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Proximate composition, levels of heavy metals and their associated risk assessment in ginger (Zingiber officinale roscoe)

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ABSTRACT

Ginger is a medicinal plant widely used as a spice and botanically categorized in the Zingiberaceae family, Zingiber genus, and officinale species. Natural and anthropogenic sources can discharge heavy metals into the environment from which they enter the human body through food, water, air, and skin contact and cause detrimental health effects. In this study, ginger samples were randomly collected from five kebeles in Ilubabor and Bunno Bedelle zones, Southwest Ethiopia, from which a composite sample was made for analysis. Microwave-assisted digestion was carried out using concentrated nitric acid in the presence of hydrogen peroxide followed by atomic absorption spectrometer detection using different Association of Analytical Chemists, AOAC methods. Proximate analysis was performed with different standard operating procedures. The mean levels of heavy metals were 0.009 mg/kg for Arsenic, Chromium, and Lead and 0.01 mg/kg for cadmium while 578.2 mg/kg was obtained for iron. Proximate composition analysis shows 82.89%, 2.00%, 0.07%%, 1.63%, 3.42%, 9.99%, and 48.59 kcal for moisture, crude protein, crude fat, ash, crude fiber, carbohydrate, and energy, respectively. The validated method had acceptable linearity (R^2 = 0.9978 - 0.9993), the limit of detection ($8.18x10^{-5}$ - 0.0075), the limit of quantification ($2.7x10^{-4}$ - 0.025), recovery (75 - 106.87%) and precision (4.77 - 13.97 %). The hazard index (HI) of the metals was less than one showing insignificant non-carcinogenic risk from exposure to a single or multiple possibly toxic elements from the studied ginger samples.

Contribution/Originality: The study aimed to quantify toxic heavy metals in ginger, a widely used and cultivated spice in the study area and analyze the associated risks on human health. The finding contributes to the knowledge on the product pollution status as it was not assessed earlier.

1. INTRODUCTION

Ginger (Zingiber Officinale) is a medicinal herb used in most of the world as a spice and it has been grown for centuries (Majithia, Sona, & Ahmedabad, 2018). It has been enormously common for culinary to relish food and create a distinctive, typical taste (Spink, Embarek, Savelli, & Bradshaw, 2019; Zagórska, Kukula-Koch, Czop, Iłowiecka, & Koch, 2023) and to treat diseases throughout India and China for more than five thousand years

(HMPC, 2012; Poudel et al., 2022). The major chemical components in ginger include 6-paradol, 6-shogaols, and, 6-gingerol which have robust antioxidant properties (Bischoff-Kont & Fürst, 2021; Teng, Seuseu, Lee, & Chen, 2019) and offer health welfare (Shivam & Sandeep, 2022). It also contains numerous organic compounds like phenolics, vitamins, lipids, organic acids, polysaccharides, and terpenes, (Jabborova et al., 2022). Ginger is used to treat inflammation, blood coagulation, low cholesterol, migraine, diabetes, arthritis, nausea, and low lipid levels (Gupta & Sharma, 2021; Ozkur et al., 2022). Proteins, fibers, carbohydrates, fats, and vitamins are also found in ginger at various levels (Sunday, 2020).

Heavy metals are elements with a relatively high density that can pose a health threat at low levels. They include lead, cadmium, zinc, mercury, arsenic, silver, chromium, copper, iron, and the platinum group elements. They have various applications in industries, agriculture, and medical technologies. Symptoms of heavy metal poisoning include nausea, abdominal pain, vomiting, shortness of breath, chills, weakness, and tingling in your hands and feet (Balali-Mood, Naseri, Tahergorabi, Khazdair, & Sadeghi, 2021; Tchounwou, Yedjou, Patlolla, & Sutton, 2012).

Cadmium and arsenic toxicity affects the kidney, lungs, reproductive systems, gastrointestinal, and liver, and it is also carcinogenic (Genchi, Sinicropi, Lauria, Carocci, & Catalano, 2020; Tchounwou et al., 2018). Persistent contact with lead sources may lead to the decreased central nervous system and kidney functions (Chan, 2003; Rubio, Balverdi, Marchisio, & Sales, 2023). It has a pronounced short and long-term effect on children and pregnant women as it damages the cognitive function and causes abortion and low birth weight (Ray & Ray, 2009; Yu et al., 2017). Though chromium exists in the 3+ and 6+ forms, the latter is more toxic and affects the respiratory system resulting in acute and chronic ailments including lung cancer (Alemu & Tegegne, 2022; Thompson et al., 2020). Iron is an important micronutrient that plays a vital role in the transport or transfer of oxygen and electrons in biochemical reactions (Galaris, Barbouti, & Pantopoulos, 2019). Unreasonably high iron consumption can irritate gastrointestinal mucosa resulting in symptoms of nausea, vomiting, and diarrhea (Uddin, Khalid, Khan, & Abbas, 2013).

The southwest (Kaffa, Ilu Aba Bora and Bunno Bedelle, Gamo Gofa, Wollaga), Southern (Sidama), and northern part (Gojjam, Gondar) are the major ginger-producing areas in Ethiopia (Kaba, Doda, & Kanido, 2020; Wagesho & Chandravanshi, 2015). It is currently being used as an important cash crop and also in the preparation of a local ‘wots’- a routinely used stew with 'Injera' as the main food. Despite the prevalent use of ginger in the country and the study area being a potential cultivator, there are no studies on risk assessment of heavy metals and their nutritional composition.

2. MATERIALS AND METHODS

2.1. Sample Collection

A total of 15 fresh commonly used Zingiber officinale roscoe samples were collected from selected districts of Ilu Aba Bora and Bunno Bedele Zones, Oromia regional state, southwest Ethiopia. Three samples from each site were collected and mixed to form a 1 kg composite sample and then packed in polyethylene bags. After washing several times with tap water it was then air-dried for seven days at room temperature and grounded with a porcelain mortar and pestle into fine particles and put in polyethylene bags and stored in a cool dried cabinet before digestion.

2.2. Chemicals and Reagents

Nitric acid (HNO_3, 70%) and hydrogen peroxide, (H_2O_2, 30% v/v) were used for the digestion of ginger samples, and distilled water. Sulphuric acid, H_2SO_4 (98%), potassium hydroxide, petroleum ether, Acetone, Copper Sulfate, and Potassium sulfate were also used. Calibration and spiking solutions were made from a 1000 mg/L of the Fe, Cr, Pb, Cd, and As nitrate salts. All chemicals used were of analytical grade and were obtained from Aldrich, Milwaukee, USA.

2.3. Method Validation

The repeatability of the method was evaluated from the ratio of standard deviation to mean recovery values of triplicate analysis. For the method accuracy different concentrations of the metals (15 µg/kg, 25 µg/kg, 35 µg/kg for As and Pb; 0.2 µg/kg, 0.6 µg/kg, 1.2 µg/kg for Cd; 0.4 µg/kg, 0.8 µg/kg, 1.6 µg/kg for Cr; 2µg/kg, 4 µg/kg, 7 µg/kg for Fe) were spiked with 1g ginger and the percent recovery were calculated by dividing the level differences after spiking and amount detected by amount spiked. Linearity was estimated in terms of the linearity coefficient (R^2) from the calibration plot of the concentration of standards vs absorbance. Method`s detection and quantification limits were determined as the ratio of three and ten folds of blank signal to the slope of calibration lines, respectively (Taghipour & Jalali, 2019; Taleuzzaman, 2018).

2.4. Proximate Analysis of Ginger

All the proximate compositions (moisture content, crude protein, crude fat, total ash, crude fiber, carbohydrate, and energy values) were determined according to procedures described in Kefale, Delele, Fanta, and Abate (2023).

2.5. Determination of Metals (Arsenic, Cadmium, Lead, Iron, and Chromium)

8 mL of HNO_3 (70%) was added to Teflon digestion vessels containing 1.0 g ginger followed by the addition of 1.0 ml H_2O_2 and transferred into the microwave digestion system at 200 ºC and kept for 2 h. After cooling the digested sample for 10 minutes it was filtered with Whatman No. 42 filter paper and diluted to 50 ml with deionized water. A similar procedure was followed for digesting the reagent blank. Both the digested ginger samples and reagent blank solution were stored in a refrigerator before analysis with atomic absorption spectrometry (Ayalew, Bhagwan, & Mesfin, 2017).

Stock standard solutions of the five metals (1000 mg/L) were used to prepare working standards in a different range of linearity Table 2 to establish calibration curves Figure 1- Figure 5 in a flame atomic absorption spectrometer. Three replicate determinations were carried out on each sample. The operating conditions of the atomic absorption spectrometer (AAS) are shown in Table 1.

Table 1. AAS working conditions.

Step	Metals	λ (nm)	SW (nm)	I (mA)	Gas types	t (s)
1	Arsenic	197.2	1.0	10.0	Normal	5
2	Cadmium	228.8	0.50	7.0	Normal	4
3	Chromium	357.9	0.2	10.0	Normal	5.0
4	Iron	429.0	0.2	10.0	Normal	2.0
5	Lead	283.3	0.5	10.0	Normal	5

Note: SW = Slit width, λ = Wave length, I = Lamp current, t = Integrated time, normal- acetylene air.

3. RESULT AND DISCUSSION

3.1. Method Validation

3.1.1. Linearity and Calibration Curves

The calibration curve indicates the retort of an instrument or method to concentrations of an analyte. Regression analysis and correlation coefficients were used to plot calibration curves and evaluate the linear dependence of instrument signal on measured concentration ranges, respectively. The levels of heavy metals were determined from their respective calibration curves after aspirating the sample solutions into the atomizer and recording absorbance values. The calibration curves and their respective equations were described in Table 1. The correlation coefficient values range between 0.9978 and 0.9993 showing that the method had an acceptable linearity. The calibration curves of standards of all heavy metals were described in Figures 1-5.

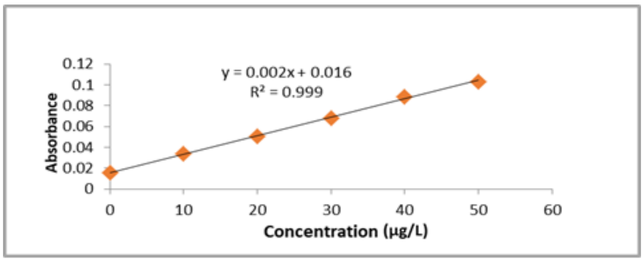

Figure 1. Calibration curve of Arsenic.

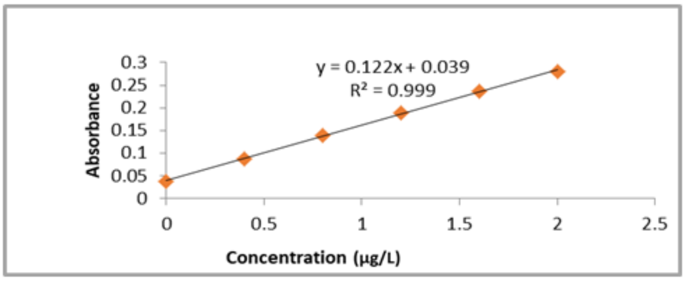

Figure 2. Calibration curve of cadmium.

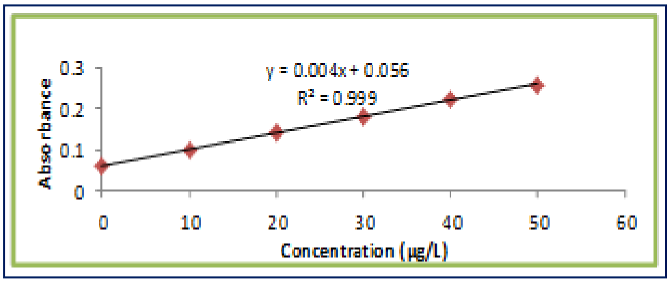

Figure 3. Calibration curve of Chromium.

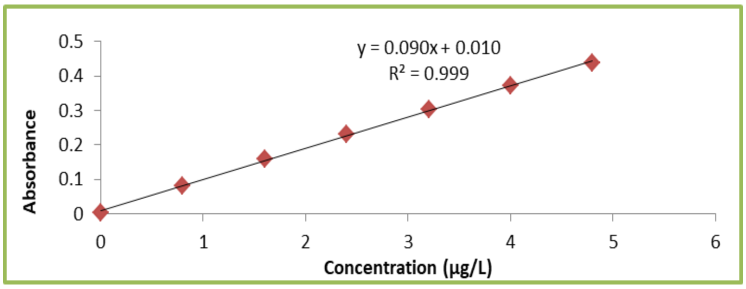

Figure 4. Calibration curve of iron.

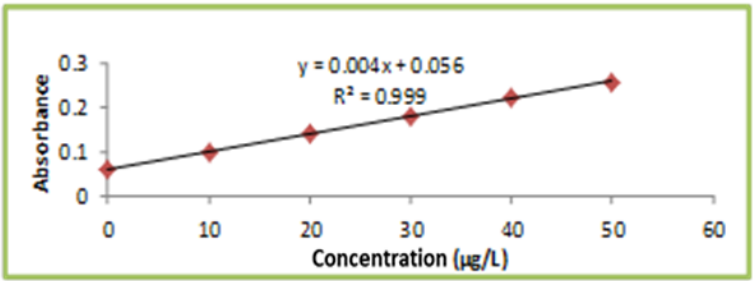

Figure 5. Calibration curve of lead.

3.1.2. Method Detection and Quantification Limits

Method`s detection and quantification limits are the ratio of three and ten folds of blank signal to the slope of calibration lines, respectively (Bernal & Guo, 2014; Taleuzzaman, 2018). The limits of detection of the studied heavy metals in ginger were 0.0022, 8.18 x 10^{-5}, 0.075, 0.0044, and 0.0025 for Arsenic, Cadmium, Chromium, Iron, and Lead, respectively. The calculated values of LoD and LoQ are shown in Table 2.

Table 2. Method validation parameters (Linearity, LOD, LOQ) of studied heavy metals in the ginger sample.

Element	Linear range (µg/ L)	Calibration curve equation	R^2	LOD (µg/ L)	LOQ (µg/ L)
Arsenic	10.00-50.00	Y = 0.0018X+ 0.0156	0.9989	0.0022	0.0073
Cadmium	0.400-2.000	Y = 0.1223X+ 0.0393	0.9992	8.18x10^{-5}	2.7x10^{-4}
Chromium	0.200-2.000	Y = 0.004X+ 0.0001	0.9976	0.0075	0.025
Iron	0.800- 4.800	Y = 0.0903X + 0.0099	0.9992	0.0044	0.015
Lead	10.00-50.00	Y = 0.004X + 0.0598	0.9992	0.0025	0.0083

3.1.3. Recovery and Precision Studies

For the method accuracy, different concentrations of the metals see Table 3 were spiked with 1.0 g ginger and digested with the same procedure used in digesting the unspiked samples. The percent recoveries were calculated by dividing the levels of the metals in ginger after spiking minus the detected amount with concentration added. The intraday precision was determined in terms of the relative standard deviation of recovery values. The ranges of percent recoveries for As, Cd, Cr, Fe, and Pb were 98.86 -101, 80-100, 95 - 106.87, 75 – 104.28, and 81.92 - 84.65, respectively. As one can see from Table 4, all the percent RSD ranges of the studied metals were also less than 15%. A validated method is accurate and precise when the recoveries and relative standard deviations are ≤ 70 - 120% and ≤ 15 - 20 %, respectively (FAO & WHO, 2023).

Table 3. Recovery and precision results of studied metals.

Analyte	Unspiked concentration (mg/kg)	Spiked concentration (mg/kg)	Recovered concentration ($\bar{X}$ ± SD) (n=3) (mg/kg)	Recovery (%) ($\bar{X}$ ± SD)	RSD (%)
Arsenic	0.009	15	14.84 ± 2.19	98.86 ± 11.5	11.63
		25	25.26 ± 1.4	101.00 ± 9.31	9.21
		35	34.62 ± 6.82	98.88 ± 10.58	10.67
Cadmium	0.01	0.2	0.21 ± 0.04	100 ± 5.17	5.17
		0.6	0.49 ± 0.01	80 ± 8.27	10.33
		1.2	1.16 ± 0.08	95.83 ± 6.01	6.27
Chromium	0.009	0.4	0.39 ± 0.15	95± 7.82	8.23
		0.8	0.84 ± 0.24	103.75 ±4.95	4.77
		1.6	1.72 ± 0.93	106.87 ± 6.24	5.84
Iron	578.2	2	580.0 ± 11.5	90 ±10.95	12.16
		7	585.5 ± 13.51	104.28 ± 9.39	9.00
		4	575.2± 12.29	75.00 ± 7.63	10.17
Lead	0.009	15	12.42 ± 4.17	82.73 ± 5.89	7.12
		25	20.49 ± 8.03	81.92 ± 10.25	12.51
		35	29.71 ± 4.64	84.65 ± 11.86	13.97

Note: Where; SD-standard deviation; $\bar{X}$ - mean; RSD- relative standard deviations of mean recoveries.

3.2. Proximate Analysis and Levels of Heavy Metals in Ginger

The quantitative analysis of proximate composition consisting of moisture content, crude protein, crude fat, total ash, carbohydrate content, and energy value (kcal.) for ginger is presented in Table 4. A comparison of the current result with different literature data is also shown in Table 4.

3.2.1. Proximate Composition of Ginger

Moisture Content: The moisture content of ginger samples was found to be 82.89 ± 0.071%. Moisture content in food can have a substantial effect on various food parameters like palate, consistency, appearance, form, and weight. It has insinuations on legal and labeling necessities, frugally significant requirements, the food`s shelf life, quality, and processing procedures, unreasonably high moisture content in a food product can increase microbial growth leading to decreased shelf life. The result in this study is higher than the moisture content of sun-dried ginger (31.30 ± 0.71%) and oven-dried ginger (15.92 ± 0.25%) as determined by Sunday (2020) and also higher than other findings Table 4.

Crude protein: Protein content evaluation is an important and common practice in food processing and proximate analysis. The crude protein content of ginger was 2.00 ± 0.03%. This result is lower than the values obtained by other researchers (Table 4). The variations could be soil chemistry, climate, plant management, and other contributing factors. It is usually assumed that a plant-based food that offers more than 12% of the total energy value from protein is considered to be a good source of protein.

Crude Fiber, fat, and carbohydrate contents: The crude fiber content determined in the current study was 3.42 ± 0.042%. A normal level of fiber in food helps in protecting undesirable effects like constipation in the gastrointestinal tract (John, Peter, Carmen, & Adam, 2019). The low fiber result in the current work doesn't have a significant effect on the use of ginger as it is not a major meal but a flavoring agent. The crude fat content determined in the current study was 0.07±0.001% while 9.99 ± 0.021% was reported for carbohydrate content. Carbohydrate is an energy source for cells and they maintenances the plasma sugar level and saves proteins in the body from being easily digested.

Ash: Ash content is an indicator of the inorganic minerals in food and it is determined based on weight differences before and after a portion of food is burnt in oxygen at high temperature. The ash content result obtained for the current study was 1.63±0.1%. It is higher than the result reported by Ia, Esekheigbe, and Je (2019) but lower than others Table 4.

Table 4. Comparison of proximate composition of ginger with various studies.

Moisture content (%)	Crude protein (%)	Crude fat (%)	Ash (%)	Crude fiber (%)	Carbohydrate (%)	Energy (Kcal/100g)	Reference
15.92±0.25 31.30±0.71	5.88±0.005-3.95±0.10	3.62±0.015 1.87±0.02	4.30±1.06-10.20±0.04	9.03±0.01-9.23±0.02		-	Sunday (2020)
72.20	8.91	11.71	0.81	1.38	2.01	-	Ia et al. (2019)
3.55-3.78	5.02-5.82	0.76-0.90	3.38-3.66	4.97-5.61	-	-	Sangwan, Kawatra, and Sehgal (2014)
3.95-4.63	11.65-12.05	9.89-17.11	4.95-7.45	8.30-21.90	39.70-58.21		Ajayi, Akomolafe, and Akinyemi (2013)
90.60±0.126 -93.40± 0.034	2.65±0.043-10.54±0.113	3.80±0.15-4.48±0.099	7.48±0.218-3.80±0.014	7.30±0.06-9.75±0.55	67.75±0.7-73.49±0.19	338.80±0.653 - 353.47±1.781	Bhati and Raghuvanshi (2021)
8.8 ± 0.14	9.01 ± 0.45	1.33 ± 0.25	6.33 ± 0.25	6.62 ± 0.12	67.81 ± 1.2	319.47±0.00	Kefale et al. (2023)
82.89 ±0.071	2.00±0.03	0.07±0.001	1.63±0.1	3.42±0.042	9.99±0.021	48.59±0.05	This study

Note: Values are means ± SD (Standard deviation) of duplicate samples.

3.2.2. Levels of Heavy Metals in Ginger

The concentrations of five elements (As, Cd, Cr, Fe, and Pb) in the digested samples of ginger were analyzed by flame atomic absorbance spectrometer. The mean levels of heavy metals were 0.009 mg/kg for Arsenic, Chromium, and Lead and 0.01 mg/kg for Cadmium while 578.2 mg/kg was obtained for Iron. The food and agricultural organization/world health organization, FAO/WHO permissible values of As, Cd, Pb, and Fe in the plant are 0.05, 0.2, 0.1, and 300 mg/kg, respectively (Kaba et al., 2020; Kusse, Zewde, & Yoseph, 2019). This indicates that the amount of iron in studied ginger samples exceeds the maximum permissible level set by the aforementioned organization. This result was higher than 140 mg/kg reported by Nkansah and Amoako (2010) and also greater than the reported value of 41.8– 89.0 mg/kg (Sunday, 2020) in Ethiopia and 32.44 mg/kg reported by Christiana and Matthews-Amune Samuel (2013). Even though there were no reports on short-term toxicity of iron from consumption of normal dietary sources, people with decreased ability to metabolize will be at threat from undue exposure to iron. It may deposit in different organs (thyroid, liver, heart, pituitary, adrenals, and pancreas) resulting in liver damage, poor adrenal functions, heart failure, or diabetes (Kusse et al., 2019). A comparison summary of the present study with different reports was presented in Table 5.

Table 5. Summary of levels of toxic heavy metals and comparison with literature data.

As (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Fe (mg/kg)	Pb (mg/kg)	Reference
-	4.63-5.43	2.17- 4.44	77.71- 81.12	ND	Getaneh, Guadie, and Tefera (2021)
0.02	0.0096	0.52	-	0.0096	Wang et al. (2021)
2.99	0.3204	1.476	-	1.9694	Mahtab et al. (2022)
0.002±0.003	0.02 ±0.007	-	-	0.17 ± 0.38	Mawari et al. (2022)
0.06± 0.04	0.10± 0.11	-	-	BLQ (0.05)	Kumar et al. (2020)
-	-	-	9.68	-	Ia et al. (2019)
-	ND	-	6.14 - 11.92	0.12 - 0.23	Kaba et al. (2020)
0.18 ± 0.01	-	-	-	0.79 ± 0.04	Blagojevic, Blagojevic, and Begovic (2015)
-	0.000054	-	0.019	-	Olusakin and Olaoluwa (2016)
-	-	-	-	1.156±0.290	Goswami and Mazumdar (2014)
0.009	0.01	0.009	578.2	0.009	This study

Note: ND-not detected; BLQ-below limit of quantification.

3.3. Health Risk Assessment

Prolonged exposure of humans to toxic heavy metals from different sources can increase the likelihood of detrimental health effects manifested in the short or long term (Boateng, Opoku, Acquaah, & Akoto, 2015; Jaishankar, Tseten, Anbalagan, Mathew, & Beeregowda, 2014). The carcinogenic and non-carcinogenic health risks of toxic heavy metals can be assessed from the hazard index calculated by considering the estimated daily intake of food samples and reference doses of the metals (Alsafran, Usman, Rizwan, Ahmed, & Al Jabri, 2021; Chonokhuu, Batbold, & Chuluunpurev, 2019).

Estimated daily intake (EDI): was calculated based on the concentrations of heavy metals detected in ginger samples and estimated average body weight of a person using the following, Equation 1.

$$EDI = \frac{Cmetal}{BW} \quad (1)$$

Where, Cmetal is the concentration (mg/kg) in ginger, IR (ingestion rate) is the mean daily intake of ginger in the local area (10 g/ day), and BW is the average body weight (Kg). The average body weight used in this study was 60kg. The target hazardous quotient (THQ): is calculated by dividing the estimated daily intake by the respective chronic reference doses of metals. It is an indicator of the non-carcinogenic risk due to the consumption of contaminated vegetables or fruit. It was calculated by Equation 2.

$$THQ = \frac{EDI}{RfD} \quad (2)$$

Where RfD is the reference dose. The reference doses for As, Cd, Cr, Fe, and Pb were 0.0003 0.001, 0.001, 0.7, and 0.002, respectively (Antoine, Fung, & Grant, 2017; Copat et al., 2013). A THQ value exceeding one indicates that there may be hostile health effects for humans due to metals exposure while a value less than one shows insignificant health risks for non-carcinogenic element (Adebiyi, Ore, & Ogunjimi, 2020; Kortei et al., 2020).

The hazard index (HI) is used to estimate the budding human health risk through ingesting multiple heavy metals. It is the individual sum of THQ and was calculated using Equation 3.

$$HI = \sum_{n=1}^{i} THQ; i = 1, 2, 3..n \qquad (3)$$

A hazard index value of less than one shows that there is a likelihood of health effects but if it is less than one there is no significant threat of non-carcinogenic effects (Ametepey, Cobbina, Akpabey, Duwiejuah, & Abuntori, 2018; Chonokhuu et al., 2019; Getaneh et al., 2021). The EDI and hazard quotient, HQ values of studied heavy metals are shown in Table 6.

Table 6. EDI, HQ, and HI values of heavy metals in adults through the consumption of ginger.

Metal	EDI	THQ
As	1.5×10^{-6}	0.0005
Cd	1.67×10^{-6}	0.00167
Cr	1.5×10^{-6}	0.0015
Fe	0.096	2.14×10^{-6}
Pb	1.5×10^{-6}	0.00075

The hazard index of metals calculated based on data in Table 6 above is 0.0044 indicating no undue non-carcinogenic risk from exposure to a single or multiple potentially toxic elements from the same food.

4. CONCLUSION

In this study, proximate analysis and determination of the content of selected heavy metals in ginger samples from Bunno Bedelle and Ilu Aba Bora zones were carried out. The collected samples were digested using the microwave technique and analyzed for metal contents by AAS using standard AOAC methods. The results show 82.89, 2.00, 0.07, 1.63, 3.42, 9.99 % (g/100), and 48.59 kcal/100g for moisture, crude protein, crude ash, crude fiber, and carbohydrate and energy values respectively. Concentrations of heavy metals were in the order: of Fe (578.2mg/kg) > Cd (0.01 mg/kg) > Cr (0.009mg/kg) = As (0.009mg/kg) = Pb (0.009 mg/kg). Except for the iron concentration, all values comply with the maximum residue limit set by FAO and WHO (2023). Moreover, the hazard index of the metals is less than one indicating no undue non-carcinogenic risk from exposure to a single or multiple potentially toxic elements from the same food. However, despite the lower levels of most of the metals studied and their low hazard index value calculated based on assumed variables, a positive result for this commonly used spice plant is not a good signal, as the cumulative effect of the toxic metals depends on the actual frequency and dose at which they are ingested. Therefore, a comprehensive study with a very large number of samples and other heavy metals from the study area needs to be conducted to get a more conclusive result. The quality offices in the districts and zones should also work very well in monitoring the levels of these toxic substances in the commonly cultivated and consumed products as they bring chronic deterioration of health and economic losses, the effects of which are not immediately visible.

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Authors' Contributions: Developed the idea, wrote draft manuscript, supervised experiments, and edited manuscript, A.K.; the experiments, contributed resources and wrote draft manuscript, T.G.; edited the manuscript, analyzed data, and wrote manuscript, K.T.; wrote and edited the manuscript, W.M. All authors have read and agreed to the published version of the manuscript.

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