

Novel risk assessment of Cd in earthworms and leeches based on bioavailability through *in vitro* digestion/Caco-2 and MDCK cells

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Abstract

Little data are available about the extent of bio-accumulation of heavy metals in traditional animal medicines (TAMs). In the present study, the oral bioavailability of cadmium (Cd) in earthworms and leeches was investigated through *in vitro* PBET digestion/Caco2 and MDKC cell models. We are the first to create an innovative assessment strategy which has capacity to offer a more precise evaluation of Cd-associated health risks in TAMs, by combinational usage of bioavailable Cd levels, the duration and frequency of the exposure to TAMs obtained by questionnaire data, as well as safety factor of TAMs. Our data showed that the percentage of bioavailability for Caco-2 cells in earthworms and leeches ranged from 3.29–14.17% and 4.32–12.61%, respectively. The percentage of bioavailability of MDCK cells in earthworms and leeches ranged from 4.83–15.74% and 6.53–15.04%, respectively. After adjusting by the bioavailability of Cd to target hazard quotient (THQ), excitingly, our findings manifested that the health risks induced by the ingestion of earthworms and leeches were acceptable in the clinic. Our novel strategy has capacity to offer a more realistic and accurate assessment of Cd-associated health risks in TAMs. Besides, our key findings suggest that bioavailability characterization cannot be ruled out and health risks should be assessed on the basis of the bioavailable Cd levels rather than total levels.

Highlights

- Oral bioavailability of Cd in traditional animal medicines (TAMs) was investigated.
- *In vitro* PBET digestion/Caco2 and MDKC cell models were developed suited to the matrix of TAMs.
- A novel health risk assessment model for TAMs was created through bioavailability characterization.

Introduction

Traditional animal medicine (TAM) is a significant type of traditional Chinese medicine (TCM). Given the unique priority of curative discoveries, TAMs have been extensively used for treating a variety of diseases in Chinese clinics for a long history. Both earthworms and leeches are commonly used TAMs, as thoroughly described in ancient literature 1700 year ago (Takahashi et al., 2010). In addition, there has been a long tradition of using earthworms and leeches for clinical purposes since ancient Egypt and medieval Europe (Whitaker et al., 2004). The proposed therapeutic benefits of earthworms include anti-arteriosclerosis, anti-tumor, anti-thrombotic, anti-arrhythmic, immune-modulatory anti-hypertension, and diuresis (Huang et al., 2018). The biological activities of leeches include anti-hypertension, relieving amenorrhea, relieving injuries, cardiovascular protective activities, and anti-cancer effects (Pan et al., 2006).

Cadmium (Cd), a ubiquitous environmental pollutant, is a global concern because of its detrimental effects on the environment and health risks to human bodies (Khana et al., 2017; Tang et al., 2017). Chronic exposure to excessive Cd can induce a series of adverse effects on humans, including obstructive pulmonary disease, reproductive deficiencies, kidney dysfunction, diabetes, osteoporosis, and fractures (Ngo et al., 2012; Chen et al., 2014; Zhang et al. 2014). Previous studies have reported that the content of Cd in TAMs was of great concern (Zuo et al., 2019). The ingestion of TAMs contaminated by Cd through food chains causes the accumulation of this toxin in human organisms, posing potential health risks to humans.

Generally, most studies on the risks associated with Cd have focused on the total Cd contents, providing valuable data on the overall contamination degree of this toxic element (Li et al. 2014; Nemati et al. 2014; Ghaleño et al. 2015; Cheng et al. 2017; Zuo et al. 2020a; Zuo et al. 2020b). However, these measurements did not reflect the actual Cd exposure levels to the human body, leading to higher exposure levels and an unreasonable overestimation of the risks to public health. Thus, it is essential to better understand the concept of bioavailability of Cd to assess its risks to humans, which involves investigating the oral bioaccessibility and bioavailability of Cd in TAMs.

Bioaccessibility is defined as the portion of pollutants that can be released from the matrix to the intestinal tract and has the potential to be absorbed by the human body during digestion (Liu et al. 2018). Recently, a variety of *in vitro* digestion approaches have been created to examine the bioaccessibility of deleterious elements in soil and food, including the model of PBET, SBET, UBM, etc (Denys et al., 2012; Wragg et al., 2011; Ruby et al., 1996). Compared with *in vivo* methods, which are time consuming, expensive, and subject to ethical complications, these *in vitro* digestion models have considerable advantages, including simplicity, high effectiveness, low cost, energy savings, and easy reproducibility (Koch et al., 2007).

Moreover, it should be mentioned that bioavailability is also quite significant. Bioavailability embodies the fraction actually absorbed by organisms and directly used for physiological functions; thus, comparatively, it can provide more reliable and accurate oral exposure (Andre et al., 2015). The Caco-2 cell line, derived from colon carcinoma, has been widely accepted as an *in vitro* model for mimicking the absorption process of the human intestinal epithelium barrier in studying the transport and retention behaviors of a variety of substances (Balimane et al., 2000). After differentiation in culture, Caco-2 cells represent the biochemical and morphological characteristics of small intestine cells, involving tight junctions, polarity, specific enzymes, and transport systems (Rousset, 1986). However, the tight junctions of this type of cell are actually tighter than those in enterocytes, thus lessening the permeability of compounds with substantial paracellular absorption (Saitoh et al., 2004; Takenaka et al., 2014). Another type of cell lines, MDCK cells, which are originally from the collecting duct of the canine kidney, are typical secretory epithelial cell lines with non-tumor features (Husted et al., 1986). Apart from being used for research on the morphology and function of renal tubular epithelial cells, MDCK cells are widely recognized as an *in vitro* tool for the evaluating drug absorption and metabolism by the Food and Drug Administration, European Medicines Agency, and other authorities (Volpe, 2008). To the best of our knowledge, scarce data is available on the bioavailability of Cd in TAMs. Additionally, little information is present related to the health risks of bioavailable Cd in TAMs. Therefore, it is of great significance to establish bioavailability models of TAMs to improve risk assessment of contaminants to uniform their clinical usage.

Considering the unique characterizations of TAMs, increasingly popular usage of TAMs worldwide, as well as associated public health concerns, our effort aimed at (1) investigating the contents of total Cd in earthworms and leeches using inductively coupled plasma-mass spectroscopy (ICP-MS), (2) unveiling the bioaccessibility of Cd in earthworms and leeches after simulating human *in vitro* digestion using a PBET model, (3) analyzing the cellular uptake of Cd in bioavailable fractions using the Caco-2 and MDCK cell models, and (4) creating the first assessment strategy suited to TAMs based on exposure to bioavailable Cd, by which we sought to provide innovative insights into health risks and safety usage of TAMs.

Material And Methods

Samples and reagents

Thirteen batches of earthworms and nine batches of leeches were collected from TCM markets and retail pharmacies from 2018 to 2019 in the provinces of Anhui, Guangxi, Hainan, Zhejiang, Jiangsu, Yunnan, Heilongjiang, Shandong, and Shanghai City (Table 1). All samples of earthworms and leeches were authenticated by Dr. Kun-zi Yu.

Table 1
Sample collection information in the present study

Type	No.	Batch No.	Location	Source
Earthworm	1	DL-1	Anhui	Pharmacy
	2	DL-2	Anhui	TCM market
	3	DL-3	Shanghai	Pharmacy
	4	DL-4	Shanghai	Pharmacy
	5	DL-5	Shanghai	TCM market
	6	DL-6	Shanghai	TCM market
	7	DL-7	Guangxi	Pharmacy
	8	DL-8	Guangxi	TCM market
	9	DL-9	Guangxi	TCM market
	10	DL-10	Hainan	Pharmacy
	11	DL-11	Hainan	Pharmacy
	12	DL-12	Hainan	Pharmacy
	13	DL-13	Zhejiang	Pharmacy
Leech	1	SZ-1	Jiangsu	Pharmacy
	2	SZ-2	Jiangsu	TCM market
	3	SZ-2	Yunnan	TCM market
	4	SZ-2	Yunnan	TCM market
	5	SZ-2	Shandong	Pharmacy
	6	SZ-2	Shandong	Pharmacy
	7	SZ-2	Shandong	TCM market
	8	SZ-2	Heilongjiang	Pharmacy
	9	SZ-2	Heilongjiang	TCM market

Double-deionized water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). The Cd standard solution was purchased from the National Standard Material Research Center (Beijing, China). The internal standard solution containing In ($m/z = 115$, 100.0 $\mu\text{g/mL}$) and tuning solutions containing Li, Y, Ce, Tl, and Co were got from Agilent (Agilent Technologies, Folsom, CA, USA). Supra-pure trace metal-grade concentrated nitric acid (HNO_3 , 65.0%) was purchased from Merck (Merck, Munchen, Germany). Unless otherwise stated, chemicals for *in vitro* PBET digestion were purchased from Sigma Chemical (St. Louis, MO, USA). For cell cultures associated with Caco-2 and MDCK cell models, fetal bovine serum (FBS), dulbecco's modified Eagle's medium (DMEM) containing glucose, and 0.25% trypsin/EDTA were obtained from Gibco (Carlsbad, CA, USA).

Determination Of The Total Cd Concentrations Via Icp-ms

0.5g homogenous powder of earthworms and leeches was placed into microwave digestion vessels for digestion with 8.0 mL of HNO_3 . After digestion, excessive acid in the digestion vessels was dispelled. The digestion solution was then diluted to 50.0 mL with Milli-Q water. The Cd contents were analyzed based on our reported methods (Zuo et al., 2019) using the Agilent 7700X ICP-MS. For quality assurance, blanks and duplicates were carried out during the determination process. The internal standard In (m/z 115) was added to the blanks, calibration standards, and samples to compensate for the signal drift and matrix effects. The mean recovery rate of the internal standard solution was $106.9 \pm 3.7\%$. The mean recovery rate of the earthworm ($n = 9$) was also determined in order to control the accuracy of the method, which was $104.6 \pm 45.2\%$.

In Vitro Digestion

The bioaccessibility of Cd in the earthworm and leech samples was investigated by applying the *in vitro* PBET model according to our published methods (Zuo et al., 2020c). The model consisted of two compartments involving a gastric and an intestinal extraction phase (Ruby et al., 1996). Briefly, during the gastric extraction stage, 0.5 g of the samples was mixed with 50 mL simulated gastric solution (pH 2.0 adjusted by HCl). Then, the mixed solutions were incubated at 37°C , shaken for 1 h and centrifuged 5 min to collect the supernatant. Further, 5 mL of nitric acid was mixed with the concentrated supernatant for digestion. Finally, the 50 mL digested solution as the gastric fraction was analyzed by ICP-MS.

In the intestinal extraction stage, the residue from the gastric extraction stage was added to 50 mL simulated intestinal solution containing pancreatin and bile salts (pH 7.0, adjusted by NaHCO_3). The solution was incubated and shaken at 37°C for 4 h, centrifuged to collect the supernatant, and concentrated to 3 mL. Then Nitric acid (5 mL) was added to the concentrated supernatant for digestion. Finally, the 50 mL digested solution as the intestinal fraction was measured by ICP-MS.

The bioaccessibility of Cd in both the gastric and intestinal phases was calculated by the following equation:

$$\text{Bioaccessibility (\%)} = \frac{\text{bioaccessible Cd contents}}{\text{total Cd contents}} \times 100\%(1)$$

Caco-2 And Mdck Cell Model

Human colon adenocarcinoma Caco-2 cells and MDCK cells were purchased from Shanghai Institutes for Biological Science of the Chinese Academy of Medical Sciences (Shanghai, China). Both cell types were maintained in DMEM supplemented with 10% (v/v) FBS and 2.3 g/L sodium bicarbonate. These two types of cells were incubated in the condition of 95% air and 5% CO₂ at 37°C. The culture medium was refreshed every 2 d, and the cells were transferred when the cell density reached 80% confluence using 0.25% trypsin/EDTA.

Cell differentiation and uptake tests were carried out in a two-chamber well (apical and basolateral) with polyester membrane (12 mm diameter, pore size 0.4 μm; Millipore, Milford, MA, USA). In this system, Caco-2 cells and MDCK cells were seeded at 5.0× 10⁴ and 4×10⁵ cells/mL, respectively. To achieve mature cells and differentiated confluent cell monolayers, the Caco2 cells and MDCK cells were incubated for 21 days and 4 days, respectively, during which the culture medium was refreshed every 2 d. The transepithelial electrical resistance (TEER) measured by Millicell ERS (WPI Corporation, USA) was the used as an indicator of the integrity of the cell monolayer. Only monolayers with TEER > 200 Ω/cm² were selected for the uptake tests.

The uptake tests were carried out at 21 d and 4 d post seeding for the Caco-2 and MDCK cells, respectively. A 0.5 mL portion of the intestinal fraction was added to the apical side, and 0.5 mL of FBS-free DMEM was added to the basolateral side. After 4 h of incubation at 37°C (5% CO₂), the mediums from the apical and basal compartments were harvested separately to measure the transport of Cd across the monolayer. Then, cell monolayers were lysed and microwave digested. The Cd content in the cell lysate was ready for retention analysis using ICP-MS. Control cells were prepared for each assay.

The cellular uptake of Cd was expressed as the content of Cd absorbed by the Caco-2 or MDCK cells (sum of retention and transport) from the intestinal fraction divided by the Cd content in the intestinal fraction. The uptake of Cd was calculated using the following formula:

$$\text{Uptake (\%)} = \frac{\text{Cd in basolateral} + \text{Cd in cell monolayer}}{\text{Cd in intestinal fraction}} \times 100\% \quad (2)$$

The bioavailability of Cd was calculated as the content of Cd absorbed by the Caco-2 or MDCK cells (sum of retention and transport) from the intestinal fraction divided by the total Cd content in TAMs. The bioavailability of Cd was calculated as follows:

$$\text{Bioavailability (\%)} = \frac{\text{Cd in basolateral} + \text{Cd in cell monolayer}}{\text{total Cd contents}} \times 100\% \quad (3)$$

Health risk assessment

To evaluate the human health risks caused by exposure to Cd stemming from the consumption of TAMs, the value of target hazard quotient (THQ) was calculated using the following formula:

$$\text{THQ} = \frac{\text{EF} \times \text{Ed} \times \text{IR} \times \text{C} \times \text{SF} \times \text{BA}}{\text{W} \times \text{AT} \times \text{RfD}} \quad (4)$$

According to our questionnaire data, the exposure frequency (EF), the exposure duration (Ed), and the safety factor (SF) for TAM was 90 days/year, 20 years and 10, respectively. Besides the average body weight (W) and average time exposed to TAMs (AT) was 60kg and 365 days/year× 70 years, respectively (Mahmood et al. 2013). IR is the ingestion rate of TAMs, according to the maximum daily ingestion rate recorded in the Chinese Pharmacopoeia (The Pharmacopoeia Commission of the People's Republic of China, 2015). C is the total Cd contents in earthworms or leeches before extraction (mg/kg). BA is the bioavailability of Cd calculated by equation (3). RfD is the oral reference dose recommended by the US EPA for Cd, which is 1 µg/kg bw/day (Mahmood et al. 2013). If THQ is less than 1, no significant hazardous health effects resulting from exposure to TAMs are expected. If THQ is larger than 1, the health risk of the exposed population cannot be ignored.

Results

Total concentration of Cd in earthworms and leeches

The levels of Cd varied remarkably from earthworms to leeches. This was likely caused by the organisms' selective bio-accumulation of different TAMs for heavy metals, their specific living environments, and their respective locations in food chains (Vicente-Martorell et al., 2009; Wang et al., 2017; Amoozadeh et al., 2014). Total contents of Cd in different batches of earthworms and leeches were illustrated in Table 2. The mean levels of Cd in 13 batches of earthworms and 9 batches of leeches were 12.649 and 1.506 mg/kg, respectively. Some previous studies have reported Cd contents in the environment and food. Gu indicated that the mean Cd levels in marine organisms at different trophic levels in the South China Sea ranged from 0.04 to 0.53 mg/kg, which were much lower than those in earthworms or leeches (Gu et al., 2018). Cui reported that the average Cd concentration in fingered citron was 0.121 mg/kg, which was 0.01 times that of earthworms and 0.08 times of leeches in the present study (Cui et al., 2018). The average Cd levels in hops (*Humulus lupulus* L.) from different geographical origins were 0.03 to 0.04 mg/L, which were notably lower than those in earthworms or leeches in the present study (Liu et al., 2019). Thus, it is necessary to study the bioaccessibility of Cd in TAMs owing to their higher concentrations.

Table 2
Total and bioaccessible contents of heavy metals in earthworm and leech
(mg/kg)

Types	No.	Total	Gastric phase(G)	Intestinal phase(I)
Earthworm	1	24.774 ± 1.242	10.249 ± 0.287	7.377 ± 0.028
	2	11.111 ± 0.393	4.591 ± 0.083	3.444 ± 0.012
	3	33.606 ± 1.875	12.624 ± 0.281	9.160 ± 0.093
	4	13.406 ± 0.986	5.688 ± 0.309	4.428 ± 0.036
	5	8.319 ± 0.238	4.168 ± 0.298	4.141 ± 0.029
	6	16.691 ± 1.267	6.581 ± 0.398	6.309 ± 0.328
	7	5.216 ± 0.087	2.376 ± 0.082	2.051 ± 0.028
	8	8.215 ± 0.036	4.863 ± 0.093	2.607 ± 0.093
	9	11.184 ± 0.023	5.597 ± 0.092	3.168 ± 0.183
	10	4.625 ± 0.292	2.473 ± 0.329	1.724 ± 0.043
	11	6.724 ± 0.983	2.030 ± 0.498	2.017 ± 0.072
	12	9.682 ± 0.129	5.679 ± 0.086	3.182 ± 0.183
	13	10.885 ± 1.875	6.893 ± 0.287	3.370 ± 0.239
Leech	1	1.896 ± 0.065	0.876 ± 0.459	0.704 ± 0.029
	2	1.692 ± 0.082	0.896 ± 0.028	0.608 ± 0.098
	3	1.329 ± 0.069	0.675 ± 0.011	0.447 ± 0.023
	4	1.682 ± 0.025	0.876 ± 0.223	0.513 ± 0.038
	5	1.876 ± 0.093	0.674 ± 0.039	0.446 ± 0.026
	6	1.523 ± 0.086	0.765 ± 0.092	0.544 ± 0.048
	7	0.986 ± 0.022	0.587 ± 0.014	0.345 ± 0.018
	8	0.893 ± 0.013	0.398 ± 0.002	0.230 ± 0.002
	9	1.679 ± 0.094	0.896 ± 0.024	0.643 ± 0.038

Bioaccessible concentration and bioaccessibility of Cd in earthworms and leeches

Table 2 showed bioaccessible Cd in gastric (G) and intestinal (I) fractions. The bioaccessible concentrations of Cd in the gastric phase were 2.030-12.624 mg/kg for earthworms and 0.398–0.896 mg/kg for leeches, respectively. The bioaccessible contents of Cd in the intestinal phase ranged from 1.724 to 9.160 mg/kg for earthworms and from 0.230 to 0.704 mg/kg for leeches, respectively. The bioaccessibility of Cd in the

intestinal phase was lower than that in the gastric phase (Fig. 1), which was in accordance with previous studies. For instance, Intawongse and Dean found that the bioaccessibility of Cd in radish roots in the small intestinal phase (24.8%) was lower than that in the gastric phase (54.9%). This discovery may result from complex factors, involving the increased pH value from the gastric phase to the intestinal phase as well as the additional precipitation of Cd owing to the supplementation of bile salts and secretin in the intestinal phase (Ruby et al. 1993). In the present study, the bioaccessibility of Cd in the gastric phase was 30.19%-66.33% for earthworms and 35.93%-59.53% for leeches, respectively. The bioaccessibility of Cd in the intestinal phase was 27.26%-49.78% for earthworms and 23.80-38.27% for leeches, respectively. Some published data on the bioaccessibility of Cd associated with the environment are currently available (Karadas and Kara, 2011; Pelfrêne et al., 2013). Generally, the bioaccessibility in the present study was comparable with previous studies. Interestingly, studies have reported that the bioaccessibility of Cd is higher than that of many other toxic metals (Poggio et al., 2009; Pelfrêne et al., 2013).

Retention and transport of Cd in Caco-2 and MDCK cells

Bioaccessibility is only associated with the maximum oral bioavailability, because absorption in the intestinal epithelia cells could decrease the portion of Cd that reaches the body circulation (Versantvoort et al., 2005). Thus, in the present study, the *in vitro* PBET digestion was advanced by coupling with Caco-2 or MDCK cell cultures to offer a more accurate estimate of the uptake (retention + transport) in the intestine. Moreover, a mass balance was tested by comparing the levels of Cd originally added to the cells with the contents of Cd obtained after the cell uptake assays. In the case of Caco2 cells, the mass balance showed that the recovery rates were from 70.53–100.79%. In the case of MDCK cells, the recoveries ranged between 70.40% and 95.47%. In the cell monolayer of Caco-2 cells, the percentage of retention for Cd ranged from 3.69–24.10% in earthworms and from 5.15–16.05% in leeches (Table 3). In the MDCK cell monolayer, the retention percentage of Cd varied from 7.26–22.63% in earthworms and from 15.01–19.46% in leeches (Table 4). For transport in the Caco-2 cells, the ratios of transport for Cd were from 4.26–20.81% in earthworms and from 3.19–19.40% in leeches (Table 3). In MDCK cells, the transport ratios ranged from 7.81–25.63% in earthworms and from 11.94–24.07% in leeches (Table 4).

Table 3
Cd retention, transport, and uptake by Caco2 cells

Sample	No.	Apical compartment		Cell monolayer (retention)		Basolateral compartment (transport)		Total cellular uptake (%)	Bioavailability(%)
		mg/kg	%	mg/kg	%	mg/kg	%		
Earthworm	1	3.679	49.87	0.876	11.87	0.763	10.34	22.22	6.62
	2	2.786	80.88	0.127	3.69	0.238	6.91	10.60	3.29
	3	6.813	74.38	1.539	16.80	0.881	9.61	26.42	7.20
	4	2.378	53.70	1.067	24.10	0.189	4.26	28.36	9.37
	5	1.942	46.90	0.896	21.64	0.283	6.83	28.47	14.17
	6	3.877	61.46	0.686	10.87	0.485	7.69	18.57	7.02
	7	1.048	51.10	0.137	6.68	0.427	20.81	27.49	10.81
	8	1.463	56.13	0.368	14.12	0.141	5.41	19.53	6.20
	9	1.856	58.59	0.216	6.82	0.320	10.10	16.91	4.79
	10	0.875	50.77	0.236	13.69	0.174	10.10	23.79	8.86
	11	1.179	58.45	0.206	10.21	0.198	9.82	20.04	6.01
	12	1.982	62.29	0.402	12.63	0.308	9.68	22.31	7.33
	13	1.664	49.38	0.439	13.03	0.383	11.36	24.39	7.55
Leech	1	0.368	52.27	0.113	16.05	0.126	17.90	33.95	12.61
	2	0.391	64.36	0.068	11.19	0.059	9.77	20.96	7.53
	3	0.233	52.08	0.035	7.82	0.052	11.60	19.43	6.54
	4	0.348	67.89	0.058	11.30	0.094	18.32	29.62	9.04
	5	0.330	73.92	0.023	5.15	0.058	12.99	18.14	4.32
	6	0.282	51.84	0.082	15.06	0.029	5.33	20.39	7.29
	7	0.192	55.66	0.052	15.08	0.011	3.19	18.26	6.39
	8	0.128	55.73	0.023	10.01	0.045	19.40	29.41	7.56
	9	0.386	60.07	0.068	10.58	0.065	10.12	20.70	7.92

Table 4
Cd retention, transport, and uptake by MCKD cells

Sample	No.	Apical compartment		Cell monolayer (retention)		Basolateral compartment (transport)		Total cellular uptake (%)	Bioavailability(%)
		mg/kg	%	mg/kg	%	mg/kg	%		
Earthworm	1	3.746	50.78	1.237	16.77	1.143	15.49	32.26	9.61
	2	1.756	50.98	0.523	15.18	0.729	21.16	36.34	11.27
	3	3.687	40.25	1.364	14.89	2.347	25.63	40.52	11.04
	4	2.118	47.83	1.002	22.63	1.108	25.01	47.64	15.74
	5	1.658	40.04	0.527	12.73	0.730	17.63	30.36	15.11
	6	3.298	52.28	0.458	7.26	0.981	15.55	22.81	8.62
	7	1.251	61.00	0.259	12.63	0.269	13.12	25.74	10.12
	8	1.189	45.61	0.267	10.24	0.388	14.90	25.14	7.98
	9	1.249	39.44	0.345	10.89	0.769	24.29	35.18	9.96
	10	0.988	57.34	0.387	22.45	0.242	14.04	36.49	13.60
	11	1.148	56.91	0.167	8.28	0.158	7.81	16.09	4.83
	12	1.368	42.99	0.548	17.22	0.753	23.65	40.87	13.43
	13	1.717	50.95	0.423	12.55	0.651	19.32	31.87	9.87
Leech	1	0.351	49.91	0.137	19.46	0.124	17.61	37.07	13.77
	2	0.324	53.33	0.118	19.42	0.112	18.44	37.86	13.59
	3	0.174	38.91	0.075	16.76	0.102	22.80	39.56	13.32
	4	0.157	30.53	0.098	19.10	0.112	21.83	40.92	12.49
	5	0.196	43.91	0.067	15.01	0.055	12.42	27.43	6.53
	6	0.274	50.30	0.098	18.00	0.131	24.07	42.07	15.04
	7	0.203	58.85	0.057	16.53	0.041	11.94	28.47	9.96
	8	0.089	38.75	0.034	14.80	0.052	22.54	37.35	9.60
	9	0.303	47.15	0.112	17.43	0.135	20.98	38.41	14.70

Uptake and bioavailability of Cd in Caco-2 and MDCK cells

The fraction of the total cellular uptake (retention + transport) for Caco-2 and MDCK cells in earthworms ranged from 10.60–28.47% and 16.09–47.64%, respectively. In leeches, the total cellular uptake fractions

ranged from 18.14–33.95% and from 27.43 % to 42.07%, respectively. Our study compared the cellular uptake of Cd in earthworms and leeches between Caco-2 and MDCK cell lines, and the results revealed that in both types of TAMs, the uptake of Cd in MDCK cells was higher than that in Caco-2 cells; this was probably caused by the difference in looseness of the tight junctions (Lee et al., 2018). Moreover, the percentage of bioavailability for Caco-2 cells in earthworms and leeches was from 3.29–14.17% and 4.32 % to 12.61%, respectively. The percentage of bioavailability of MDCK cells in earthworms and leeches ranged from 4.83 % to 15.74% and 6.53 % to 15.04 %, respectively.

To the best of our knowledge, little valuable data on the bioavailability of detrimental metals in TCMs or TAMs are available, whereas the bioavailability of toxic metal elements associated with food, water, or soil has been investigated in several researches. Generally, the bioavailability of Cd in our study was comparable with those found in previous studies. For example, Fu and Cui reported that the bioavailability of Cd ranged from 4.0–15.9% for the vegetables (Fu and Cui, 2013). However, the bioavailability of Cd in the present study was higher or lower than some other studies reported on CdCl₂ solution and infant food (Eklund et al., 2003; Chan et al., 2007). Besides, in an *in vivo* experiment, the bioavailability of Cd was 0.8% and 0.6% in the kidneys and livers of rats, respectively (Yannai and Sachs, 1993). The uptake of Cd by Caco2 or MDCK cells is influenced by many factors. First, the gene expression of Cd in cells is directly associated with its uptake efficiency. The augmented uptake of Cd was attributed to the upregulation of MRP1 gene expression (Öhrvik et al., 2013). In addition, the uptake of Cd is related to the activity of its ionic form in solution. Studies have demonstrated that labile complexes of Cd enhanced bioavailability of metals in cells, possibly by the mechanism of alleviating diffusive limitations (Verheyen et al., 2012). Moreover, the temperature, pH, and other environmental factors greatly influence the uptake of Cd (Reeves and Chaney, 2008). Additionally, the uptake of Cd can be evidently modulated by the organism's requirement for essential metals, including Zn, Fe, and Ca. Because Cd is a non-essential element, specific carrier proteins/channels for transporting Cd are unlikely to exist. However, experimental evidence have suggested that proteins of essential metals, such as divalent metal transporter 1, were responsible for the transport of Cd. When these nutrient elements were in short supply, the accumulation and toxicity of Cd are enhanced (Vesey, 2010). Another study by Reeves and Chaney revealed that absorption of Cd from food could be decreased if the calcium contents were high (Reeves and Chaney, 2008). Furthermore, different types of matrixes seemed to play a significant role in the differences of bioavailability (Fu and Cui, 2013).

Conclusion

Excitingly, our study built up an innovative assessment strategy of Cd-associated health risks of TAMs based on *in vitro* PBET digestion/Caco2 and MDCK cell models for the first time. Moreover, the duration and frequency of exposure to TAMs was acquired from questionnaire information, and the safety factor of TAMs was applied to establish assessment models applicable to TAMs. Taken together, by the comprehensive usage all of the above, we figured out to innovate assessment approaches that were able to scientifically and precisely determine the health risks of Cd in TAMs. Our key findings suggested that health risks should be assessed based on the bioavailable Cd levels rather than the total levels to offer a more realistic and accurate assessment of toxins to humans. Importantly, an overestimation of health risks or wastage of medicine resources may be avoided. We hope the novel health risk assessment strategy applicable to TAMs based on *in*

vitro digestion/cellular uptake opened a window for the bio-accumulation of Cd in organisms as well as toxicological evaluation of TAMs, in order to improve public health by guiding the usage of TCMs in the clinic.

Abbreviations

TAM: Traditional animal medicines; PBET: the physiologically based extraction test; ICP-MS: inductively coupled plasma mass spectrometry; FBS: fetal bovine serum; DMEM: Dulbecco's modified eagle medium; TEER: transepithelial electrical resistance; THQ: target hazard quotient

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data are fully available without restriction.

Declaration of competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SCM and LCH designed the study. TTZ, FYL, and FG conducted the experiments. TTZ analyzed the data. TTZ wrote the manuscript. SCM, LS, SXX and BL revised the manuscript. All authors read and approved the final manuscript.

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Figures

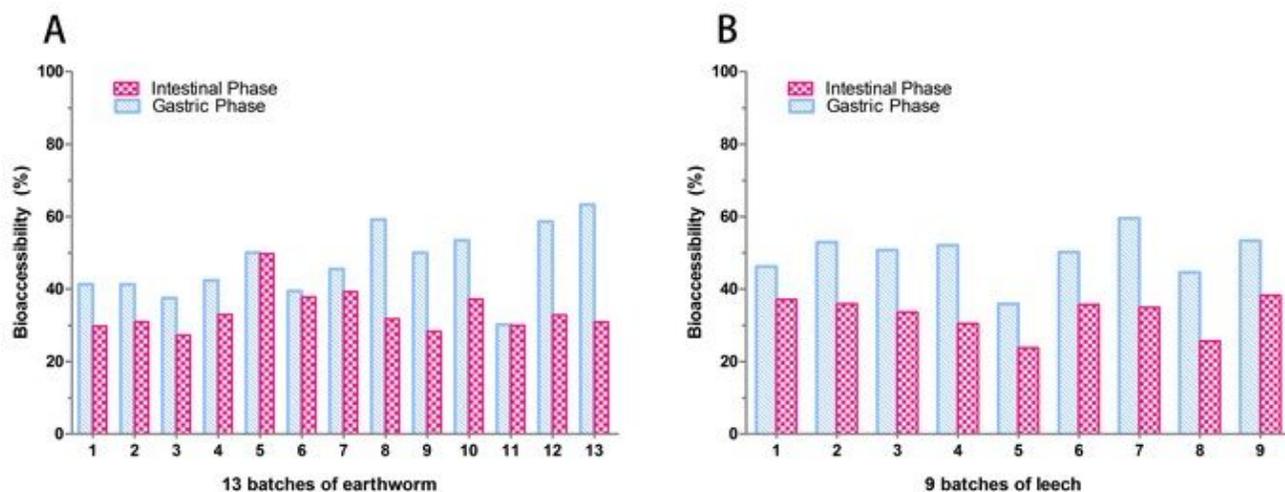


Figure 1

Percentage bioaccessibility of Cd in Gastric Phase and Intestinal Phase, respectively. A: Percentage bioaccessibility of Cd in 13 batches of earthworm samples. B: Percentage bioaccessibility of Cd in 9 batches of leech samples.

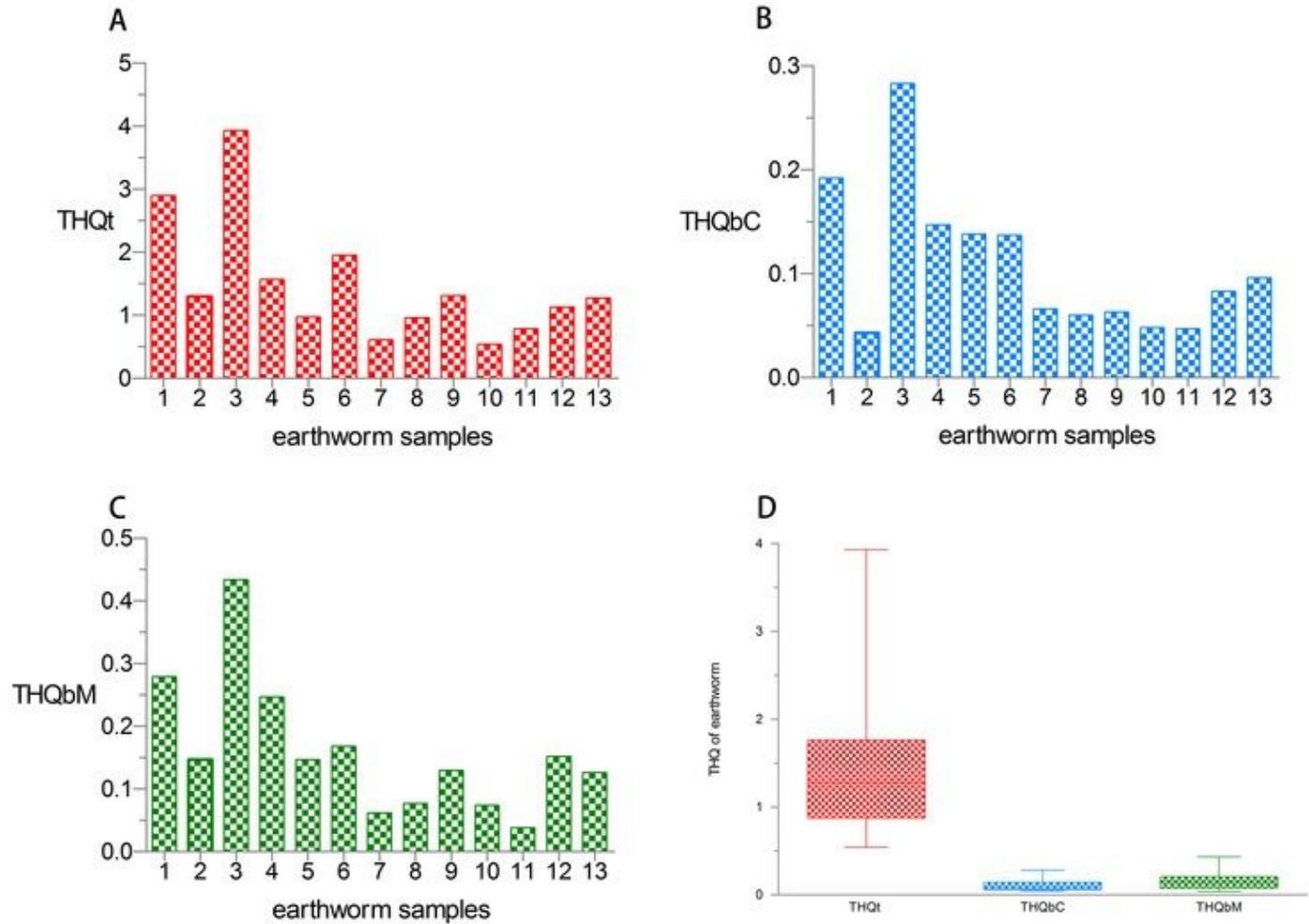


Figure 2

THQ for 13 batches of earthworm samples. A: THQt for earthworm calculated based on total concentration. B: THQbC for earthworm calculated based on bioavailable concentration from Caco2 cells. C: THQbM for earthworm calculated based on bioavailable concentration from MDCK cells. D: Comparison between THQt, THQbC, and THQbM.

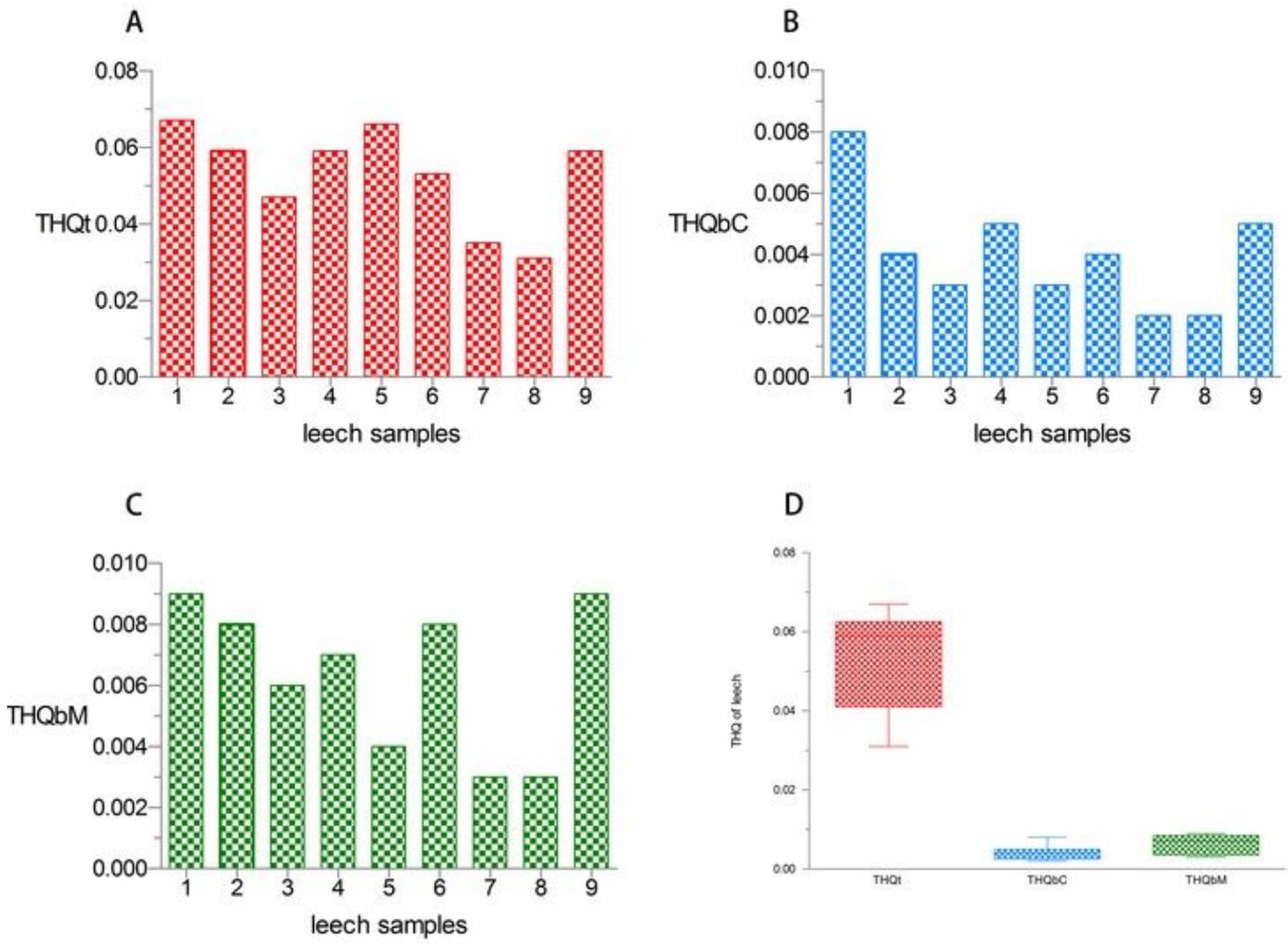


Figure 3

THQ for 9 batches of leech samples. A: THQt for leech calculated based on total concentration. B: THQbC for leech calculated based on bioavailable concentration from Caco2 cells. C: THQbM for leech calculated based on bioavailable concentration from MDCK cells. D: Comparison between THQt, THQbC , and THQb