



Exposure of human population to heavy metals through consumption of selected vegetables collected from local markets of north-western Indian Himalaya

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Abstract

Local anthropogenic activities such as market, vehicular and agricultural activities contribute heavy metals to vegetables and consumption of such vegetables can pose health risk to human population. Therefore, in the present study, concentrations of heavy metal in frequently consumed vegetables in the Kullu valley of north-western Indian Himalaya and associated human health risks were assessed. Four vegetables namely, cauliflower (*Brassica oleracea* L. var. *botrytis*), tomato (*Solanum lycopersicum* L.), radish (*Raphanus sativus* L.) and cabbage (*Brassica oleracea* L. var. *capitata*) were collected from the local markets of Kullu valley and were analyzed for Cu, Zn, Cd and Pb using an atomic absorption spectrophotometer. The results showed that mean concentrations of Zn, Cd and Pb were found to be maximum in *R. sativus* (49.5 mg/kg dw, 2.7 mg/kg dw and 2.5 mg/kg dw, respectively) and Cu in *B. oleracea* var. *botrytis* (38.2 mg/kg dw). The concentration of heavy metals is significantly influenced by vegetables, sampling locations and their interaction ($p < 0.05$). Except *B. oleracea* var. *capitata*, consumption of all the test vegetables can cause health risk to local population as health risk index value exceeds a unit for Cd. The results further showed that heavy metal load is found highest for *S. lycopersicum*, followed by *R. sativus* and *B. oleracea* var. *capitata* and least for *B. oleracea* var. *botrytis*. The study concludes that local anthropogenic Activities contribute heavy metal significantly to the tested vegetables and Cd can pose health risk to the human population by consuming these vegetables.

Keywords: heavy metals, vegetables, kullu valley, cd, health risk, human population

Introduction

Heavy metal pollution has arisen as a global problem because of evolutionary, ecological, nutritional and environmental reasons (Nagajyoti *et al.*, 2010; Adameic *et al.*, 2016; Khorshid and Thiele, 2016) [24, 2, 18]. Heavy metals are non-biodegradable, cytotoxic, persistent and biotoxic in nature may have harmful or beneficial effects on plants, animals and human life depending on their concentration in the medium (Zhang *et al.*, 2012; Chatterjee *et al.*, 2007) [41, 7]. Heavy metals released from both natural and anthropogenic activities get accumulated in different components of the environment e.g., water, soil and atmosphere and entered to the human's body through dietary intake (Zhang *et al.*, 2012; Marti-Cid *et al.*, 2008) [41, 23].

Now a day, due to increased concentration of heavy metals in the environment, vegetables take up heavy metals by absorbing them from contaminated soil, air and irrigation water (Wang *et al.*, 2005; Singh and Kumar, 2006; Singh *et al.*, 2010; Nawaz *et al.*, 2021) [39, 34, 32, 25] and pose risk to both the food safety and human health (Sharma *et al.*, 2008; Sipter *et al.*, 2008) [31, 35]. Further, the inedible parts are responsible for a wide range of problems in animals and humans who consume it (Khan *et al.*, 2008; Alam *et al.*, 2003) [17, 3]. Heavy metals are easily accumulated in edible parts of the leafy vegetables as compared to grain or fruit crops (Mapanda *et al.*, 2005; Ruzaidy and Amid, 2020) [22, 28]. The concentration of heavy metals in different parts of vegetables varied significantly due to their morphological and physiological

nature (Singh and Kumar, 2006; Sharma *et al.*, 2009) [34, 30]. Atmospheric depositions, transport and marketing systems of vegetables play an important role in elevating the level of heavy metals in vegetables (Sharma *et al.*, 2008; Ali and Al-Qahtani, 2012; Kulkarni, 2017) [31, 4, 19].

Consumption of heavy metal contaminated foods affect many biochemical processes and leads to nervous, kidney, cardiovascular and bone disease (WHO, 1992; Jarup, 2003) [40, 15]. Pb and Cd have shown their carcinogenic effect on humans (Trichopoulos, 1997) [36]. Many countries have set up regulations to control the emission of heavy metals in the environment as well as for vegetables to reduce the human health risk. Earlier studies have reported that the concentrations of Cd, Pb and Ni in vegetables grown on sewage effluent irrigated soil had exceeded the safe limits set by national and international agencies (Singh, 2018) [33]. Vegetables grown in location close to industries have higher concentrations of Pb, Cd, Ni, Co and Cr (Naser *et al.*, 2018) [26]. Sharma *et al.* (2009) [30] found that transportation and marketing of vegetables have increased the level of heavy metals in vegetables. Study conducted by Kumar *et al.* (2019) [21] found that the concentrations of Cu and Cd in radish, cabbage, beet root, coriander, spinach, brassica and fenugreek collected from 3 local markets of Lucknow, India ranged between 4.6 - 16.6 and 0.05 - 2.6 $\mu\text{g/g}$, respectively. Heavy metal concentration in 32% of vegetable samples collected from the study areas were higher

than the permissible limit set by FAO/WHO (Kumar *et al.*, 2019)^[21]. Except Pb, Concentrations of Zn, and Cu in *Citrullus colocynthis* and *Irvingia wimbolu* from South-East Nigerian markets were found below the permissible limit set by NAFDAG (Anukwuorji *et al.*, 2020)^[6]. Pb concentration was found higher in branded spices such as black pepper, red pepper, turmeric and garam masala than the non – branded spices marketed in Multan city of Pakistan and the females above 24 years old were more exposed to the risk due to higher rate of spices intake (Aakhtar *et al.*, 2020)^[11].

The present study was carried out to quantify the concentration of heavy metals such as Cu, Zn, Cd and Pb in frequently consumed vegetables of family Brassicaceae (cauliflower, *Brassica oleracea* L. var. *botrytis*; radish, *Raphanus sativus* L. and cabbage, *Brassica oleracea* L. var. *capitata*) and Solanaceae (tomato, *Solanum lycopersicon* L.), grown locally and sold in local markets of Kullu valley. The generated data were compared with national and international safe limits and also used to calculate the risk indices to assess the health risk associated with heavy metals to the local people.

Table 1: Characteristics of markets sites located in Kullu valley of north-western Indian Himalaya

Site ID	Study sites	Geographical location			Characteristics
		Latitude	Longitude	Altitude (ft)	
M1	Nagwain	31°48'50.46"N	77°11'.042"E	3395	HT, DP, RA, CA
M2	Bajaura	31°51'1.02"N	77°09'47.22"E	3625	HT, MP, RA, CA
M3	Bhuntar	31°52'54.12"N	77°09'2.16"E	3604	HT, DP, RA, CA
M4	Kullu	31°57'43.80"N	77°06'36.84"E	3969	HT, DP, RA, CA
M5	Katrain	32°06'12.60"N	77°08'38.04"E	4746	HT, MP, RA, CA
M6	Patlikul	32°06'52.56"N	77°08'48.54"E	4869	HT, SP, RA, CA
M7	Naggar	32°06'40.98"N	77°09'46.80"E	5520	MT, MP, RA, CA
M8	Manali	32°14'37.26"N	77°11'22.80"E	6270	HT, DP, RA, CA

Heavy traffic (HT), Moderate traffic (MT), Dense population (DP), Moderate population (MP), Sparse population (SP), Commercial area (CA), Residential area (RA), Industrial area (IA)2.3

Materials and Methods

Study Area

The present study was carried out in the Kullu valley of north-western Indian Himalaya, located on the banks of Beas River. Based on the supply of locally produced vegetables, traffic congestion, industrial, residential and non-residential activities, eight market sites namely, Nagwain, Bajaura, Bhuntar, Kullu, Katrain, Patlikul, Naggar and Manali were selected for the present study. Geographical location, traffic load, population load and land use at selected markets of north-western Indian Himalaya are given in Figure 1 and Table 1.

Sampling and Processing

Vegetables selected for the present study were morphologically different from each other e.g., cauliflower and cabbage are inflorescenced with large surface areas which may receive more

aerial deposits. Radish and tomatoes are root and fruit vegetables, respectively. Approximately 1 kg of fresh vegetables produced locally and sold in markets was collected from three shops of a market site during the sampling period i.e., January – December 2017. The vegetable samples were collected from each study site once in a couple of months, six times during the sampling period. Samples were immediately kept in a pre-distilled water-washed polyethylene bags and were brought back to the laboratory and processed. The uneatable portions of the vegetables were removed and edible portion was chopped into small pieces by using a stainless-steel knife. The samples were then oven dried at 80 °C till a constant weight was achieved. The oven dried samples were powdered or crushed using stainless steel blender and passed through a 2mm size sieve. The resulting fine powder was kept at room temperature for further analysis.

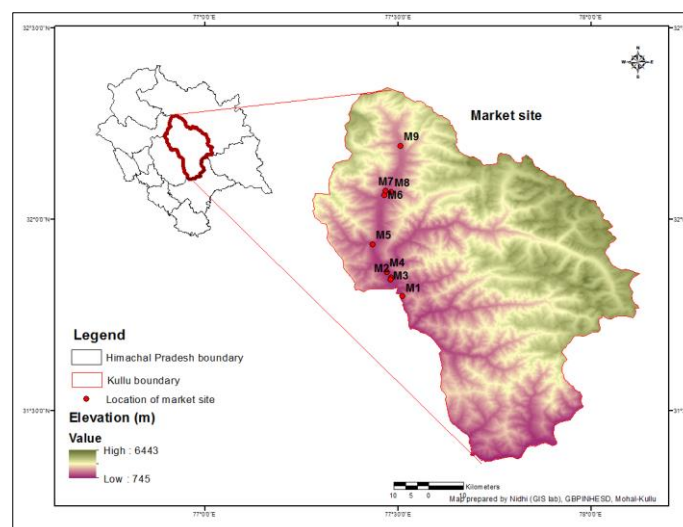


Fig 1: Map showing the geographical locations of different market sampling sites in Kullu valley of north-western Indian Himalaya

Digestion of Samples

Dry powders of vegetables (1.00 ± 0.001 g) were taken into a 100 ml acid-washed beaker and digested in 15 ml of tri-acid mixture (70% HNO_3 , 70% H_2SO_4 and 65% HClO_4 in 5:1:1 ratio) at 80 °C till the transparent solution was achieved (Allen *et al.*, 1986)^[5]. After cooling, the digested samples were filtered using Whatman number 42 filter paper and the filtrate was finally maintained to 25 ml with double distilled water. Determination of Cu, Zn, Cd and Pb concentrations in the filtrates were achieved by atomic absorption spectrophotometer (Model Analyst 800, Perkin-Elmer). The instrument was fitted with a specific lamp with a particular metal. The measurements were made using a hollow cathode lamp of Cu, Zn, Cd and Pb at wavelengths of 324.8nm, 213.9 nm, 288.8 nm and 213.3 nm, respectively. The values of the detection limits for Cu, Zn, Cd and Pb were 0.001 $\mu\text{g/ml}$, 0.008 $\mu\text{g/ml}$, 0.005 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$, respectively. Standard solutions and blank samples were frequently run to check the sensitivity of the instrument.

Quality Control and Assurance

Quality assurance and all precautionary measures were taken into consideration to avoid the errors in methodology. All the chemicals used in the present study were purchased from Merck Pvt. Ltd., India. High quality polyethylene containers were used for the collection of vegetable samples before the analysis. All laboratory wares used in this study were washed properly and rinsed with double distilled water before use.

Data Analysis

The obtained data were used to calculate the daily intake of heavy metals (DIM), health risk index (HRI) and heavy metal pollution index (HPI) to assess the risk associated with heavy metal contaminated vegetables. Health risks associated with contaminated vegetables were further assessed by comparing data with the safe limits set by PFA and EU/WHO/FAO for the different heavy metals (Singh *et al.*, 2010)^[32].

Daily Intake of Heavy Metals

Daily intake of Cu, Zn, Cd and Pb ($\mu\text{g/person/day}$) by human population by consuming the vegetables was calculated by multiplying the concentration of heavy metals in vegetables with consumption rate of vegetable (Cui *et al.*, 2004)^[8]. Daily intake of test vegetables by the local population was obtained through a formal survey conducted in and around the study sites. An interview of 20 adult individuals of 40-60 years age group, and of 60-70 Kg body weight was conducted regarding their daily consumption rate of each vegetable using a semi-structured questionnaire.

Each individual represented a household having minimum 2 individuals, thus 160 individuals were effectively interviewed. An average consumption rate of each tested vegetable was calculated.

Health Risk Index

Health risk index (HRI) of heavy metals for the local population from the consumption of contaminated vegetables was assessed and calculated as the ratio of estimated exposures due to daily heavy metal intake through vegetables and reference oral dose (R_{FD}), United States Environmental Protection Agency (USEPA, 2002; Cui *et al.*, 2004)^[37, 8]. Oral reference doses were 0.04, 0.3 and 0.001 mg/kg/day for Cu, Zn and Cd, respectively (USEPA, 2002)^[37] and 0.004 mg/kg/day for Pb (USEPA, 1997). $HRI < 1$ means the exposed population is unlikely to experience obvious effect, whereas $HRI > 1$ means there may be adverse effect on human health, with an increasing probability as the value increases.

Heavy Metal Pollution Index

To compare the total heavy metal load in tested vegetables, heavy metal pollution index (HPI) was calculated using the equation given by Usero *et al.* (1997)^[38].

$$HPI = (C_1 \times C_2 \times C_3 \dots \times C_n)^{1/n}$$

Where, C is the concentration of n heavy metal in vegetables

Statistical Analysis

The generated data on were used to find the mean, standard error, minimum and maximum. Data were also analyzed for the ANOVA test to assess the effect of variables such as sites, vegetables and their interaction on heavy metal concentration. SPSS software was used for all the statistical analysis.

Results and Discussion

Heavy Metals in Vegetables

The market wise variations in concentration of heavy metals in different vegetables marketed in the Kullu valley of north-western Indian Himalaya is shown in Figure 2. The mean concentrations of heavy metals (mg/kg dw) in tomato, cauliflower, cabbage and radish were 16.1, 38.2, 9.8 and 31.4, for Cu, 39.9, 49.3, 32.3 and 49.8, for Zn, 1.5, 0.68, 0.82 and 2.7 for Cd and 1.3, 1.3, 1.7 and 2.5 for Pb, respectively (Table 3). Concentration of Cu, Zn, Cd and Pb in vegetables varied significantly due to site, vegetable species and their interaction (Table 4). The vegetables sold in market areas such as Mohal, Manali, Nagwain, Kullu, Bhunter and Bajaura were found highly contaminated with heavy metals. Cu and Zn concentrations were maximum in cauliflower at Mohal (62.2mg/kg dw) and at Nagwain (75.2 mg/kg dw), respectively. Cd and Pb concentrations were maximum in Radish at Bhunter (5.9 mg/kg dw) and Manali (6.58mg/kg dw), respectively. The mean concentration of Cu, Zn, Cd and Pb in radish exceeded the permissible limit of Indian standard. Mean concentrations of both Cu and Zn in cauliflower exceeded the permissible limit of Indian standard (Table 3).

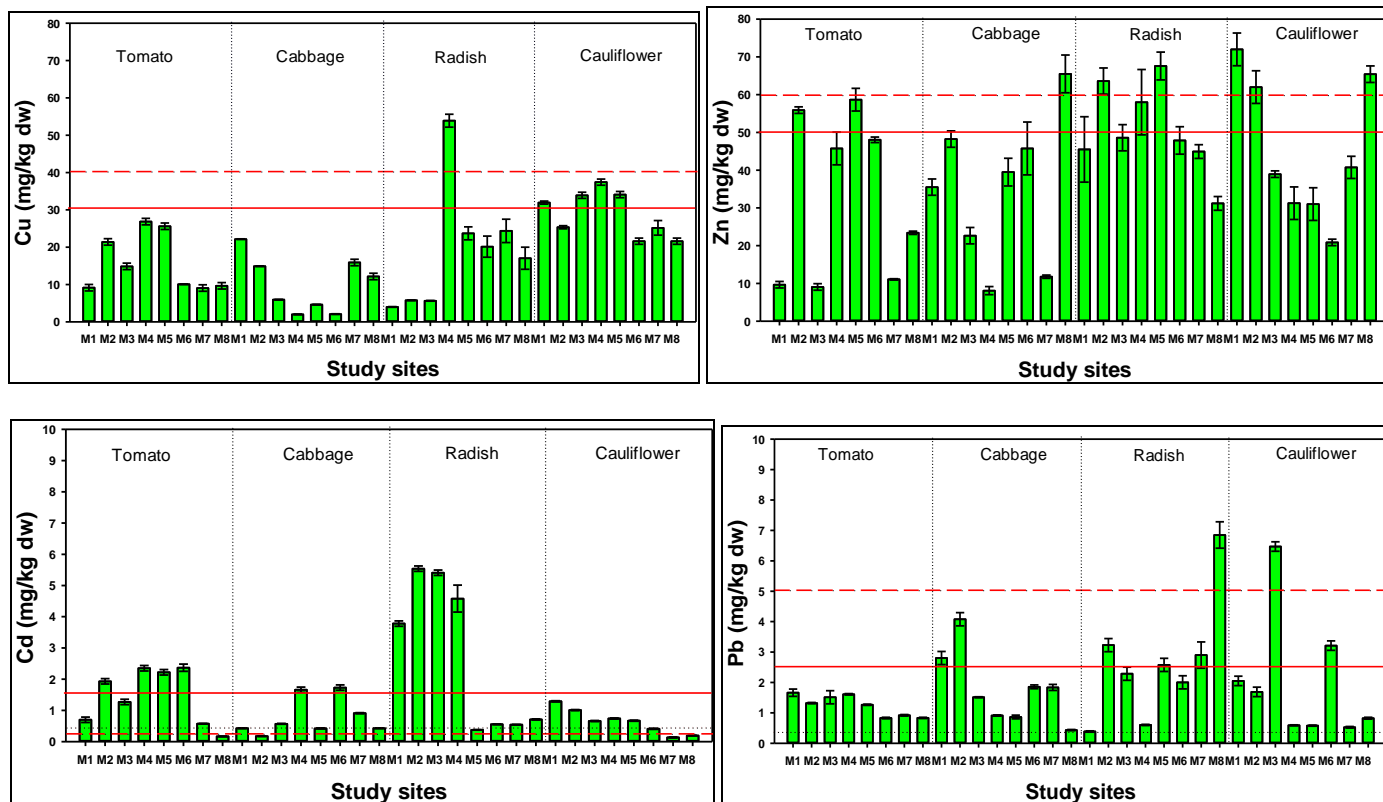


Fig 2: Site wise variations in the concentrations of heavy metals in vegetables of Kullu valley of north-western Indian Himalaya (Red line i.e., solid, long dash and dotted showed Indian, WHO/FAO and EU standards, respectively)

Table 2: Heavy metal concentration (mg/kg Dw) in vegetables produced and sold in urban markets of north-western Indian Himalaya during January to December 2017

Heavy metals	Vegetables				Safe limit		
	Tomato (n= 45)	Cauliflower (n= 45)	Cabbage (n= 60)	Radish (n= 60)	PFA Act ^a	EU ^b	WHO/FAO ^c
Cu	16.10 (9 - 26.9)	38.18 (20.2 - 64.9)	9.82 (2 - 22.3)	31.4 (22.8 - 54)	30	-	40
Zn	39.9 (20.3 - 58.9)	49.3 (21 - 75.9)	32.3 (17.5 - 56.3)	49.8 (5.9 - 66.3)	50	-	60
Cd	1.5 (0.2 - 3)	0.7 (0.1 - 1.4)	0.8 (0.2 - 1.8)	2.7 (0.4 - 6)	1.5	0.3	0.2
Pb	1.3 (0.7 - 2)	1.3 (0.1 - 4)	1.7 (0.2 - 4.3)	2.5 (0.4 - 6.9)	2.5	0.2	5
HPI	2.17 (5.1-8.9)	0.6 (5.6-9.4)	1.45 (3.8-5.9)	1.52 (6.9-12.1)			

^a Awashthi, 2000; ^b European Union Commission, 2006; ^cWHO/FAO, 2007

n is the number of samples collected from the local market during sampling periods (January – December 2017)

The results of present study indicated that the local anthropogenic activities such as transportation, vehicular (traffic congestion, damaged road, vehicle parking, etc.), market (watering, chemical treatment, etc.), agricultural activities (overuse of pesticides, fertilizers, etc.) have significant influence on heavy metal concentration in the vegetables. A study conducted by Sharma *et al.* (2008) ^[31] showed that atmospheric deposits significantly contribute heavy metal contamination to vegetables sold in local markets. The variation in heavy metal concentrations in different vegetables ascribed to their morphological characteristics such as rough/plain surface areas, exposure to atmospheric and vehicular activities. The higher concentration of heavy metals in radish may be ascribed to more accumulation of heavy metals and their sequestration in root. The significant variations in site wise heavy metal concentration may be due to the location study sites to populated areas or near the vehicle stops. Throughout the world,

studies to assess the heavy metal concentration in food items like fruits, vegetables, cereals, etc. have been carried out. Study conducted by Sharma *et al.*, (2009) ^[30] in Varanasi, India reported that the concentration (mg/kg dw) of Zn, Cd and Pb in cauliflower collected from local market ranged from 25.2 to 94.3, 0.80 to 4.30 and 0.90 to 2.40, respectively, which was higher than the range of heavy metals reported in the present study except for Pb which was found between 0.11-3.98. Das and Das (2018) ^[9] reported the mean concentration of Cu (13.02 mg/kg dw) in vegetables collected from street side market in Kolkata, India was less as compared to those (38.18 mg/kg dw) reported in the present study, whereas, mean concentration of Zn (52.95 mg/kg dw) was higher than those (49.77 mg/kg dw) reported in the present study. Mean concentration of Cu and Cd was higher in vegetables collected from market in Saudi Arabia as compared to those found in present study (Naser *et al.*, 2018) ^[26]. Ngumbu *et al.*,

(2017)^[27] showed that the range of Cd and Pb concentration in tomato purchased from market in Monrovia, Liberia was 0.040 mg/kg dw to 0.051 mg/kg dw and 0.091 mg/kg dw to 0.144 mg/kg dw, respectively which was less than the range i.e. 0.16 mg/kg dw to 2.97 mg/kg dw and 0.72 mg/kg dw to 1.99 mg/kg dw found in tomato in the present study.

Safe Limits Exceedence

The generated data on the concentration of heavy metals in vegetables at different market sites were compared with safe limits set by national (PFA acts 1954) and international (EU and WHO/FAO guideline) agencies to assess the risk associated with heavy metals in tested vegetables, consumed by local people of Kullu valley of the north-western Indian Himalaya (Figure 2). Cu concentration in tomato and cabbage collected from market sites was found below the safe limit set by national and international standards at all the market sites. Cu concentration in cauliflower was exceeded the national safe limit at Manali (64.26 mg/kg dw), Naggar (57.86 mg/kg dw), Mohal (37.52 mg/kg dw), Kullu (34.77 mg/kg dw), Bhuntar (33.07 mg/kg, dw) and Nagwain (32.39 mg/kg, dw), whereas exceeded international safe limit at Manali (64.26 mg/kg dw) and Naggar (57.86 mg/kg dw). Cu concentration in radish was exceeded the national safe limit at Mohal (53.58 mg/kg dw), Bhuntar (38.52 mg/kg dw) and Manali (34.61 mg/kg dw), and international safe limits at Mohal (53.58 mg/kg dw). Zn concentration in tomato exceeded the international safe limits at Kullu (57.81 mg/kg dw) and Bajaura (55.11 mg/kg dw). Zn concentration in cauliflower exceeded the national safe limits at Nagwain (75.18 mg/kg dw), Naggar (55.90 mg/kg), Manali (69.08 mg/kg dw) and Bajaura (65.18 mg/kg dw) and international safe limits at Nagwain (75.18 mg/kg dw), Manali (69.08 mg/kg dw) and Bajaura (65.18 mg/kg dw). Zn concentration in Cabbage at Kullu (55.69 mg/kg dw) exceeded national safe limit.

Table 3: Influence of vegetables, sites and their interaction on heavy metals in Kullu valley of north-western Indian Himalaya (Results of two-way ANOVA test)

Heavy metals	Vegetables	Sites	Vegetables × Sites
df	3	7	21
Cu	6021***	425.7***	504.4***
Zn	515.17***	324.77***	172.18***
Cd	828.6***	260***	183.1***
Pd	209.6***	994.55***	150.1***

Level of significance: *** = p<0.001

Table 4: Daily intake of heavy metals by human population through consumption of vegetables produced and sold in north-western Indian Himalaya

Vegetables	Heavy metals			
	Cu	Zn	Cd	Pb
Tomato	0.03	0.08	3.17	2.74
Cauliflower	0.06	0.08	1.13	2.15
Cabbage	0.01	0.03	0.73	1.54
Radish	0.01	0.02	1.15	1.07
Total	0.12	0.22	6.17	7.48
PTDI ^a	3	60	60	214

^a Joint FAO/WHO Expert Committee on Food Additives (1999)

*Daily intake of Cu and Zn (mg/person/day) and Cd and Pb (µg/person/day)

PTDI; Potential tolerable daily intake

Cd concentration in tomato was found above the national safe limit at Mohal (2.6 mg/kg dw), Patlikul (2.5 mg/kg), Kullu (2.25 mg/kg dw) and Bajaura (1.76 mg/kg dw). Cd concentration in tomato, cabbage, cauliflower except at Naggar and radish exceeded the international standards (Figure 2). Pb concentration in all the test vegetables at all sites, except in radish at Manali (6.58 mg/kg dw) was found below the WHO/FAO safe limits (Figure 2). But, Pb exceeded the EU safe limit at all the market sites except cauliflower in Naggar (0.21 mg/kg dw) and cabbage (0.28 mg/kg dw) in Kullu.

The concentration of heavy metals in most of the tested vegetables collected from market sites exceeds by both the national or international safe limits. It may be ascribed to heavy vehicular load as almost all the markets situated on the side of National Highway (NH). This NH leads to one of the best holiday destination in India (Kullu-Manali). Apart from this, the transportation and use of pesticides and chemical fertilizers in agriculture play an important role in accumulation of heavy metals in tested vegetables during its growth and development.

Risk Associated with Heavy Metals Contaminated Vegetables

The average consumption (on dry weight basis) rate of tomato, cauliflower, cabbage and radish by the local people of Kullu valley, north-western Indian Himalaya were found as 2.12 g/person/day, 1.66 g/person/day, 0.88 g/person/day and 0.42 g/person/day, respectively. The concentrations of heavy metals in vegetables were used as representative of contaminants and daily intake of heavy metals was calculated for the human people. Total daily intake of Cu, Zn, Cd and Pb by the human people via tested vegetables were found to be 0.12 mg/day/person, 0.0.22 mg/day/person, 6.17 µg/day/person and 7.48 µg/day/person, respectively (Table 4). Further the results showed that the total daily intake of Cu, Zn, Cd and Pb via the tested vegetables was found below the PTDI (FAO/WHO, 1999)^[12] values.

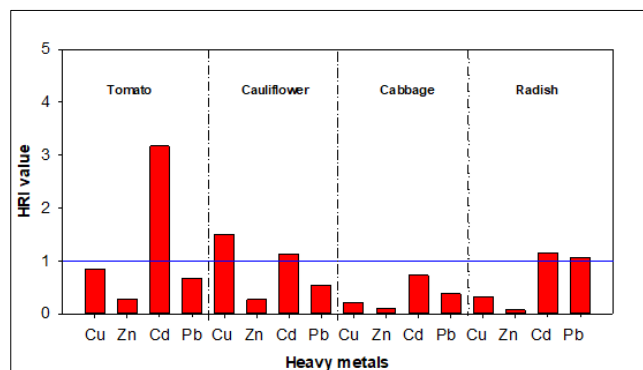


Fig 3: Health risk index of heavy metals for different vegetables produced and sold in north-western Indian Himalaya

Estimated exposure and health risk index were calculated to assess the health risk associated with heavy metal contaminated vegetables grown locally in different production sites and sold in urban markets of Kullu Valley to local population and presented in Table 4 and Figure 3. Health risk index was found more than a unit for Cu in cauliflower and Cd in tomato, cauliflower and

cabbage. In the present study, Zn and Pb concentrations in the tested vegetables did not found to cause any health risk to local population. Except cabbage, the HRI of Cd via tested vegetables had exceeded a unit and long-term consumption of such vegetables may post risk to the human population of Kullu valley. However, Cu, Zn and Pb of test vegetables showed HRI values below 1 and may not post risk to the human population (Figure 3). Sharma *et al.* (2008)^[31] found that Cd and Cu contaminated vegetables posed health risk to human population of Varanasi, India. Vegetables grown in the wastewater irrigated areas of Varanasi, India are also posing serious health risk associated with Cd, Pb and Ni to local population. Study of Elbagermi *et al.* (2012)^[10] showed that there is no health risk associated with dietary intake of heavy metal contaminated vegetables produced locally and sold in Misurata Area of Libya.

In the present study, heavy metal load on each vegetable collected from market sites was assessed in term of heavy metal pollution index (HPI) and are shown in Table 2. HPI value was found maximum 2.17, 1.52, 1.45 and 0.6 respectively for tomato, radish, cabbage and cauliflower. The HPI values of each test vegetables were observed more than a unit suggests that these vegetables may cause more human health risks due to higher accumulation of heavy metals in vegetables. Singh *et al.* (2010)^[32] also found higher HPI in vegetables like cabbage, palak, brinjal and lady finger in a study conducted in Dinapur, Varanasi, India.

Long-term exposure to heavy metal contaminated vegetables through dietary intake can result their accumulation in soft tissues of human body which ultimately caused health hazards. The results of present study indicate that the current anthropogenic activities in Kullu valley of north-western Indian Himalaya potentially contaminate the vegetables with heavy metals such as Cu, Zn, Cd and Pb. Among these heavy metals, Cd needs more attention as it poses more health risk as compared to the other heavy metals. Thus, the present study suggests that concentrations of heavy metals in vegetables and other crops produced locally and sold in markets should be monitored regularly as well as awareness programme should be promoted among the local stakeholders to prevent the health risk associated with heavy metal contamination.

Acknowledgement

Directors, GBPNIHE, Almora for research facilities and Science Engineering Research Board (grant no: SERC/LS-10-135/2010) New Delhi, India for the financial support to the present work are gratefully acknowledged.

Conflict of Interest

The authors declare that there is no conflict of interest for this publication.

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