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Bioaccessibility of Heavy Metals in Raw and Processed *Alternanthera sessilis* and *Centella asiatica*: An *In Vitro* Study

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ABSTRACT

Heavy metals cause considerable hazards to food safety due to their long-term presence in the environment and their ability to accumulate within the food chain. Upon consumption, even small amounts of these metallic substances can have harmful effects on human health. The current investigation aimed to assess the *in vitro* bioaccessibility of Ni, Cd, Cr, Pb, and Cu in two commonly consumed green leafy vegetables in Sri Lanka, namely *Alternanthera sessilis* and *Centella asiatica*. Composite samples of *A. sessilis* and *C. asiatica* were randomly collected from the Western Province, Sri Lanka. The edible parts of each leafy vegetable sample were divided into three test portions of 200 g and subjected to the following treatments: Treatment 1 - raw sample, Treatment 2 - cooked sample, and Treatment 3 - stir-fried sample. The *in vitro* bioaccessibility of heavy metals in raw, cooked, and stir-fried samples was determined using a physiology-based extraction test (PBET). In contrast to overall concentrations of heavy metals in *A. sessilis* and *C. asiatica*, the bioaccessible fractions of metals were significantly lower for raw, stir-fried, and cooked samples (P<0.05). Moreover, significant differences were detected in metal concentrations between intestinal and gastric stages. The average bioaccessibility (%) of Cu was considerably greater in the intestinal stage, while Cr, Cd, Pb, and Ni were more elevated (P<0.05) in the gastric stage. Additionally, cooking and stir-frying reduced the bioaccessibility of metals compared to the raw samples.

INTRODUCTION

Ensuring food safety and security has become a global priority. Recently, there has been increasing focus on food safety, leading to extensive research into the health risks linked to consuming foods contaminated with pesticides, heavy metals, and other agrochemicals. Heavy metals, recognized as harmful environmental pollutants, are especially prevalent in areas with significant human activity. The accumulation of these metals in organisms through the food chain presents a serious threat to human health. Even in trace amounts, metals in soil, water, and air can have detrimental effects on various living organisms (Suruchi & Khanna, 2011; Tchounwou et al. 2012). Numerous studies worldwide have highlighted the ingestion of heavy metals by humans through the food chain (Islam et al. 2007; Mawari et al. 2022). The toxicity of most heavy metals arises from their ability to dissolve in water, causing harm even at low concentrations in both humans and animals. Furthermore, the body's inefficient elimination of these toxic metals exacerbates their potential for harm.

In Sri Lanka, the high consumption of green leafy vegetables (GLVs), whether raw or cooked, is driven by their rich nutritional value, affordability, and accessibility. However, studies by Rathnayaka et al. (2004), Premarathna et al. (2011), and Kananke et al. (2014-2018) have revealed elevated heavy metal concentrations in GLVs and other crops across various regions of the country. Leafy vegetables are particularly effective at absorbing heavy metals from contaminated soil, water, and atmospheric deposits. This issue is especially concerning in highly urbanized areas such as Colombo and Kalutara Districts in the Western Province, where GLV production and distribution are significant. Urban environments near roadways often experience pollution from exhaust fumes containing high levels of metals from vehicles. Additionally, rapid urbanization and increased traffic have contributed to rising concentrations of hazardous metals in these regions.

Assessing the potential health risks of consuming metal-contaminated plants is critical, and one approach to this is evaluating oral bioaccessibility. Oral bioaccessibility involves replicating the transfer of metal contaminants from plants to the human gastrointestinal system in a laboratory setting. This method enables researchers to estimate the health risks associated with consuming contaminated food crops. Bioaccessibility refers to the portion of a substance that is released in the digestive system, making it available for absorption by the intestines and entry into the bloodstream (Ma et al. 2024). Several in vitro methods, often referred to as physiologically based extraction tests (PBET) or simulated gastrointestinal extraction processes, have been developed to mimic human digestion (Intawongse & Dean, 2008; Yin et al. 2017; Ma et al. 2024). These in vitro methods are typically preferred over in vivo approaches due to their speed, cost-effectiveness, accuracy, and reduced use of experimental animals (Kulkarni et al. 2007; Intawongse & Dean, 2008; Tremlova et al. 2012; Hu et al. 2013; Omar et al. 2013; Ma et al. 2024).

This study aimed to assess the oral bioaccessibility of heavy metals in Alternanthera sessilis and Centella asiatica, the two most widely consumed green leafy vegetables in Sri Lanka, using a

simulated gastrointestinal extraction method. Previous research has reported high metal contamination in these plants, particularly those cultivated in urban areas. However, the bioaccessibility of these metals through the consumption of both fresh and processed forms of these vegetables has not yet been investigated in Sri Lanka. This gap in knowledge forms the basis of the present research.

2. MATERIALS AND METHODS

Chemicals and apparatus

The reagents used in this research were of analytical grade or higher and complied with the necessary standards. Concentrated HCl, sodium bicarbonate, acetic acid, pancreatin, pepsin, sodium malate, bile salts, lactic acid, and sodium citrate were sourced from Sigma Aldrich (St. Louis, Missouri, USA). Trace element standards for Ni, Cd, Cr, Pb, and Cu were also obtained from Sigma Aldrich (St. Louis, Missouri, USA). Certified reference material (CRM) GBW10015 – Spinach was obtained from the National Research Center for Certified Reference Materials (NRCCRM), Beijing, China.

Collection of samples

A preliminary investigation was carried out in the Colombo and Kalutara regions of the Western Province, Sri Lanka, to identify the primary cultivation areas of *A. sessilis* and *C. asiatica*. A structured questionnaire was used to collect information on cultivation practices from farmers in the selected production sites. Five locations were chosen for the collection of leafy vegetable samples: Wellampitiya, Kolonnawa, Kottawa, Piliyandala, and Bandaragama (Fig. 1).

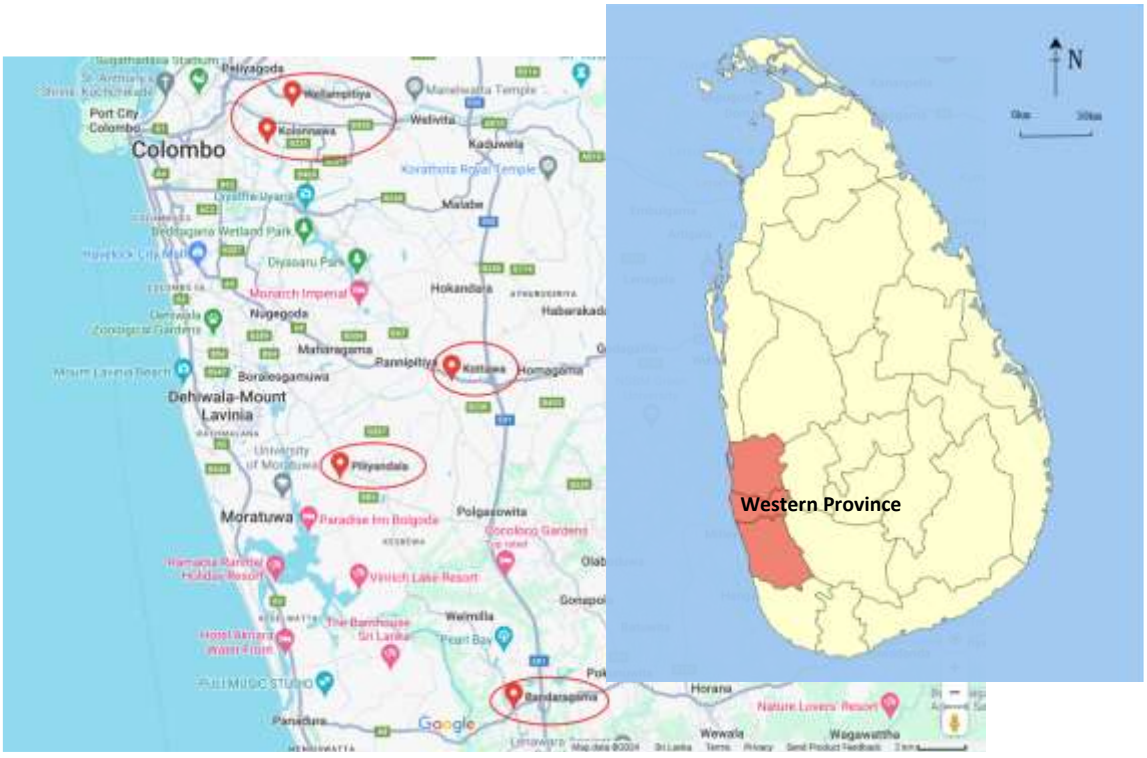

Fig. 1: *A. sessilis* and *C. asiatica* sample collection sites (Piliyandala, Kolonawa, Wellampitiya, Kottawa, Bandaragama) identified in the Western Province, Sri Lanka.

From each leafy vegetable (*A. sessilis* and *C. asiatica*), 40 random samples were collected by obtaining eight samples per each location. The samples (Fig. 2) were carefully transported to the laboratory in clean polyethylene bags. The samples were then sorted and cleaned using tap water to remove any impurities. The edible portions of each sample were divided into three test portions, each weighing 200 g. These portions underwent different treatments before analyzing the levels of toxic metals (Ni, Cd, Cu, Cr, and Pb) using the *in-vitro* extraction method. Treatment 1 involved using a fresh sample. Treatment 2 involved cooking the sample by finely cutting and mixing 200 g of cleaned and sorted plant material with 50 mL of coconut milk. Cooking was carried out on an electric hot plate set to maintain a temperature of 95 ± 2°C, monitored using a calibrated digital thermometer. Each sample was cooked for 12 minutes to ensure consistency across treatments. In Treatment 3, 200 g of finely chopped, cleaned, and sorted plant material was stir-fried with 15 mL of coconut oil in an uncovered stainless-steel pan using an electric hot plate set to maintain a temperature of 95 ± 2°C, monitored with a calibrated digital thermometer. All samples were stir-fried for 12 minutes.

Fig. 2: (a) *Alternanthera sessilis* and (b) *Centella asiatica* species collected from the cultivation areas

Assessment of *in vitro* bioaccessibility of heavy metals in plant samples using a physiology-based extraction test (PBET)

The *in vitro* bioaccessibility of the plant samples was assessed using the PBET method, as outlined by Intawongse & Dean (2008), with minor modifications.

***Gastric stage**:* Gastric solution was prepared by combining 1.25 g of pepsin, 0.42 mL of lactic acid, 0.5 g of sodium citrate, and 0.50 mL of acetic acid. Deionized water was added to achieve a final volume of 1 L in a volumetric flask. The pH of the solution was carefully adjusted to 2.5 (± 0.05) using concentrated HCl.

Subsequently, 0.3 g of raw or treated plant sample was mixed with 30 mL of gastric solution in a 250 mL beaker. The beaker was kept in a shaking water bath at 37 °C and mildly agitated at 100 rpm for one hour. After centrifugation (at 3000 rpm for 10 minutes), a 5 mL aliquot was taken and analyzed for trace elements using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

***Intestinal stage**:* To the remaining reaction mixture, 5 mL of fresh gastric solution was added, and the pH was adjusted to 7 using a saturated $NaHCO_3$ solution. The mixture was supplemented with 15 mg of pancreatin and 52.5 mg of bile salts. The sample was gently agitated (100 rpm) in a temperature-controlled water bath at 37 °C for 2 hours. After centrifugation (3000 rpm / 10 minutes), a 5 mL aliquot was taken and subjected to metal analysis using ICP-OES.

Analysis of total heavy metal concentrations in A. sessilis and C. asiatica

Each plant sample was analyzed for total metal concentrations (Ni, Cd, Cu, Cr, and Pb) using the AOAC 999.11 technique (AOAC, 2022). Oven-dried samples were subjected to dry ashing at 550 °C in a muffle furnace. The resulting ash was treated with concentrated nitric acid to convert the elements into a chemically detectable form. The solution was then filtered and appropriately diluted. Finally, the element concentrations in the solution were quantified using ICP-OES.

***Bioaccessibility (%) were calculated as follows**:*

Bioaccessibility (%) = 100 × Y/Z, where Y represents the element concentration of the bioaccessible portion (mg/100 g of sample) and Z represents the total metal concentration (mg/100 g of sample).

Validation of Analytical Method:

To validate the analytical procedure, the certified reference material (CRM) GBW10015 – Spinach was used. The accuracy and reliability of the method were assessed through quality control evaluations and figures of merit. The limit of detection (LOD) for each element was determined based on the slope of the calibration curve and three times the standard deviation (SD) of ten replicate blank measurements. The LOD values obtained for the ICP-OES were 0.025 µg/g for Ni, 0.005 µg/g for Cd, 0.010 µg/g for Cr, 0.050 µg/g for Pb, and 0.015 µg/g for Cu. For further quality assurance, total concentrations of Ni, Cd, Cr, Pb, and Cu were quantified in the GBW10015-spinach reference material. The measured concentrations of metals showed strong agreement with the certified values, with recovery rates ranging from 96% to 104%, thereby confirming the method's accuracy and robustness.

Data analysis:

The heavy metal data were analyzed using Microsoft Excel to generate descriptive statistics, including the mean, minimum, maximum, and standard deviation. Statistical analysis was performed using one-way ANOVA to determine the effect of different cooking treatments on the bioaccessibility of heavy metals in *A. sessilis* and *C. asiatica* samples. Differences among means were considered significant at $p < 0.05$, and post-hoc comparisons were carried out using Tukey's HSD test.

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3. RESULTS AND DISCUSSIONS

In vitro bioaccessibility of heavy metals in *A. sessilis* and *C. asiatica*

In recent years, *in vitro* screening techniques have been progressively refined and enhanced to evaluate the bioavailability and bioaccessibility of nutrients in foods. The bioavailability of a nutrient, which refers to the quantity that is absorbed and becomes available for physiological functions, is influenced by several factors, including digestion, nutrient release from the food matrix, absorption by intestinal cells, and transport to body tissues. In contrast, bioaccessibility is the fraction of a nutrient that, once consumed, is potentially available for absorption, determined primarily by the digestion process and the subsequent release from the food matrix (Etcheverry et al. 2012). Unlike bioaccessibility, bioavailability, which is associated with a physiological or metabolic outcome, cannot be directly quantified using *in vitro* methods. Additionally, factors that influence nutrient absorption—such as an individual's nutrient status, genotype, age, physiological state (e.g., pregnancy, lactation, or obesity), chronic or acute illnesses, gastric acid secretion, and other intrinsic factors—cannot be addressed in *in vitro* experiments (Etcheverry et al. 2012).

To evaluate bioaccessibility, an *in vitro* digestion model is utilized to mimic the human digestive system. This procedure generally consists of a two-step (or three-step) digestion process, which includes both gastric (stomach) and intestinal digestion stages. Initially, samples are acidified to pH 2 (representing the gastric pH of an adult), and pepsin is added during the gastric digestion phase. Acidifying the samples is essential, as pepsin loses its activity and denatures at pH levels exceeding 5. Before initiating intestinal digestion, the pH of the samples is adjusted to a range of 5.5–6. Next, pancreatin (a blend of pancreatic amylase, lipase, trypsin, and ribonuclease) and bile salts (emulsifiers) are introduced, and the pH is adjusted to a final range of 6.5–7. After digestion, the intestinal mixture is centrifuged to separate the supernatant from the precipitate. The soluble components in the supernatant are measured using spectrophotometry. The solubility percentage is calculated by dividing the amount of soluble component by the total amount of element in the sample (Etcheverry et al. 2012). Tables 1 and 2 present the percentage of heavy metal bioaccessibility in the gastric and intestinal phases of raw, cooked, and stir-fried samples of *A. sessilis* and *C. asiatica* collected from different regions in the Western Province, Sri Lanka. The bioaccessibility in the oral phase was excluded since no metals were detected in either plant during this phase. The data show that the overall metal concentrations in the analyzed samples were significantly higher in the Wellampitiya, Kolonnawa, and Kottawa regions compared to Piliyandala and Bandaragama. However, the proportion of heavy metals

potentially absorbed by the body was substantially lower than the total metal concentrations detected in the samples. Additionally, the concentrations of these metals in the gastric and intestinal phases varied considerably, likely influenced by pH differences, phase composition, and the intrinsic properties of the vegetables themselves. Previous studies suggest that metal extraction during each phase can be affected by numerous factors, both *in vitro* and *in vivo*, including sorption, pH, precipitation processes, food type, particle size, residence time, mixing speed, and other physiological variables (Intawongse & Dean, 2008). Since the concentration of the investigated metals in several earlier studies was below the detection limit of the *in vitro* extraction technique, bioaccessibility could not be determined (Jayawardene et al. 2010).

Alternanthera sessilis: The bioaccessibility of Ni in the raw, cooked, and stir-fried samples varied in the gastric phase from 7.9% to 16.9%, 0% to 14.9%, and 0% to 11.9%, respectively. In the intestinal phase, the bioaccessibility of Ni ranged from 1.3% to 7.6%, 0% to 5.6%, and 0% to 6%, respectively. The bioaccessibility of Cd in the gastric phase varied from 0% to 13.9% for the raw sample, 0% to 9.3% for the cooked sample, and 0% to 7% for the stir-fried sample. In the intestinal phase, Cd was only detected in the raw samples, with bioaccessibility ranging from 0% to 5.8% across different locations. For Cr, the bioaccessibility in the gastric phase ranged from 0% to 12.6%, 0% to 7.9%, and 0% to 8.8% for the raw, cooked, and stir-fried samples, respectively. In the intestinal phase, the bioaccessibility of Cr ranged from 0% to 4.1%, 0% to 5.9%, and 0% to 1.9% for the raw, cooked, and stir-fried samples, respectively. The bioaccessibility of Pb in the gastric and intestinal phases varied as follows: for the raw sample, 0% to 15.3% and 0% to 10.8%, for the cooked sample, 0% to 20.7% and 0% to 10.2%, and for the stir-fried sample, 0% to 16.2% and 0% to 3.5%, respectively. Unlike other metals, the bioaccessibility of Cu was lower in the gastric phase (0% to 20.1%, 0% to 15.2%, and 0% to 13.6%) compared to the intestinal phase (21.7% to 35.0%, 23.5% to 31.8%, and 15.0% to 28.4%) for the raw, cooked, and stir-fried samples, respectively (Table 1).

Centella asiatica: The bioaccessibility of Ni in the gastric and intestinal phases for raw, cooked, and stir-fried samples varied as follows: 0% to 11% and 0% to 4.8%, 0% to 9.8% and 0% to 4.9%, and 0% to 9.3% and 0% to 3.7%, respectively. Cd was detected only in the gastric phase of the raw samples, ranging from 0% to 11.1%. The bioaccessibility of Cr in the gastric phase ranged from 0% to 2.6%, 0% to 2.3%, and 0% to 2.2%, while in the intestinal phase, it ranged from 0% to 1.0%, 0% to 1.0%, and 0% to 0.7% for the raw, cooked, and stir-fried samples, respectively. Pb bioaccessibility in the gastric phase varied from 0% to 9.9%, 0% to 10%, and 0% to 9.4% for the raw, cooked, and stir-fried samples, respectively. In the intestinal phase, Pb bioaccessibility ranged from 0% to 2.2%, 0% to 3.1%, and 0% to 2.1%, respectively, for the raw, cooked, and stir-fried samples. Higher Cu concentrations were detected in the intestinal phase (8.1% to 39.4%, 0% to 35.8%, and 0% to 31.6%) compared to the gastric phase (0% to 11.4%, 0% to 11.5%, and 0% to 16.0%) for the raw, cooked, and stir-fried samples, respectively (Table 2).

Table 1: *In vitro* bioaccessibility of heavy metals in raw and processed *Alternanthera sessilis* at the gastric and intestinal phases.

Area	Metal	Total metal content (mg kg^{-1})	Bioaccessibility of Raw samples				Bioaccessibility of Cooked samples				Bioaccessibility of Stir-fried samples			
			Gastric Phase		Intestinal Phase		Gastric Phase		Intestinal Phase		Gastric Phase		Intestinal Phase	
			(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%
Piliyandala	Ni	3.02±1.02	0.51±0.04	16.9	0.23±0.04[d]	7.6	0.45±0.05	14.9	0.16±0.02	5.3	0.36±0.06	11.9	0.18±0.07	6.0
N=8	Cd	0.20±0.01	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	0.82±0.20	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Pb	0.24±0.04	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cu	7.85±2.21	ND	-	2.75±0.99	35	ND	-	2.50±1.00	31.8	ND	-	2.22±1.00	28.3
Wellampitiya	Ni	8.42±2.25	0.68±0.06	8.1	0.56±0.07	6.7	0.51±0.06	6.1	0.46±0.01	5.5	0.5±0.06	5.9	0.15±0.01	1.8
N=8	Cd	0.36±0.06	0.05±0.00	13.9	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	4.59±1.11	0.22±0.03	4.8	0.19±0.01	4.1	0.11±0.00	2.4	0.06±0.00	1.3	0.09±0.00	2.0	ND	-
	Pb	3.14±0.98	0.48±0.04	15.3	0.34±0.03	10.8	0.65±0.04	20.7	0.32±0.04	10.2	0.51±0.09	16.2	0.11±0.01	3.5
	Cu	15.40±3.23	2.32±0.78	15.1	3.34±1.01	21.7	1.58±0.99	10.3	4.23±1.45	27.5	0.87±0.07	5.6	3.67±1.02	23.8
Kolonnawa	Ni	11.35±2.76	1.52±0.61	13.4	0.21±0.02	1.9	1.01±0.44	8.9	0.64±0.08	5.6	0.86±0.09	7.6	0.03±0.00	0.3
N=8	Cd	0.86±0.04	0.11±0.01	12.8	0.05±0.00	5.8	0.08±0.00	9.3	ND	-	0.06±0.00	7.0	ND	-
	Cr	6.74±2.22	0.85±0.07	12.6	0.23±0.01	3.4	0.53±0.12	7.9	0.27±0.01	4.0	0.59±0.07	8.8	0.13±0.02	1.9
	Pb	3.05±1.01	0.36±0.02	11.8	0.12±0.00	3.9	0.22±0.07	7.2	ND	-	0.15±0.03	4.9	0.05±0.00	1.6
	Cu	10.30±2.99	1.12±0.77	10.9	3.13±0.98	30.4	0.26±0.08	2.5	3.12±1.09	30.3	0.05±0.00	0.5	2.24±1.09	21.7
Kottawa	Ni	9.15±1.87	1.11±0.60	12.1	0.12±0.08	1.3	1.10±0.53	12.0	0.22±0.08	2.4	1.03±0.10	11.3	0.14±0.04	1.5
N=8	Cd	0.22±0.05	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	5.05±1.23	0.31±0.07	6.1	0.15±0.06	3.0	0.33±0.01	6.5	0.30±0.01	5.9	0.27±0.07	5.3	0.05±0.00	1.0
	Pb	3.89±1.00	0.34±0.08	8.7	0.15±0.07	3.9	0.31±0.00	8.0	0.20±0.00	5.1	0.25±0.08	6.4	0.02±0.00	0.5
	Cu	10.90±2.45	2.23±0.80	20.5	3.23±0.97	29.6	1.39±0.99	12.8	2.56±0.89	23.5	1.22±0.12	11.2	1.63±0.42	15.0
Bandaragama	Ni	2.02±0.98	0.16±0.02	7.9	0.05±0.00	2.5	ND	-	ND	-	ND	-	ND	-
N=8	Cd	0.11±0.01	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	0.78±0.07	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Pb	0.31±0.05	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cu	6.96±1.45	1.12±0.67	16.1	2.36±0.89	33.9	1.06±0.50	15.2	2.03±0.87	29.2	0.95±0.09	13.6	1.98±0.65	28.4

ND = not detected: N = sample size: WHO/FAO permissible limits: Ni = 4 mg/kg, Cd = 0.2 mg/kg, Cr = 2.3 mg/kg, Pb = 0.3 mg/kg and Cu = 40 mg/kg

Table 2: *In vitro* bioaccessibility of heavy metals in raw and processed *Centella asiatica* at the gastric and intestinal phases.

Area	Metal	Total metal content (mg kg^{-1})	Bioaccessibility of Raw samples				Bioaccessibility of Cooked samples				Bioaccessibility of Stir-fried samples			
			Gastric Phase		Intestinal Phase		Gastric Phase		Intestinal Phase		Gastric Phase		Intestinal Phase	
			(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%	(mg kg^{-1})	%
Piliyandala	Ni	2.11±1.01	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
N=8	Cd	0.15±0.05	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	0.72±0.12	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Pb	0.27±0.09	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cu	10.60±2.12	0.85±0.06	8.0	1.04±0.87	9.8	0.56±0.34	5.3	0.98±0.41	9.2	0.66±0.22	6.2	1.01±0.09	9.5
Wellampitiya	Ni	14.06±3.12	1.41±0.07	10.0	0.65±0.12	4.6	1.23±0.71	8.7	0.69±0.25	4.9	1.31±0.45	9.3	0.52±0.02	3.7
N=8	Cd	0.32±0.08	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	5.82±2.03	0.11±0.02	1.9	0.06±0.00	1.0	0.10±0.02	1.7	0.06±0.01	1.0	0.13±0.54	2.2	0.04±0.00	0.7
	Pb	5.90±2.01	0.58±0.07	9.8	0.12±0.07	2.0	0.59±0.12	10.0	0.11±0.07	1.9	0.45±0.09	7.6	0.09±0.10	1.5
	Cu	16.26±5.21	1.86±0.06	11.4	4.69±1.23	28.8	1.33±0.76	8.2	3.65±1.10	22.4	1.03±0.29	6.3	4.52±1.21	27.8
Kolonnawa	Ni	16.55±5.25	1.53±0.70	9.2	0.64±0.20	3.9	1.36±0.45	8.2	0.13±0.04	0.8	1.22±0.33	7.4	0.10±0.02	0.6
N=8	Cd	0.45±0.11	0.05±0.00	11.1	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	11.33±2.31	0.29±0.02	2.6	0.05±0.01	0.4	0.26±0.08	2.3	ND	-	0.21±0.13	1.9	0.03±0.00	0.3
	Pb	10.23±1.98	1.01±0.08	9.9	0.22±0.11	2.2	0.98±0.45	9.6	0.32±0.10	3.1	0.96±0.32	9.4	0.21±0.09	2.1
	Cu	21.22±6.86	2.31±0.89	10.9	8.36±2.33	39.4	2.45±1.01	11.5	7.60±2.01	35.8	3.40±1.06	16.0	6.70±1.23	31.6
Kottawa	Ni	11.23±2.45	1.12±0.70	10.0	0.54±0.12	4.8	1.10±0.40	9.8	0.25±0.02	2.2	0.95±0.11	8.5	0.10±0.03	0.9
N=8	Cd	0.30±0.08	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	4.36±2.00	0.08±0.01	1.8	ND	-	ND	-	ND	-	ND	-	ND	-
	Pb	3.21±1.98	0.30±0.05	9.3	0.06±0.01	1.9	0.10±0.02	3.1	0.06±0.00	1.9	0.09±0.01	2.8	ND	-
	Cu	16.22±5.43	0.83±0.12	5.1	4.83±1.12	29.8	1.21±0.76	7.5	3.65±1.01	22.5	0.56±0.12	3.5	4.20±0.99	25.9
Bandaragama	Ni	1.45±0.98	0.16±0.05	11.0	ND	-	ND	-	ND	-	ND	-	ND	-
N=8	Cd	0.15±0.07	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cr	0.63±0.12	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Pb	0.30±0.12	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
	Cu	5.32±0.57	ND	-	0.43±0.22	8.1	ND	-	ND	-	ND	-	ND	-

ND = not detected: N = sample size: WHO/FAO permissible limits: Ni = 4 mg/kg, Cd = 0.2 mg/kg, Cr = 2.3 mg/kg, Pb = 0.3 mg/kg and Cu = 40 mg/kg

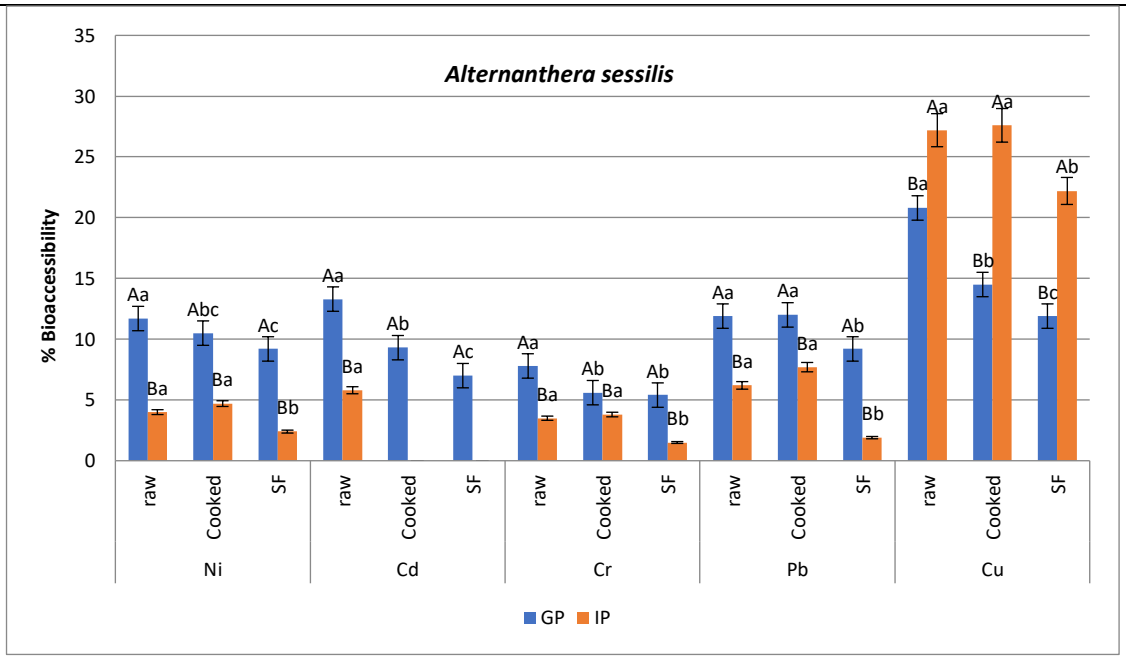

Fig. 3: Average bio-accessibility (%) of heavy metals in raw, cooked and stir-fried *A. sessilis* in the gastric and intestinal phases (GP = Gastric Phase and IP = Intestinal Phase)

Different uppercase letters indicate significant differences (P<0.05) between GP and IP for each treatment within a specific heavy metal, while different lowercase letters indicate significant differences (P<0.05) among treatments (raw, cooked, stir-fried) for each heavy metal within GP and IP

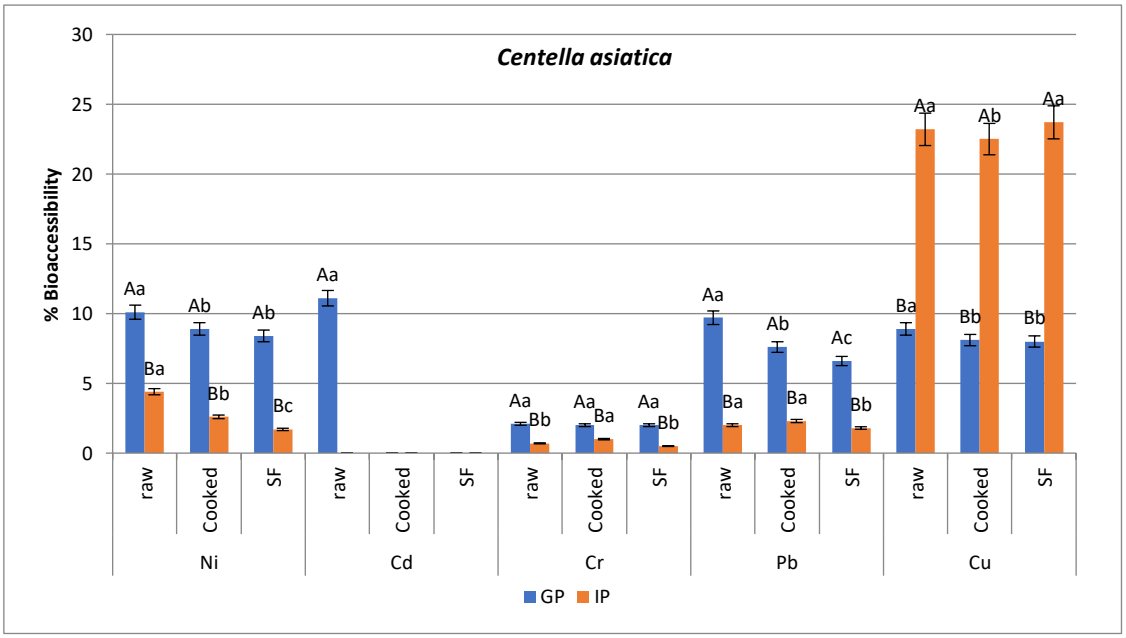

Fig. 4: Average bio-accessibility (%) of heavy metals in raw, cooked and stir-fried *C. asiatica* in the gastric and intestinal phases (GP = Gastric Phase and IP = Intestinal Phase)

Different uppercase letters indicate significant differences (P<0.05) between GP and IP for each treatment within a specific heavy metal, while different lowercase letters indicate significant differences (P<0.05) among treatments (raw, cooked, stir-fried) for each heavy metal within GP and IP

As demonstrated in Figs. 3 and 4, the bioaccessibility of heavy metals in *Alternanthera sessilis* and *Centella asiatica* analyzed in this study reveals distinct patterns. On average, the bioaccessibility

percentages of Ni, Cd, Cr, and Pb were significantly higher ($p<0.05$) during the gastric phase than in the intestinal phase, except for Cu.

In the gastric phase, the acidic environment and the high concentration of hydrogen ions (H^+) facilitate the dissolution of metals. The formation of complex ions with chloride (Cl^{-1}) helps maintain the solubility of metals (Ruby et al. 1996). Gastric enzymes and organic acids further assist in enhancing the solubility of these elements in the stomach. Conversely, in the intestinal phase, metal concentrations tend to decrease due to binding or precipitation caused by the higher pH (Chaney et al. 1989; Wang et al. 2024). Some studies suggest that gastric-phase extractions alone can provide a reliable estimate of bioaccessibility (Gasser et al. 1996; Hamel et al. 1998). However, this research included both gastric and intestinal phases to offer a more comprehensive understanding of the digestive system's processes.

The absorption site for Cu in humans remains contested, with some studies indicating higher bioaccessibility in the gastric phase (Pan et al. 2016; Wang et al. 2024), while others suggest increased absorption during the intestinal phase (Chaney et al. 1989). Several factors affect Cu absorption, such as its chemical form (e.g., Cu acetate and Cu sulfate are more bioavailable than Cu oxide), along with dietary enhancers like citrate, phosphate, and animal proteins. On the other hand, inhibitors such as Zn, Cd, phytates, and sugars may reduce Cu absorption.

Despite the high total concentrations of heavy metals in *A. sessilis* and *C. asiatica*, their bioaccessible fractions were considerably lower ($p<0.05$) across raw, cooked, and stir-fried samples. This indicates that total heavy metal concentrations do not directly correlate with the amounts available for absorption by the human body. Literature suggests that typical dietary absorption rates are about 5% for Cd, 0.4-2.5% for Cr, 30-40% for Cu, 10% for Pb (40-50% in children), and 1-10% for Ni, with the remainder excreted through urine or feces (Jaishankar, 2014). In our study, the average bioaccessible fractions of heavy metals in *A. sessilis* and *C. asiatica* were as follows: Gastric phase: 9.98% Ni, 9.46% Cd, 3.93% Cr, 9.70% Pb, and 13.8% Cu; Intestinal phase: 3.51% Ni, 5.81% Cd, 1.97% Cr, 3.15% Pb, and 33.6% Cu. These results are consistent with previous findings.

The methods of cooking influenced the bioaccessibility of heavy metals, with cooking and stir-frying generally reducing bioaccessibility when compared to raw samples. For example, Cd bioaccessibility was

significantly lower in cooked and stir-fried *A. sessilis* and *C. asiatica*. Yang et al. (2012) pointed out that Cd bioaccessibility is dependent on its chemical binding forms and the characteristics of the food. However, differences in bioaccessibility for other metals among raw, cooked, and stir-fried samples were less pronounced ($p<0.05$). Stir-frying resulted in the lowest bioaccessibility for most metals of concern. Various factors such as the type of green leafy vegetable, food composition (e.g., antinutrients like phytates and tannins), cooking methods, processing temperature, and the cooking medium (e.g., coconut milk or oil) likely influence metal extraction during digestion.

Studies by Intawongse & Dean (2008), Jayawardene et al. (2011), and Hu et al. (2013) offer valuable perspectives on heavy metal bioaccessibility across different food matrices. Hu et al. (2013) found patterns similar to this study, showing higher bioaccessibility for Ni, Cd, Cr, and Pb during the gastric phase, with Cu bioaccessibility being higher during the intestinal phase. Intawongse & Dean (2008) observed that metals in vegetables were mostly in insoluble forms at neutral pH but became soluble in the acidic gastric phase. Jayawardene et al. (2010) reported comparable findings in medicinal plants, noting significantly higher bioaccessibility of Pb, As, and Cd during the gastric phase.

Other research supports the finding that low pH in the gastric phase enhances metal solubility. Turner & Ip (2007), Ovca et al. (2011), and Tremlova et al. (2012) found reduced bioaccessibility in the intestinal phase due to factors such as antinutrient binding, complex formation, or precipitation. For example, phytates and tannins can form insoluble complexes with metals, reducing their bioaccessibility.

Conversely, some studies, such as those by Kulkarni et al. (2007) and De Lima et al. (2014), reported greater bioaccessibility in the intestinal phase. Kulkarni et al. (2007) observed higher bioaccessibility of K, Mn, Zn, and Fe during intestinal digestion in wheat products. Yin et al. (2017) found varying bioaccessibility across gastric and intestinal phases in vegetables from Beijing markets, with Cu showing significantly higher bioaccessibility in the intestinal phase.

Pan et al. (2016) studied health risks related to heavy metals in vegetables grown near a waste incinerator, finding bioaccessible fractions that aligned with the results in this study for Cd, Cr, Cu, Ni, and Pb in different gastrointestinal phases.

4. CONCLUSIONS

This research emphasizes the significance of evaluating bioaccessibility rather than just total heavy metal content when assessing potential health risks. While *Alternanthera sessilis* and *Centella asiatica* from urban areas in Sri Lanka exhibited elevated total heavy metal concentrations, their bioaccessible fractions were significantly lower, indicating limited absorption by the human body. These findings suggest that total heavy metal concentrations do not directly reflect the amounts available for absorption. Ongoing monitoring of metal contamination and the adoption of safer farming practices are essential to mitigate risks. Future research are recommended to include a broader range of foods and incorporate *in vivo* studies to provide a more comprehensive understanding of dietary exposure to heavy metals.

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