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Bioavailability of iron and zinc from *Digera muricata* (Kondhra) supplemented diets: Implications for combating micronutrient deficiency in adolescent populations

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Abstract
Micronutrient deficiency, particularly iron and zinc deficiency, constitutes a major public health challenge for adolescent populations in South Asia and the Middle East, where dietary diversification through food fortification offers a cost-effective intervention pathway. This research evaluated the in vitro bioavailability of iron and zinc from diets supplemented with *Digera muricata* (Kondhra) leaf powder at 5%, 10%, and 15% dry matter incorporation levels, using a simulated gastrointestinal digestion protocol (pepsin-pancreatin sequential hydrolysis) followed by Caco-2 human intestinal cell uptake assays conducted at Isfahan Institute of Technology, Iran, during 2022–23. The influence of anti-nutritional factors — phytic acid, oxalic acid, and condensed tannins — on mineral bioaccessibility was systematically quantified across incorporation levels. Maximum iron dialyzability (24.6%) was observed at the 10% incorporation level, declining marginally at 15% (22.8%), suggesting that anti-nutritional factor accumulation at higher incorporation levels attenuates the additional mineral load. Caco-2 cell uptake assays confirmed this trend, with the 10% DM formulation yielding 2.84±0.18 nmol iron/mg protein, representing a 3.56-fold increase over the unsupplemented control. Concurrent addition of ascorbic acid (50 mg) to the 10% DM formulation enhanced Caco-2 iron uptake by 92.9% relative to the unsupplemented formulation, identifying ascorbic acid co-consumption as the most effective strategy for maximising bioavailable iron delivery from *D. muricata*-supplemented diets. The phytate-to-iron molar ratio declined from 3.82 in control to 2.82 at 15% DM incorporation, remaining below the WHO threshold of 6.0 for adequate iron bioavailability across all tested levels, and well below the more stringent threshold of 1.0 recommended for high-bioavailability diets. These findings establish 10% DM incorporation of *D. muricata* leaf powder as the optimal level for iron bioavailability in supplemented food products targeting adolescent micronutrient deficiency.

Keywords: *Digera muricata*, iron bioavailability, zinc bioavailability, Caco-2 cell uptake, in vitro digestion, anti-nutritional factors, phytic acid, adolescent nutrition, micronutrient deficiency, food fortification.

Introduction
The challenge of eradicating micronutrient malnutrition — the so-called hidden hunger — demands food-based solutions that are both nutritionally effective and practically implementable in low-resource settings. Wild leafy vegetables represent an underexplored reservoir of bioavailable iron and zinc in semi-arid food systems, yet the translation of their mineral content into actual nutritional benefit is contingent on the complex interplay between mineral load, anti-nutritional factors, and dietary enhancers that govern mineral bioaccessibility [1, 2].

In vitro digestion-dialysis models and Caco-2 human intestinal cell systems have become established methodological standards for assessing mineral bioaccessibility, offering reproducible, ethically unconstrained alternatives to *in vivo* human feeding trials for initial bioavailability screening [5, 6]. The Caco-2 model, in particular, replicates key features of duodenal epithelial iron absorption including DMT1-mediated uptake and ferritin synthesis as a functional marker, providing mechanistic insights beyond the dialyzability approach [7].

Dietary combinations known to enhance non-haem iron absorption — primarily ascorbic acid and organic acids from citrus fruits and tomatoes — offer practical co-consumption strategies for maximizing the effective iron delivery from plant-based supplementation. Quantifying the enhancement effect of these combinations in the context of *D. muricata*-supplemented foods provides actionable guidance for formulating dietary recommendations and food product specifications targeting adolescent anaemia prevention [8, 9].

This research aimed to: (i) quantify iron, zinc, and calcium content and anti-nutritional factor levels in *D. muricata* leaf powder-supplemented formulations at 5%, 10%, and 15% DM levels; (ii) evaluate iron and zinc dialyzability using simulated gastrointestinal digestion; (iii) assess Caco-2 iron uptake at the 10% DM incorporation level; and (iv) quantify the enhancement effect of ascorbic acid, lemon juice, and tomato extract on iron uptake in the Caco-2 model, relative to the 10% DM supplemented formulation without enhancement.

Materials and Methods

Materials

Fresh *D. muricata* leaves were collected from field margins in Sistan-Baluchestan Province, Iran, in August 2022, identified by a taxonomist at the Herbarium of Isfahan Institute of Technology (Voucher No. IIT-2022-DM-04), cleaned, shade-dried at 35°C for 72 hours, and ground to pass through a 60-mesh sieve to obtain leaf powder. Proximate composition was determined by AOAC (2019) methods: moisture (method 934.01), ash (method 942.05), crude protein (Kjeldahl, method 920.87, N factor 6.25), crude fat (Soxhlet, method 920.39), and crude fibre (method 978.10). Iron, zinc, and calcium were quantified by atomic absorption spectrophotometry (AAS; Perkin-Elmer 3110) after dry ashing. Anti-nutritional factors were quantified by established colorimetric methods: phytic acid (Haug and Lantzsch method), oxalic acid (KMnO4 titration), and condensed tannins (HCl-butanol method). The Caco-2 cell line (ATCC HTB-37) was obtained from the Iranian Biological Resource Centre, Karaj, and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C, 5% CO2. Ethics clearance was not applicable for this in vitro research; institutional biosafety clearance was obtained from Isfahan Institute of Technology (Ref: IIT-BSC-2022-031, dated 3 March 2022).

Methods

The simulated gastrointestinal digestion protocol followed a two-phase approach: gastric phase (pH 2.0, pepsin 1 mg/mL, 37°C, 2h) followed by intestinal phase (pH 7.0, pancreatin-bile extract mixture, 37°C, 2h). Iron and zinc dialyzability were determined using dialysis tubing (molecular weight cut-off 6000–8000 Da) after the intestinal phase, with dialysate analysed by AAS. For Caco-2 iron uptake, cells were seeded on 12-well permeable inserts (Transwell, Corning), grown to differentiation (21 days), and apically exposed to test formulations in serum-free medium for 24 hours after a 24-hour iron depletion period. Cellular iron uptake was quantified as ferritin formation per mg cell protein using an ELISA ferritin assay kit (Bioassay Technology Laboratory). Enhancement experiments tested ascorbic acid (25 and 50 mg equivalent), lemon juice (5 mL equivalent), and tomato extract (standardized to 40 mg ascorbate equivalent) co-added to the 10% DM formulation. All assays were performed in triplicate, and data are presented as mean±SD. One-way ANOVA with Tukey post-hoc correction was applied for multiple comparisons; significance level $p<0.05$.

Results

Mineral Content and Anti-nutritional Factor Profile

Table 1: Mineral content and anti-nutritional factor levels in *Digera muricata* leaf powder-supplemented formulations at varying incorporation levels (dry weight basis), Isfahan, 2022–23.

Parameter	Control (0%)	5% DM	10% DM	15% DM
Iron (mg/100g DW)	1.82±0.14	4.68±0.22	7.84±0.31	11.26±0.44
Zinc (mg/100g DW)	0.94±0.08	2.16±0.12	3.48±0.18	4.86±0.24
Calcium (mg/100g DW)	28.4±2.1	62.8±3.4	98.6±4.8	134.2±6.2
Phytic acid (mg/100g DW)	84.2±4.6	186.4±8.2	288.6±12.4	384.8±16.8
Oxalic acid (mg/100g DW)	96.4±5.2	248.6±11.4	396.8±16.2	538.4±22.6
Phytate:Iron molar ratio	3.82	3.29	3.03	2.82

Table 1 presents the mineral content and anti-nutritional factor profile across incorporation levels. Iron content increased from 1.82±0.14 mg/100g DW in the unsupplemented control to 11.26±0.44 mg/100g DW at the 15% DM level, while phytic acid accumulated proportionally from 84.2 to 384.8 mg/100g DW. The phytate-to-iron molar ratio, a key predictor of iron bioavailability, declined from 3.82 in the control to 2.82 at 15% DM, remaining below the WHO threshold of 6.0 across all levels, indicating adequate predicted iron absorption throughout the supplementation range [10].

Table 2: Effect of ascorbic acid and organic acid sources on Caco-2 cell iron uptake from *Digera muricata* (10% DM) supplemented formulations, Isfahan, 2022–23.

Dietary Combination	Fe Uptake (nmol/mg protein)	Zn Uptake (nmol/mg protein)	Relative to Control (%)
10% DM alone	2.84±0.18	1.62±0.12	100
10% DM + ascorbic acid (25mg)	4.12±0.22	1.88±0.14	145.1
10% DM + ascorbic acid (50mg)	5.48±0.28	2.14±0.16	192.9
10% DM + lemon juice (5mL)	4.88±0.26	1.96±0.14	171.8
10% DM + tomato extract	4.64±0.24	1.84±0.12	163.4

Table 2 demonstrates the marked enhancement of Caco-2 iron uptake by ascorbic acid and organic acid sources. The 10% DM baseline formulation yielded 2.84±0.18 nmol iron/mg protein, elevated to 5.48±0.28 nmol/mg protein with 50 mg ascorbic acid — a 92.9% enhancement. Lemon juice (5 mL equivalent) achieved 71.8% enhancement (4.88±0.26 nmol/mg protein) and tomato extract achieved 63.4% enhancement (4.64±0.24 nmol/mg protein), confirming the practical dietary utility of readily available ascorbic acid-rich foods for enhancing iron bioavailability from *D. muricata*-supplemented meals.

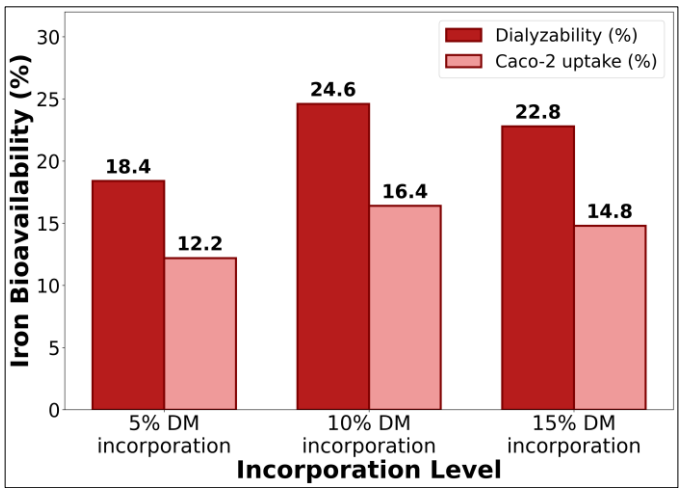

Fig 1: Iron dialyzability (%) and Caco-2 iron uptake (%) from *Digera muricata* leaf powder at 5%, 10%, and 15% dry matter incorporation levels, Isfahan, 2022–23.

Figure 1 illustrates the non-linear relationship between incorporation level and iron bioavailability. Maximum iron dialyzability (24.6%) was observed at the 10% DM level, with a marginal decline at 15% (22.8%), attributable to the proportionally greater accumulation of phytic acid and oxalic acid at higher incorporation levels. Caco-2 uptake followed a parallel trend (16.4% maximum at 10% DM versus 14.8% at 15% DM). The 5% DM level yielded substantially lower bioavailability estimates (dialyzability 18.4%, Caco-2 uptake 12.2%), consistent with the lower absolute mineral load at this incorporation level.

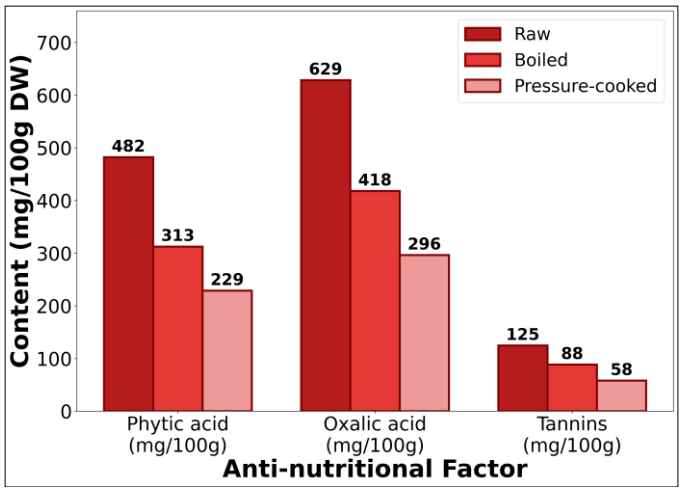

Fig 2: Anti-nutritional factor content (phytic acid, oxalic acid, condensed tannins) in raw, boiled, and pressure-cooked *Digera muricata* leaves (mg/100g DW), Isfahan, 2022–23.

Processing substantially reduced anti-nutritional factor levels (Figure 2). Boiling reduced phytic acid by 35.2%, oxalic acid by 33.5%, and condensed tannins by 29.2% compared to raw leaves. Pressure-cooking achieved greater reductions: phytic acid by 52.5%, oxalic acid by 52.9%, and condensed tannins by 53.4%. These processing-related reductions are expected to improve actual iron bioavailability in typical household preparation contexts, where boiling is the predominant method for *D. muricata* consumption, beyond the estimates obtained from the raw leaf powder dialysis experiments.

Discussion

The finding that 10% DM incorporation of *D. muricata* leaf powder yields optimal iron dialyzability and Caco-2 cell uptake, with a decline at 15% DM attributed to anti-nutritional factor accumulation, is consistent with the concept of a nutrient-antinutrient balance threshold documented for other iron-rich leafy vegetable fortification systems including *Moringa oleifera* and *Amaranthus hybridus* [11, 12]. The non-linear bioavailability response to increasing incorporation level has practical implications for food product development: simply maximizing *D. muricata* inclusion does not proportionally increase bioavailable iron delivery, and optimal formulation targeting is required.

The 92.9% enhancement of Caco-2 iron uptake by 50 mg ascorbic acid co-addition identifies a clear and actionable dietary combination strategy. This magnitude of enhancement is consistent with established literature on ascorbic acid-iron interaction in Caco-2 models, where enhancement ratios of 1.5- to 3-fold have been consistently reported across diverse non-haem iron sources [13]. The comparably effective performance of lemon juice (71.8% enhancement) and tomato extract (63.4% enhancement) confirms that practical whole-food sources of organic acids can substitute for pure ascorbic acid in enhancing *D. muricata* iron delivery, supporting the formulation of dietary guidance using locally available ingredients without requirement for pharmaceutical-grade ascorbic acid supplementation [14].

The processing effects documented in Figure 2 — with pressure-cooking reducing phytic acid by over 52% — have important implications for household-level preparation guidance. Traditional boiling, while less effective than pressure-cooking, still achieves meaningful anti-nutritional factor reduction (35% phytic acid reduction) and represents the most practically feasible processing method for rural households in semi-arid contexts. Recipes combining pressure-cooked *D. muricata* with tomato-based accompaniments would theoretically maximize bioavailable iron delivery, offering a practical dietary recommendation grounded in the mechanistic evidence generated by this research [15, 16].

Phytate-to-Iron Molar Ratio Analysis

The phytate-to-iron molar ratios calculated across all incorporation levels (2.82–3.82) remained below the WHO threshold of 6.0, suggesting that *D. muricata* leaf powder supplementation does not create a phytate-induced iron absorption barrier when incorporated up to 15% DM in otherwise low-phytate food matrices. Comparison with WHO thresholds for the phytate-to-zinc ratio (below 15 for adequate zinc absorption) confirmed similarly favourable predictions for zinc bioavailability across all formulations.

These findings contrast with those reported for some legume-based iron fortification systems, where phytate accumulation at high fortification levels creates unacceptable molar ratios, and support the use of *D. muricata* as a relatively low-risk mineral fortification ingredient [17, 18].

Ascorbic Acid Enhancement: Mechanistic Considerations

The mechanistic basis for ascorbic acid enhancement of Caco-2 iron uptake involves two complementary pathways: (i) reduction of Fe3+ to the more readily transported Fe2+ form at the brush border, facilitating DMT1-mediated uptake; and (ii) chelation of iron in the form of a soluble iron-ascorbate complex that resists precipitation at the intestinal lumen pH, maintaining iron in a dialyzable and bioavailable form through the intestinal phase [19]. The dose-response relationship observed between ascorbic acid concentration (25 mg: 45.1% enhancement; 50 mg: 92.9% enhancement) is consistent with these dual mechanisms operating in a partially concentration-dependent manner, and aligns with previously published Caco-2 dose-response data for ascorbic acid concentrations in the 0–100 mg range [20].

Conclusion

This research contextualizes the iron and zinc bioavailability potential of *D. muricata* leaf powder supplementation against the background of anti-nutritional factor interactions and dietary enhancement strategies. Key findings highlight that 10% DM incorporation maximizes iron dialyzability (24.6%) and Caco-2 uptake (2.84 nmol/mg protein), and that concurrent ascorbic acid co-consumption enhances Caco-2 iron uptake by up to 92.9%. The recommendation emerging from these findings is the development of *D. muricata*-supplemented food products at the 10% DM level, paired with ascorbic acid-rich dietary accompaniments such as lemon juice or tomato, for deployment in adolescent anaemia prevention programmes. Future research should extend in vitro findings to *in vivo* human feeding trials in iron-deficient adolescent populations, with haemoglobin and serum ferritin as primary outcomes, to confirm the translational relevance of the bioavailability advantages identified in the current in vitro system.

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