
201 Administered 24-Hour Recall (ASA24®). After all recalls were collected in each
202 supplementation period, dietary data were extracted, and total caloric intake (kcal), protein (g),
203 fat (g), carbohydrate (g), iron (mg), ascorbic acid (mg), meat protein (oz.), sugar (g), fiber (g), Zn
204 (mg), and Cu (mg) content were averaged from system calculated amounts for each participant.
205 Food intake logs were downloaded from the ASA24® for manual calculation of
206 proanthocyanidins and polyphenols. During this process, a research assistant reviewed all dietary
207 data for each participant using an electronic spreadsheet (Microsoft Excel). Food items were
208 referenced from United States Department of Agriculture (USDA) tables pulled into an
209 electronic spreadsheet, and total proanthocyanidin (condensed tannin, 45) and polyphenol (46)
210 amounts were calculated and summated for each recall. From these summations, averages were
211 calculated. All assessments and calculations were reviewed by the project coordinator before
212 analyses were completed.
213
214 *Salivary PRP measurement*
215
216 *Acidified saliva sample preparation*
217 Frozen salivary samples were thawed overnight in a refrigerator. Before sample analysis,
218 consistency in chromatogram output with duplicate samples was verified, and samples were
219 analyzed in a single run. For PRP extraction, 900 µl of saliva was mixed with 10 µl of 10%
220 trifluoroacetic acid (TFA) in water, centrifuged for 5 min at 8000 RPM, and the supernatant was
221 filtered through a 0.2 µm PVDF syringe filter as described previously (47). Before samples were
222 analyzed, it was verified that there was no PRP peak loss with use of syringe filters by testing in

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223 trial HPLC runs. The supernatant was then analyzed by high performance liquid chromatography
224 (HPLC).

225 *HPLC parameters and equipment*

226 All reagents were analytical grade. Acetonitrile, TFA, and HPLC grade water were
227 purchased from Fisher Scientific. Ninety µl of salivary supernatant was injected into a Fisher
228 2.1x150 mm, 5µm BioBasic C8 analytical column at a flow rate of 0.3 ml/min for 49 minutes at
229 40◦C with an autosampler (Shimadzu SIL) on a HPLC system containing a LC20AB pump
230 (Shimadzu), and a Shimadzu SPD-M20A PDA system. Detection of PRPs was carried out at 214
231 nm (47, 48). Mobile phase consisted of 0.2% TFA in HPLC grade water (A) and 0.2% TFA in
232 80/20 acetonitrile and HPLC grade water (B) (41, 47-49). A linear gradient was applied from 0-
233 39 minutes from 0-54% (B), then from 39-49 minutes at 54-100% B to elute late proteins (41,
234 47-49). After each run, the column was washed and stabilized with initial conditions by
235 increasing linear gradient back to 100% A over 10 minutes.

236

237 ***Statistical analyses***

238

239 Data were analyzed using SAS statistical software (SAS Studio version 3.6, Cary, North
240 Carolina), statistical significance was set at $p < 0.05$. All data are presented as mean ± standard
241 deviation (SD). Before analysis, all data were analyzed for normality and homogeneity of data in
242 Q-Q plots and with Levene's tests. Variables that were non-normal (proanthocyanidin
243 monomers, dimers, total proanthocyanidin, ascorbic acid, sugar, and iron intake) were log
244 transformed, and determined to be normal before further analysis. Log-transformed variables

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245 were included in stepwise variable selection in adjusted model building (below). All log-
246 transformed data were back-transformed for results presentation.
247
248 *Sample size*
249 A paired t-test sample size calculation (SAS studio version 3.6, Cary, NC) determined
250 that four participants would be needed to detect a change in iAUC of 41%, which was observed
251 in a similarly designed antinutritional factor adaptation study (16) as statistically significant with
252 80% power and at an α-level of 0.05.
253
254 *Demographic data, washout, and randomization order analysis*
255 Week 0 demographic and nutritional intake data were analyzed by analysis of variance by
256 supplementation period. Randomization order and previous dose effect were analyzed by Chi-
257 square testing to assess for bias in supplementation period order, or previous effect of
258 supplementation period. Changes between previous and next supplementation period during
259 washout were analyzed for hemoglobin, ferritin, iAUC for serum iron, and % max iron
260 absorption by analysis of variance.
261
262 *Hematological outcomes analysis*
263
264 *Regression analysis of hematological outcomes*
265 Linear regression of raw outcomes data was used to determine whether four weeks of
266 multiple daily tannin supplementation would change iron absorption or status within or between
267 supplementation periods. In regression analysis, differences between supplementation periods

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268 were analyzed for % max iron absorption, ferritin, and hemoglobin at Weeks 0 and 4 (to analyze
269 for within dose responses). Multiple regression was used to adjust models for repeated
270 (participant) and random (ferritin, CRP, dietary intake, weight, and age) covariates after stepwise
271 selection for significant ($p < 0.05$) variables. To maximize analysis of individual iron
272 bioavailability and status within different supplementation periods, individual movements
273 (increase, decrease, or maintain) in dose-responses (hemoglobin, ferritin, % max iron absorption,
274 and serum iron iAUC) were analyzed with Chi-square testing and Fisher's exact tests.
275
276 *PRP and astringency outcomes analysis*
277
278 *PRP changes with tannin supplementation and correlations with iron bioavailability*
279 To determine whether salivary PRP production would be inducible by tannin
280 consumption both over time and in a dose-dependent manner, salivary proteins were divided into
281 type by retention times (41, 47-49), peak mAu were recorded for each, and protein subtypes were
282 aggregated to quantify total salivary proteins and PRPs. Salivary protein subtypes were further
283 analyzed by proportion to total mAu from the equation:
284

$$PRP\ subtype\ proportion = PRP\ type\ (area\ sum) \div Total\ PRP\ area$$

285
286 Or

$$Total\ PRPs = Total\ PRP\ area\ \div Total\ salivary\ protein\ area$$

287

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288 Differences in salivary protein production from Week 0 to 4 within doses were analyzed
289 by multiple factor analysis of variance. To determine whether PRP production would impact iron
290 bioavailability with tannin consumption, Pearson's product-moment correlations were used to
291 determine correlations between % max iron absorption, iAUC for serum iron, randomization
292 order, and PRP types.
293
294 *Astringency perception, connections to salivary protein production, and iron bioavailability*
295 We determined whether astringency perception was changed within or between tannin
296 doses using Chi-square testing and Fisher's exact tests by allocated, and previous dose.
297 Connections between salivary protein production, iron bioavailability and astringency were
298 analyzed by Pearson's product-moment correlations.
299
300 **Results**
301
302 ***Week 0 demographics***
303 Mean participant age was 26 ± 1.2 yrs, and ranged from 20-35. All participants were
304 occasional (2-3 drinks/month) or moderate (2-3 drinks/week) alcohol consumers. Aside from one
305 participant, who consumed a vegan diet, and took vitamin B_{12} supplements, no participants took
306 vitamin or mineral supplements during the study period. The average BMI of participants was 24
307 ± 2.4 kg/m^2 (range 18.2-28.9). Participant weights (kg) did not significantly change between
308 tannin doses nor from Week 0 to Week 4 of each supplementation period.
309
310 ***Supplementation order and outcomes measures***

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311 With our randomization procedure, six of the eleven participants were randomized to 1.5
312 g tannin doses during the first supplementation period, and 0.03 g doses during the second
313 supplementation period. Incremental AUC for serum iron ($p = 0.118$), hemoglobin ($p = 0.87$),
314 and ferritin ($p = 0.15$) were not different by order of tannin dose in any supplementation period.
315 Supplementation order did significantly positively impact % max iron absorption following the
316 1.5 g tannin dose when taken in the third versus the first supplementation order ($p = 0.046$),
317 meaning that lower doses taken before the 1.5 g dose led to significantly improved iron
318 bioavailability during that intervention period. There were no significant differences in Week 0
319 to Week 4 dose-responses for hemoglobin, ferritin, serum iron iAUC, or % max iron absorption
320 when accounting for previous dose by Chi-square testing (results shown in Supplementary
321 Material). There were no significant changes in hemoglobin ($p = 0.993$), ferritin ($p = 0.982$),
322 iAUC for serum iron ($p = 0.984$), or % max iron absorption ($p = 0.998$) at each Week 0-time
323 point, and previous tannin dose did not affect outcomes changes during washout for hemoglobin
324 ($p = 0.68$), ferritin ($p = 0.511$), % max iron absorption ($p = 0.735$), or iAUC for serum iron ($p =$
325 0.137). No salivary protein measurements were significantly correlated with tannin dose order
326 (ps > 0.62).
327
328 ***Study dietary intake***
329 There was wide variability in nutrient consumption during supplementation periods, but
330 there were no significant differences in total calorie, macronutrient, meat, fiber, or micronutrient
331 consumption between tannin doses (**Table 1**). Despite wide variability in nutrient consumption,
332 individual macronutrient and micronutrient intake were not different between tannin doses. Iron
333 intake was 7-18% less than the recommended dietary allowance (RDA) of 18 mg in all

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334 supplementation periods, ascorbic acid exceeded the RDA by 15-80%. While not significant,
335 dietary proanthocyanidin intake (apart from supplements) trended towards lower amounts in the
336 0.03 g (69.1 ± 78.9 mg) and 0.25 g (82.3 ± 85.1 mg) doses compared to the 1.5 g dose (123.2 ±
337 136.6 mg; *p*s > 0.09). On average, 0.03 g, 0.25 g, and 1.5 g tannin supplements constituted 2, 8,
338 and 35-fold the average dietary proanthocyanidin intake for their respective supplementation
339 period.

340 ***Supplementation period iron absorption, hematological indices of iron status***

341

342 *Unadjusted regression outcomes*

343 Individual level data are included in the Supplementary Material. There were no changes
344 in unadjusted iron bioavailability (by iAUC and % max iron absorption) within, or between,
345 tannin supplementation periods (**Table 2**). In addition, there were no differences at Week 0 (p =
346 0.82) or Week 4 (p = 0.92) unadjusted serum iron iAUC or Week 0 ($p = 0.82$) or Week 4 (p =
347 0.62, Table 2) % max iron absorption between tannin doses. Hemoglobin and ferritin values
348 were not different at Week 0 or Week 4 for any tannin dose (Hb: Week 0: $p = 0.838$,; Week 4: p
349 = 0.68 and ferritin: Week 0: $p = 0.855$,; Week 4 $p = 0.575$, , Table 2). There were no significant
350 differences in hemoglobin ($p = 0.90$), ferritin ($p = 0.81$), % max iron absorption ($p = 0.39$), or
351 serum iron iAUC ($p = 1.0$) for improvement, deterioration, or maintenance by any tannin dose
352 through Chi-square testing (**Table 3**).

353

354 *Stepwise linear regression analysis and adjusted regression models*

355 To test the impact of dietary and individual physiological differences (iron status,
356 anthropometric, salivary protein) on iron bioavailability and status, we employed stepwise

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357 regression analysis to establish significant covariates to build an adjusted model for
358 hematological outcomes. Covariates that were significantly positively associated with serum iron
359 iAUC and % max iron absorption included bPRP and cystatin production (**Table 4**). Significant
360 covariates that were negatively associated with serum iron iAUC and % max iron absorption
361 included aPRP and total salivary protein production, higher rating of 0.2 mg/dL astringency
362 testing, and total meat consumption. Significant covariates positively associated with ferritin
363 levels included bPRP production, and zinc consumption.

364 Significant covariates for each outcome measure were added to the linear regression for
365 adjusted outcomes analysis. Following the full adjustment for significant covariates, neither
366 serum iron iAUC or % max iron absorption was statistically different between, or within, each
367 tannin dose (Table 4, **Figures 3, 4**). There were no significant differences in adjusted
368 hemoglobin or ferritin values within, or between, tannin supplementation periods (Table 4).
369

370 *Correlations between salivary protein production and iron absorption with tannin*
371 *supplementation*

372 There were no significant correlations between total salivary protein production and iron
373 absorption (by % max iron absorption and iAUC for serum iron) during the study. In all tannin-
374 doses, and when combining all data from 4-week supplementation periods, bPRP production was
375 significantly and positively correlated with % max iron absorption at Week 0 and Week 4 (**Table**
376 **5**). There were more positive correlations with Week 4 0.03 and 0.25 g dose-max iron absorption
377 % and bPRP production than the 1.5 g dose (Table 5), suggesting that bPRP production was
378 potentially important to enhance iron bioavailability for lower, but not higher tannin doses. Week
379 0 and Week 4 aPRP production was significantly negatively correlated with iron absorption in

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380 each supplementation period (Table 5). Total gPRP production was significantly negatively
381 correlated with iron bioavailability at Week 4 in the 1.5 g supplementation period (Table 5).
382 Statherin production was non-correlated with iron absorption, while cystatin was overall
383 significantly positively correlated with iron absorption (Table 5).
384

385 ***Astringency testing***

386

387 *Astringency ratings with tannin consumption*

388 Astringency ratings did not change among participants with changes in tannin doses, and
389 were not statistically affected by order of tannin dose in Chi-square testing (ps > 0.09), except for
390 the lowest astringency doses, which were rated significantly lower after 1.5 g tannin doses (p =
391 0.047; Table 6). There were no significant effects of tannin dose on changes in ratings of
392 astringency (ps > 0.126), however, overall ratings of astringency were lower for the 0.15 mg/dL
393 astringency dose due to supplementation with the 1.5 g tannin dose compared to the 0.03 g
394 tannin dose ($p = 0.013$).
395

396 *Astringency ratings with PRP production*

397 Cystatin and bPRP production were correlated with lower astringency sensationin all
398 alum doses. In correlations from individual participants with astringency ratings at the highest
399 alum concentration (0.2 mg/dL), there were significant, positive correlations between total
400 salivary proteins (7 of 11 participants, $r > 0.49$, $p < 0.05$) and astringency, and negative
401 relationships between bPRPs (9 of11 participants, r = -0.32 to -0.81, p = 0.001 – 0.21), cystatins
402 (9 of 11 participants, r = -0.2 to-0.76, p = 0.03 – 0.28) and astringency.

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403

404 **Discussion**

405

406 The primary objectives of this trial were to determine the effect of long term dose-
407 response condensed-tannin supplementation on iron bioavailability, and status, and to understand
408 the effect of salivary proteins on iron bioavailability during prolonged condensed tannin
409 consumption. Secondarily, the study assessed astringency as a potential marker for adaptation to
410 tannins and iron bioavailability.

411

412 ***Hematological outcomes and tannin supplementation periods***

413 Overall, our results support the hypotheses of no significant reductions in iron
414 bioavailability or status with three supplementation periods of long-term, multiple-daily tannin
415 supplements over four weeks. Despite non-significant negative trends in Week 0 iron absorption
416 with 1.5 g (highest) compared to 0.25 and 0.03 mg (lowest) doses, hemoglobin and ferritin were
417 maintained in all groups throughout supplementation periods (Table 2). There were no
418 differences in ferritin or CRP measurements within individuals, or among tannin doses
419 throughout the study (Table 2). To our knowledge, this is the first study that has quantified
420 effects of long-term, dose-response condensed tannin effects on iron bioavailability and status.
421 Our findings of no significant changes in iron bioavailability or status within or among tannin
422 doses are contrary to previous single meal studies using black tea (6, 50, 51), which contain
423 theaflavins and thearubigins (52), or in trials using tannic acid (3, 4, 51). Tannic acid and tea
424 tannins may bind to salivary proteins and chelate iron differently than condensed tannins
425 (proanthycyanidins), which are typically larger in size and consumed within a complex food

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426 matrix (53, 54). Condensed tannin models in humans and rats (55-58), also found no changes in
427 iron bioavailability or status with tannin consumption over time are consistent. In contrast, dose-
428 dependent inhibition of grape seed extract on iron bioavailability has been reported in Caco-2
429 cells (59). There have been similar discrepancies in *in vivo* and *in vitro* models cited previously.
430 Iron status in pigs consuming red (higher tannins) and white beans (lower tannins) resulted in no
431 difference in iron status outcomes, while the Caco-2 cell model found higher iron bioavailability
432 from white than red beans (60). Inconsistencies between long-term in vivo and *in vitro* studies
433 may be partially a result of the complexity of factors contributing to human and animal digestion,
434 including salivary proteins, which likely are not accounted for in simulated digestion. In
435 addition, the single-meal digestion simulation used in Caco-2 cells might have the same
436 limitations as short-term bioavailability studies. The discrepancies between long-
437 termconsumption studies compared to short-term bioavailability studies and Caco-2 findings
438 may suggest that caution needs to be exercised when using the evidence from the latter types of
439 research to predict chronic consumption *in vivo* iron outcomes.
440 The current study is the first that we know of that has quantified the effects of multiple daily,
441 multi-dose condensed proanthocyanidins on iron bioavailability or status. A similar study found
442 that 4-week tea supplementation, similar to this study's 0.25 g supplement dose, resulted in
443 significantly lower ferritin levels in non-anemic and anemic women (31), suggesting that there
444 may be differences in the impact of tannin-type on iron status. Interestingly, in a study observing
445 effects from green leafy vegetables on hemoglobin, significant improvements were seen after
446 only three weeks (61). While supplementation levels in the present study at 1.5 g were 50 x
447 greater than 0.03 g and 10 x greater than 0.25 g tannin doses, iron absorption was only modestly
448 reduced in the 1.5 g supplementation period (equivalent to consuming 100g of high tannin

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449 sorghum three times daily), and there were no significant reductions in iron status over time.
450 There were no changes in iron absorption, ferritin, or hemoglobin over time in any adjusted or
451 unadjusted models, suggesting that condensed tannin intake at any dose did not affect iron
452 absorption. While other studies have noted reduction in iron bioavailability with condensed
453 tannin intake (4, 62), the current study is the first that we know of to isolate supplementation of
454 proanthocyanidins outside of other antinutritional factors commonly consumed concurrently *in*
455 *vivo*.
456
457 ***PRP production and iron bioavailability***
458 The current study is the first, to the best of our knowledge, that has assessed correlations
459 between salivary protein production and iron bioavailability, or investigated long-term tannin
460 supplementation effects on salivary profiles in humans. Overall, our hypotheses that salivary
461 PRP production would be inducible in higher compared to lower tannin doses, and that PRP
462 production would impact iron bioavailability with tannin consumption were partially supported.
463 There were not significant changes in PRP or salivary protein production within or among tannin
464 doses, however, there were significant correlations between PRPs, non-PRP salivary proteins,
465 and iron bioavailability, suggesting that participants producing higher quantities of total salivary
466 proteins, bPRPs, and cystatins improved iron absorption with condensed tannin intake.
467 Correlations between bPRPs, cystatins, and iron absorption tended to be stronger at Week 4 in
468 lower doses, suggesting that salivary protein subtypes may change with regular tannin
469 consumption to improve iron bioavailability, but are not likely the only physiological adaptation
470 when consuming higher tannin doses. In caco-2 cells, bPRPs have been found to inhibit uptake
471 of small tannin molecules through formation of insoluble complexes, but this process was

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472 mediated in part by sodium-glucose transporter-1 (SGLT-1) and multidrug resistance protein
473 (MRP2) (63). It may be that bPRPs signal changes in these receptors that mediate tannin
474 absorption and iron related sequelae.

475 Binding of bPRP to polyphenols may be preferential versus other PRP subtypes (64), and
476 production of larger bPRPs that would efficiently bind to tannins are most likely genetically
477 determined (64-66). This idea may help to explain the wide variability in iron absorption among
478 participants and age-related changes in iron absorption with tannin consumption. For example, in
479 pre-term infants, salivary protein profiles vary widely from adults (49), and bPRPs are almost
480 non-existent, which may affect tolerance of the former population to tannins.

481 Contrary to findings that bPRPs supported iron bioavailability with tannin consumption,
482 gPRP and aPRP production, especially at Week 0 for each tannin dose, were significantly
483 negatively correlated with iron bioavailability. Negative impacts of these PRP subtypes on iron
484 bioavailability could mean that individuals producing higher levels of aPRP or gPRP proteins
485 less efficiently absorb iron, especially when initially exposed to increased concentrations of
486 tannins and until other homeostatic protective mechanisms are employed. This is the first time, to
487 the best of our knowledge, that aPRP and gPRP interactions with tannins over time have been
488 determined in vivo. It may be that aPRP and gPRP are upregulated with tannin consumption, but
489 do not bind to condensed tannins effectively, thereby increasing protein-iron chelation. Further,
490 aPRP and gPRP may be effectively inhibited by carbohydrate consumption (67) compared to
491 bPRP, meaning that individuals producing more of these proteins may less effectively prevent
492 tannin-iron chelation.

493

494 ***Astringency as a predictor of iron bioavailability with tannin consumption***

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495

496 Our secondary hypotheses that: 1) astringency perception would be changed with tannin
497 consumption over time, 2) salivary proline-rich protein production would predict astringency,
498 and 3) astringency could be used as a surrogate marker for iron bioavailability, based on PRP
499 expression with consumption were partially supported by our findings. Astringency ratings did
500 not change within or among tannin doses throughout the study, except for the highest (1.5g)
501 tannin dose. Astringency ratings were lower with higher tannin concentrations, and were also
502 significantly negatively correlated with bPRP and cystatin production, suggesting that reductions
503 in ratings of very astringent, or bitter foods, may help predict iron bioavailability with tannin
504 exposure. Despite this, we did not find consistent associations between iron bioavailability and
505 astringency ratings within or between supplementation periods.

506

507 **Limitations**

508 There are several important limitations that must be considered when interpreting results
509 from the current study. It must be acknowledged that tannin-supplementation limits the
510 generalizability of these findings to tannins within foods, which commonly co-exist with other
511 antinutritional factors, such as phytic acid. Tannin-rich food commodities may also confer
512 different effects with antinutritional-food matrix interactions. Beyond tannin supplement
513 limitations themselves, several factors, including above RDA ascorbic acid intake, and challenge
514 meal ascorbic acid supplementation may have inhibited tannin effects on iron bioavailability as
515 seen previously (62), although iron bioavailability has been inhibited with similar doses of
516 ascorbic acid in test meals elsewhere (16). In addition, the population assessed in this study
517 consisted of non-anemic, pre-menopausal adult women with a sufficient and varied diet. Given

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518 that women were non-anemic, and Week 0 ferritin stores ranged from 7-100 ng/mL, it is possible
519 that 4 weeks may not have been long enough for multimeal supplementation to impact iron
520 status, however antinutritional factors have been shown to change serum ferritin and
521 hemoglobin in as little as 2-4 weeks (31, 68-71). Iron bioavailability in our study was less than
522 the 9% suggested previously for the model employed here (40), and although the present study
523 findings are consistent with many studies testing effects of antinutritional factors (3-8, 16, 40),
524 low iron bioavailability may have impacted the sensitivity of iron absorption curves between
525 supplements (40). Similarly, variability in iron absorption limited the power of our sample size,
526 and may have diminished the small impact of tannins on iron absorption observed here. Despite
527 this, it is important to consider the lack of concentration-dependent effect from tannins on
528 *individual* study participants that had limited variability in iron absorption throughout the
529 supplementation periods. Individual results from crossover design support our findings overall
530 (Supplemental materials). It may be problematic to generalize these findings to a clinical
531 population, for example, anemic women and children, who may have a different response to
532 tannin exposure.

533 Participants noted that they experienced increased salivary flow rates during 1.5 g
534 compared to 0.03 tannin doses, although flow rates were not measured quantitatively. It is also
535 important to note that while concentrations of PRPs themselves did not change through the
536 study, subjective experiences of salivary flow rates among participants was greater at Week 4 in
537 higher, 0.25 and 1.5 g, supplementation periods than at Week 0. Previous research findings have
538 indicated that salivary flow along with PRP concentration have given more accurate estimates of
539 total production than concentration alone (72). Salivary flow rate has been found to be an
540 independent factor in reducing ratings of astringency along with salivary protein concentration

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541 (73) suggesting that in our study, total PRP production may have been increased with increasing
542 salivary flow rates. Lack of measurement of salivary flow rate is a limitation in PRP-iron
543 outcomes analysis because we were not able to assimilate total PRP quantification from a pre-
544 determined 2 ml salivary sample (which was obtained over varying time spans). Lastly, we
545 grouped salivary types based on elution times. This has been previously employed (47), but is
546 not an accurate representation of salivary protein quantification.
547

548 **Future directions**

549 Foremost, better characterization of proanthocyanidin-phytic acid interactions on iron
550 bioavailability and salivary protein production needs to be explored, including the effects of
551 mixed antinutritional factor outcomes regarding iron bioavailability over time. Mixed diets have
552 conferred different findings in the past (74) than those presented in the current research study,
553 and understanding nutrient interactions may be key to understanding these discrepancies. In
554 addition, effects of tannins in anemic populations, who may have disease burden or dietary
555 deficiencies, need to be explored.

556 Due to the complexity of PRP subtypes, determining which specific bPRP and cystatins
557 improve iron bioavailability with tannin challenge may enable diet-specification in both children
558 and adults (64). Determination of PRP genetic makeup in anemic and non-anemic tannin
559 consumers may help to determine those with tannin-binding subtypes, and protein production
560 could later be determined based on findings. More studies are needed to determine the effects of
561 tannin supplementation on iron bioavailability in infants, and the effects of different tannin types
562 (tannic acid, theaflavins and thearubigins) on salivary proteins. Further comparison of oral and

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563 enteric tannin exposure may help to determine non-salivary determinants of physiological tannin

564 mediation.

565

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566 **Conclusions**

567 Long-term condensed tannin supplementation did not impair iron bioavailability, ferritin,
568 or hemoglobin levels in non-anemic, premenopausal women. Iron absorption following tannin
569 supplementation was positively correlated with bPRP and cystatin production, and tannin
570 supplementation was associated with significantly reduced ratings of astringency over time.
571 These findings suggest that individual physiology may need to be accounted for when
572 considering nutritional impact on iron bioavailability and status. Given the lack of impact of
573 condensed tannins on iron status over time, these results suggest that efforts to remove
574 condensed tannins from the diet to increase iron bioavailability and status may need to be
575 reconsidered.

576

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581 throughout the study, and edited the manuscript. KK analyzed salivary proteins on HPLC and
582 edited the manuscript. SR conceived the experiment and edited the manuscript, MH conceived
583 and edited the manuscript. BL conceived and oversaw the experiment, analyzed data, and edited
584 the manuscript.

585

586

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Tables

Table 1: Dietary intake of calories, macronutrients, micronutrients, and proanthocyanidins by supplementation period

Supplementation period	0.03 g	0.25 g	1.5 g
	Mean ± SD	Mean ± SD	Mean ± SD
N	11	11	11
Kcal/day	2186.2 ± 570.9	2230.5 ± 640.6	1957.8 ± 348
Protein (g/day)	80.8 ± 27.2	79.7 ± 21.6	71.6 ± 16.6
Fat (g/day)	90.3 ± 30	93.5 ± 27.9	71.1 ± 19.5
Carbohydrates (g/day)	259.3 ± 96.2	268.4 ± 109.9	252.1 ± 126.2
Meat (oz/day)	3.61 ± 2.5	3.81 ± 2.6	3.6 ± 1.9
Sugar (g/day)	122.8 ± 57.9	127.7 ± 69.3	120.0 ± 99.8
Fiber (g/day)	21.2 ± 12	19.2 ± 10	21.6 ± 14.3
Iron (mg/day)	15.1 ± 6.6	15.7 ± 7.2	14.7 ± 6.2
Ascorbic acid (mg/day)	109.7 ± 87.9	80.9 ± 66.9	110.4 ± 142.8
Zinc (mg/day)	12.6 ± 5	12.8 ± 5.6	10.2 ± 2.5
Copper (mg/day)	1.5 ± 0.69	1.5 ± 0.91	1.4 ± 0.61
Monomers (mg/day)	8.0 ± 7.5	18.2 ± 23.4	16.1 ± 21.8
Dimers (mg/day)	8.6 ± 6.6	13.5 ± 15.7	14.8 ± 17.3
Trimers (mg/day)	5.3 ± 4.5	6.7 ± 8.0	8.9 ± 12.5
4-6 mers (mg/day)	15 ± 16.3	16.6 ± 19.4	27.5 ± 36.3
7-10 mers (mg/day)	9.6 ± 12.7	9.0 ± 9.8	15.7 ± 18.7
Polymers (mg/day)	22.7 ± 39.5	18.4 ± 25.7	40.3 ± 51.3

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Total proanthocyanidin intake (mg/day)	69.1 ± 78.9	82.3 ± 85.1	123.2 ± 136.6
Total polyphenol intake (mg/day)	1106.6 ± 531.1	1139.6 ± 647.3	1108.9 ± 590

SD: standard deviation,

No significant differences ($p > 0.05$)

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Table 2: Unadjusted iron bioavailability, status, and inflammatory markers at Week 0 and Week 4 of each supplementation period

	0.03 g Week 0	0.03 g Week 4	0.25 g Week 0	0.25 g Week 4	1.5 g Week 0	1.5 g Week 4
	Mean 95% CI	Mean 95% CI	Mean 95% CI	Mean 95% CI	Mean 95% CI	Mean 95% CI
% Max iron absorption	12.7 (7.5, 17.9)	10.7 (5.4, 15.9)	12.1 (6.9, 17.3)	12.4 (7.2, 17.6)	11.2 (6.0, 16.5)	10.3 (5.0, 15.5)
iAUC for serum iron (µg/dL*hr)	2155 (612, 3696)	2269 (727, 3810)	2461 (919, 4003)	2769 (1228, 4311)	2237 (696, 3779)	2277 (735, 3819)
Hemoglobin (g/dL)	13.2 (13.0, 13.4)	13.3 (13.1, 13.5)	13.3 (13.1, 13.5)	13.4 (13.2, 13.6)	13.4 (13.1, 13.5)	13.3 (13.2, 13.6)
Ferritin (ng/ml)	35.4 (28.4, 42.4)	42.3 (35.3, 49.3)	35.8 (28.8, 42.8)	37.3 (30.3, 44.3)	40.0 (33.0, 47.0)	44.5 (37.5, 51.5)
CRP (mg/dL)	0.2 (0.0, 0.5)	0.3 (0.0, 0.5)	0.3 (0.1, 0.5)	0.2 (0.0, 0.4)	0.2 (0, 0.4)	0.3 (0.1, 0.6)

CI: confidence interval, iAUC: incremental area under the curve; CRP: C-reactive protein.

No significant differences ($p > 0.05$).

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Table 3: Comparison of improvement, maintenance, or deterioration of iron bioavailability and status within each supplementation period

	Supplementation period	Improvement	Maintenance	Deterioration	Fisher's exact (*p*)
% max iron absorption	0.03 g	5	0	6	0.394
	0.25 g	8	0	3	
	1.5 g	5	0	6	
	Total	18	0	15	
iAUC serum iron µg/dL*hr	0.03 g	6	0	5	1.0
	0.25 g	6	0	5	
	1.5 g	6	0	5	
	Total	18	0	15	
Hemoglobin (g/dL)	0.03 g	6	2	3	0.896
	0.25 g	5	2	4	
	1.5 g	4	4	3	
	Total	15	8	10	
Ferritin (ng/dL)	0.03 g	7	1	3	0.816
	0.25 g	5	1	5	
	1.5 g	6	0	5	
	Total	18	2	13	

iAUC: incremental area under the curve

No significant differences: $p < 0.05$

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Table 4: Estimation of iron bioavailability and status due to supplementation period, time, and significant covariates

		B	SE *B*	*β*	t	*p*
% max iron absorption	Model				6.92	<0.0001
	Constant	48.9	22.7	0	2.16	0.004
	Supplementation period	-0.36	1.5	-0.023	0.81	0.82
	Week 0	1.3	1.9	0.07	0.70	0.49
	Hemoglobin	-4.2	1.7	-0.20	-1.54	0.034
	Ferritin	-0.06	0.03	-0.23	-2.00	0.023
	CRP	-26.1	3.7	-0.74	-5.91	<0.0001
	bPRP	10.22	3.02	0.25	2.00	0.023
	aPRP	-16.9	6.5	-0.21	1.86	0.012
	Cystatin	0.0016	0.0004	0.06	2.51	0.0008
iAUC serum iron µg/dL*hr	Model				9.81	<0.0001
	Constant	10281	1869.2	0	5.5	
	Supplementation period	65.94	299.6	0.022	0.22	0.83
	Week 0	-210.6	379.4	-0.05	-0.56	0.58
	CRP	-2091.3	514.5	-0.40	-4.06	0.0002
	bPRP	0.0042	0.001	0.31	3.12	0.003
	Total salivary Protein	-6606	1731	-0.38	-3.82	0.0004
	Meat	-185.7	78.3	-0.23	-2.37	0.022
	0.2 astringency	-237.3	238.4	-0.31	-2.83	0.0066
Hemoglobin (g/dL)	Model				5.54	<0.0001
	Constant	12.4	0.27	0	41.55	<0.0001

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	Supplementation period	0.17	0.11	0.14	1.55	0.126
	Week 0	-0.05	0.13	-0.03	-5.12	<0.0001
	CRP	-0.97	0.19	-0.47	4.14	0.0005
	Ferritin	0.007	0.001	0.36	3.32	0.0001
	Fat	0.008	0.002	0.32	-3.82	0.002
Ferritin (ng/dL)	Model				6.47	<0.0001
	Constant	-213.7	69.5	0	-3.33	0.0016
	Supplementation period	-5.44	5.64	-0.11	-0.97	0.83
	Week 0	-0.54	7.06	-0.01	-0.08	0.58
	Hemoglobin	22.95	4.49	0.50	4.60	0.0002
	bPRP	38.76	14.4	0.29	2.69	0.003
	%max iron	-0.70	0.40	-0.19	-1.72	0.0004
	Zinc	-2.24	0.85	-0.32	-2.63	0.022

SE: standard error; CRP: C-reactive protein; bPRP: basic proline rich protein; aPRP: acidic proline rich protein; iAUC: area under the curve; Zn: zinc

Significance: $p < 0.05$

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Table 5: Correlations between % max iron absorption and salivary proteins at Week 0 and Week 4 of each supplementation period

Dose	Week 0/ Week 4	*N*	bPRP R (*p*)	aPRP R (*p*)	gPRP R (*p*)	Statherin R (*p*)	Cystatin R (*p*)	Total R (*p*)
0.03 g	Week 0	11	0.218 (0.518)	-0.18 (0.596)	-0.131 (0.70)	-0.241 (0.475)	0.290 (0.387)	-0.078 (0.82)
	Week 4		0.605* (0.049)	-0.198 (0.56)	0.184 (0.587)	-0.055 (0.872)	-0.007 (0.985)	0.09 (0.793)
0.25 g	Week 0	11	0.25 (0.46)	-0.645* (0.03)	-0.204 (0.547)	-0.245 (0.469)	0.326 (0.328)	0.047 (0.892)
	Week 4		0.489 (0.07)	0.057 (0.876)	0.111 (0.76)	0.112 (0.757)	0.138 (0.704)	0.158 (0.66)
1.5 g	Week 0	11	0.297 (0.438)	0.075 (0.861)	0.391 (0.298)	0.01 (0.80)	0.201 (0.60)	0.46 (0.182)
	Week 4		0.173 (0.611)	-0.483 (0.133)	-0.595* (0.05)	0.10 (0.767)	0.114 (0.739)	-0.076 (0.825)
Total			0.366* (0.003)	-0.20* (0.028)	-0.23 (0.06)	0.07 (0.57)	0.27* (0.03)	0.20 (0.11)

basic proline-rich protein: bPRP, acidic proline-rich protein: aPRP, glycosylated proline-rich protein (gPRP); $*p < 0.05$

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Table 6: Mean astringency ratings, and changes from Week 0 to Week 4 of supplementation periods

Dose*	0.03 g Week 0	0.03 g Week 4	0.25 g Week 0	0.25 g Week 4	1.5 g Week 0	1.5 g Week 4	Fisher's exact Week 0 to 4
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	p
0.03 mg/dL	1.4 (1.1, 1.6)	1.1 (0.9, 1.3)	1.2 (0.9, 1.4)	1.3 (1.0, 1.5)	1.2 (0.9, 1.4)	1* (0.8, 1.2)	0.31
0.07 mg/dL	2.2 (1.8, 2.5)	2.3 (1.9, 2.6)	2 (1.7, 2.4)	1.9 (1.6, 2.3)	2.1 (1.7, 2.4)	1.8* (1.5, 2.2)	0.50
0.15 mg/dL	3.3 (2.8, 3.7)	3.4 (2.9, 3.8)	3.2 (2.7, 3.6)	3.5 (3, 3.9)	2.4 (1.9, 2.8)	3.3+ (2.8, 3.7)	0.126
0.25 mg/dL	4.2 (3.7, 4.7)	4.4 (3.9, 4.8)	4.3 (3.8, 4.7)	4.5 (4, 5)	3.3 (2.9, 3.8)	4.4 (3.9, 4.8)	0.55

CI: confidence interval

*$p < 0.05$ Week 4 vs. Week 0. + $p < 0.05$, 0.03 g vs. 1.5 g supplementation period.

Scale 1 = not astringent, 5 = extremely astringent

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Figure Legends:

Figure 1: Enrollment allocation.

Forty-eight potential participants responded to the study call and were screened; the study was conducted on a rolling basis. Twelve participants were initially enrolled, and during study duration, two participants dropped out due to relocation, one due to time commitment in the study, another due to intolerance to blood draws, and one due to supplement intolerance. These participants all dropped out after supplementation period I of the study was completed, and another four participants were recruited on a rolling basis from the initially screened pool of 48.

Figure 2: Supplementation periods (A) and supplementation period activities (B).

The study consisted of three supplementation periods for each participant, a high (1.5 g), medium (0.25 g), or low (0.03 g) condensed tannin supplement was provided for four weeks. Two to three-week washout periods between supplementation periods aimed to stabilize salivary protein and iron biomarkers. Supplementation periods consisted of Week 0 and Week 4 meal challenges, salivary collection, and astringency testing. At midpoint of each supplementation period, there were 3 24- hour dietary recalls (2 weekday, and 1 weekend day) collected from each participant.

Figure 3: Mean adjusted and unadjusted individual level iron absorption at Week 0 and Week 4 of each supplementation period.

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Adjusted for hemoglobin, ferritin, C-reactive protein, basic proline-rich proteins, and acidic proline-rich proteins, (red) and unadjusted (black). There were no significant differences (*p*s > 0.05) in iron absorption at any dose of condensed tannin before or after supplementation periods.

Figure 4: Individual level incremental area under the curve for serum iron at Week 0 and Week 4 of each supplementation period.

Mean regression-adjusted for C-reactive protein, basic proline-rich protein, total salivary protein, meat consumption, and rating of highest level of astringency: red; and unadjusted: black. There were no significant differences (*p*s > 0.05) in iron absorption within tannin supplementation periods.

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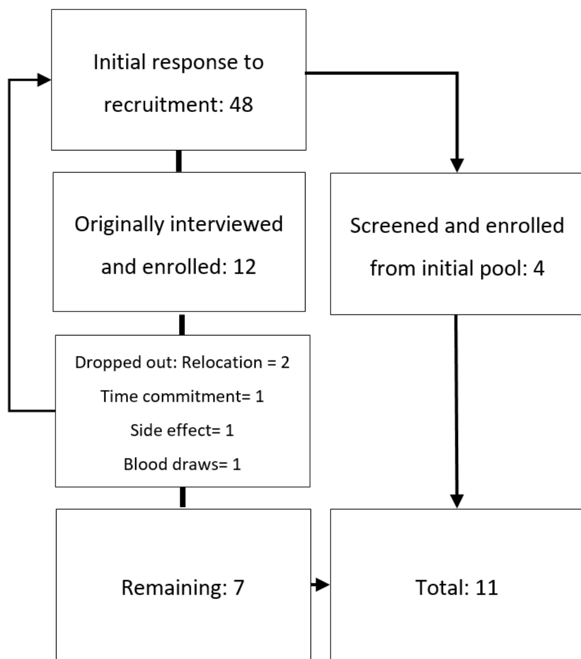

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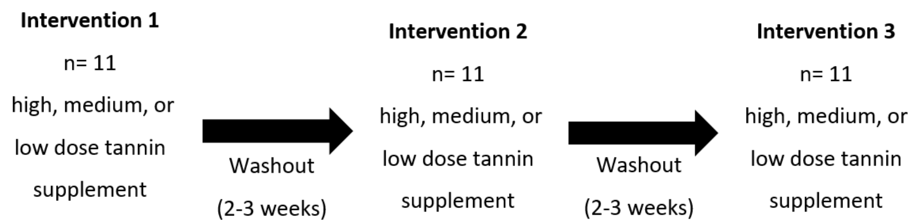

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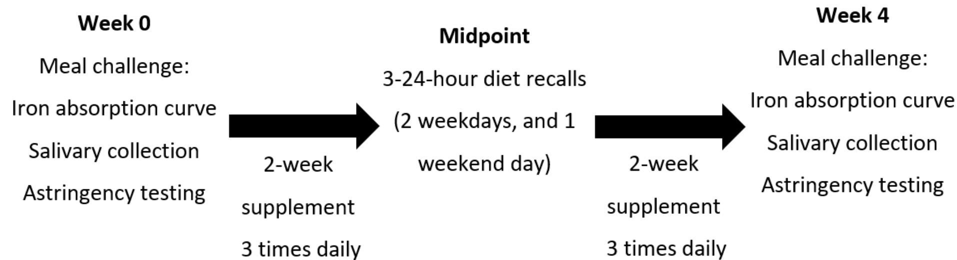

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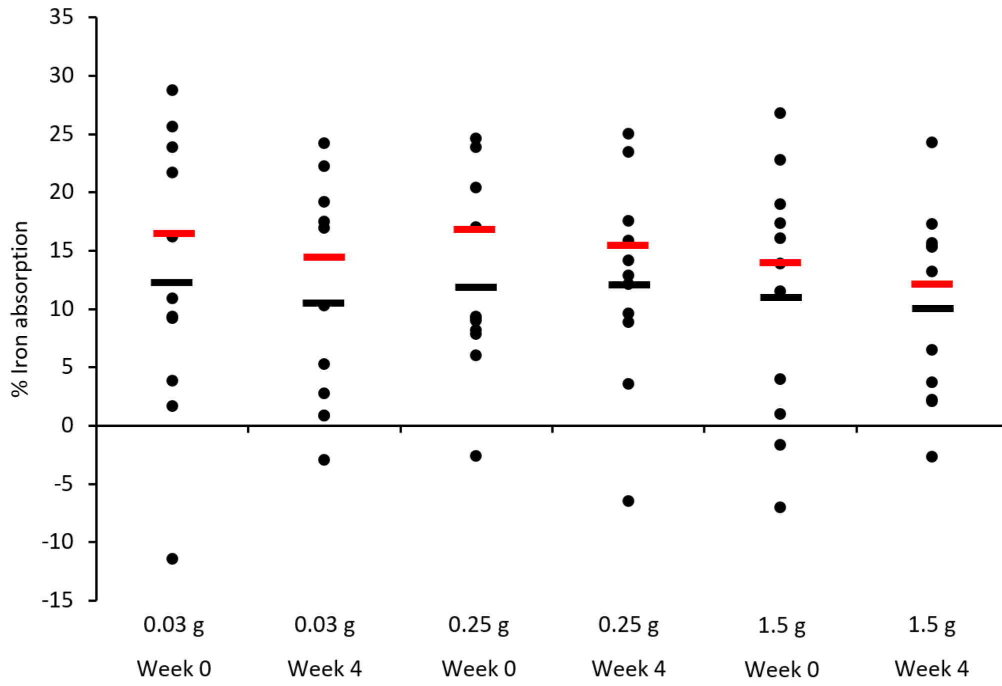

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