The oral bioaccessibility and gingival cytotoxicity of metal(loid)s in wild vegetables from mining areas: Implication for human oral health

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Background: Heavy metal(loid)s are frequently detected in vegetables posing potential human health risks, especially for those grown around mining areas. However, the oral bioaccessibility and gingival cytotoxicity of heavy metals in wild vegetables remain unclear.

Methods: In this study, we assessed the total and bioaccessible Cr, As, Cd, Pb, and Ni in four wild vegetables from mining areas in Southwest China. In addition, the cytotoxicity and underlying mechanisms of vegetable saliva extracts on human gingival epithelial cells (HGEC) were studied.

Results: The Plantago asiatica L. (PAL) showed the highest bioaccessible Cr, As, Cd, and Pb, while the greatest bioaccessible Ni was in Taraxacum mongolicum (TMM). The Pteridium aquilinum (PAM), Chenopodium album L. (CAL), and TMM extracts decreased cell viability, induced apoptosis, caused DNA damage, and disrupted associated gene expressions. However, PAL extracts which have the highest bioaccessible heavy metals did not present adverse effects on HGEC, which may be due to its inhibition of apoptosis by upregulating p53 and Bcl-2.

Conclusion: Our results indicated that polluted vegetable intake caused toxic effects on human gingiva. The heavy metals in vegetables were not positively related to human health risks. Collectively, both bioaccessibility and toxic data should be considered for accurate risk assessment.

KEYWORDS
wild vegetables, heavy metal(loid)s, oral bioaccessibility, gingival cytotoxicity, health risk
Introduction

Heavy metal(loid)s are wildly distributed in the environment, such as soil, and plants (1). Anthropogenic activities are the main contributors to heavy metal(loid)s in the environment (2). Smelting and mining operations are the most important anthropogenic sources of heavy metal(loid)s. Mining of polymetallic ores associated with sulfide minerals, lead–zinc, and copper can release Cr, As, Cd, Pb, and Ni into the environment (3, 4), which may result in their accumulation in the surrounding plants such as wild vegetables.

Wild vegetables are uncultivated but edible plants and distributed around the world. By supplying the body with protein, minerals, vitamins, and certain hormone precursors, wild vegetables play an important role in the human daily diet, especially in some developing countries such as China, Russia, and Thailand (5). Also, some of them provide important health benefits because of their nutritional and pharmacological characteristics (6). Plantago asiatica L. and Taraxacum mongolicum are traditional medicinal and representative wild vegetables in China for their variously beneficial functions, such as anti-oxidative, anti-inflammatory, and anti-microbial properties. In addition, Pteridium aquilinum and Chenopodium album L. are popular wild vegetables as well due to affluent nutrients such as polyunsaturated fatty acids, vitamin C, and carotene (7–9). Since the wild vegetables are uncultivated by humans and the heavy metal(loid)s in them are not regulated and monitored, consuming the wild vegetables that grew up on contaminated sites would lead to human health risks (4). In addition, the bioaccessibility of heavy metal(loid)s was employed to accurately assess exposure health risks as an in vitro assay by predicting the in vivo bioavailable fractions of the heavy metal(loid)s (10, 11).

Among exposure pathways, oral ingestion is an important contributor. The oral cavity is the original phase of the alimentary system, which contains various organs, such as teeth, tongue, and salivary glands. Ingesting heavy-metal(loid)-contaminated foods could lead to acute toxicities or chronic accumulations in human tissues and contribute to certain diseases (12). For instance, periodontitis is a representative inflammatory disease in the gingival tissues (13). Gingival epithelial cells play important roles in maintaining oral immunologic homeostasis and protecting oral organs from inflammatory damage (14). Ingestion of heavy-metal(loid)-contaminated wild vegetables may expose gingival epithelial cells to heavy metal(loid)s, especially during the chewing procedure. As such, the adverse effects of heavy metal(loid)s on human gingival epithelial cells and the underlying molecular mechanisms are still unclear. However, heavy metal(loid)s have been demonstrated to cause cytotoxicity, induce apoptosis and DNA damage, and alter related genes expressions (e.g., Caspase-3, p53, and GADD45a) to human oral mucosa cells, gastric adenocarcinoma cells, and colonic epithelial cells (15–17).

As one of the known vegetable kingdoms, Yunnan has abundant plant resources such as wild vegetables. Residents consume wild vegetables purchased from markets or collected by themselves, especially in spring. To evaluate the oral health risk of heavy metal(loid)s consumption of wild vegetables from mining areas, in this study, we investigated the pollution levels of Cr, As, Cd, Pb, and Ni in four common edible wild vegetables as well as their corresponding growing soils from mining areas in Yunnan, Southwest China. Also, the toxic effects of wild vegetable extracts on human gingival epithelial cells were assessed by analyzing cell morphology, viability, apoptosis, intracellular DNA damage, and the expressions of related genes. Overall, this study aims to provide a refining health risk assessment method of the wild vegetable extracts including heavy metal(loid)s on human gingiva via ingestion exposure.

Materials and methods

Study area and sampling

The study area is an industrial district in the central region of Yunnan Province, Southwest China, which as a site for Cu mining and smelting (Figure 1), it is influenced by a subtropical plateau monsoon climate, holding an annual average temperature of 21.2°C, the average annual precipitation is 560.9 mm with a relative humidity of 76%. The rainy season comes in May every year, the plants will have a better living environment since then. The Cu mining and smelting sites had more than 2,000 years, which caused intermittent water and soil loss, along with discharged/ emitted wastewater, solid wastes, and dust. Heavy metal(loid)s would be accumulated in soils and plants surrounding the mines. However, there are few studies on the pollution status and health risk of heavy metal(loid)s in local wild edible vegetables.

Four sampling sites (S1–S4) were selected in the vicinity of the mines in Xiaojiang River Basin, Dongchuan District, Kunming city, Yunnan province, Southwest China. And four common types of local wild edible vegetables (eight samples) including Pteridium aquilinum (PAM), Plantago asiatica L. (PAL), Chenopodium album L. (CAL), and Taraxacum mongolicum (TMM), as well as their corresponding growing soils (eight samples) were collected from S1–S4 in June 2021. All wild vegetable samples were washed three times with distilled water and dried to constant weight at 75°C, then crushed with a porcelain mortar and pestle, and kept in a refrigerator before analyses. All soil samples were air-dried and grounded to pass through a 100-mesh nylon sieve before analyses.
Measurement of metal(loid)s contents

The total contents of heavy metal(loid)s in soils were analyzed by an X-ray fluorescence spectrometer (XRF, Emax 500). Due to XRF with a high limit of detection, the total and bioaccessible concentrations of heavy metal(loid)s in wild vegetables were determined by inductively coupled plasma mass spectrometry (ICP-MS, ICAPQR, Thermo Fisher Scientific, USA) (18). For total contents, wild vegetable samples (0.10 g, dry weight) were digested with concentrated HNO$_3$ (Guaranteed Reagent) and 30% H$_2$O$_2$ (Guaranteed Reagent), then analyzed by ICP-MS. Based on total concentrations of heavy metal(loid)s in all wild vegetables, we selected four wild vegetable samples for the following experiments.

Bioaccessible heavy metal(loid)s in wild vegetables were extracted using artificial saliva (pH = 7, Fusayama, Shanghai Lianshuo Biological Technology, China) (19). The compositions of the artificial saliva include NaCl, KCl, CaCl$_2$, NaH$_2$PO$_4$, and urea. Briefly, wild vegetable samples (0.50 g, dry weight) were dissolved in 20 mL artificial saliva and extracted at 150 rpm and 37°C in an oscillator for 24 h. After that, each extract was centrifuged and the supernatant was passed through a 0.22 µm sterile syringe filter (Corning, NY, USA), and collected in two sterile tubes, respectively. The content of the supernatant in one tube was applied for ICP-MS analysis, while the supernatant in another tube was used for cell experiments.

Cell culture and exposure

Human gingival epithelial cells (HGEC) were purchased from the American type culture collection (Manassas, VA, USA). HGEC were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in a humidified atmosphere with 5% CO$_2$ at 37°C, and the media was substituted with fresh medium every alternate day.

Before each exposure, the logarithmic growth phase HGEC was trypsinized and reseeded into 6/24/96-well plates overnight to allow cell attachment for use. Each wild vegetable extract was mixed with DMEM 1:4 to obtain the exposure solution. The diluted extracts were added to the 6/24/96-well plates and incubated for 24 h, with a blank artificial saliva solution as the control.

Cytotoxicity and apoptosis assay

To determine the impacts of extracts on HGEC proliferation, HGEC were cultured in a 96-well plate after overnight incubation at the density of $8 \times 10^3$ cells/100 µL/well. After exposure to the extracts for 24 h, cellular morphology was recorded by an inverted microscope (TS-100, Nikon, Japan). Simultaneously, a cell counting kit-8 assay kit (CCK-8, Yi Fei Xue Biotechnology, China) was employed to analyse cell proliferation. To calculate cell viability, the absorbance was
measured at 450 nm with a microplate reader (Molecular Devices LLC, USA).

Annexin V-FITC/PI apoptosis assay kit was used for cell apoptosis analysis. In brief, HGEC were seeded in 6-well plates (1 × 10⁶ cells per well) with DMEM for 24 h. After that, the spent DMEM was replaced with the exposure solution and treated for 24 h. After the exposures, cells were harvested and washed with cold phosphate buffer saline (PBS) three times and then immersed in 500 μL of PBS binding buffer (containing 5 μL Annexin V-FITC and 5 μL of PI-staining solution) for 15 min at room temperature in the dark. Approximately 1 × 10⁶ cells were loaded with a CyFlow® Cube 6 Flow cytometer (Sysmex Partec, Germany) to assess cell apoptosis, and then analyzed by FlowJo Version 10.0.6 software (BD Biosciences, USA).

DNA damage assay

Direct immunofluorescence staining was employed to assay the DNA damage of HGEC induced by extracts. Briefly, HGEC were replated into 24-well plates at 5 × 10⁴ cells/well for 1 day and then exposed to extracts for 24 h at 37°C. Cells were rinsed three times with PBS, fixed by 4% formaldehyde for 30 min, and permeabilized by 10% Triton X-100 for 15 min. After being washed three times with PBS, cells were blocked with 1% bovine serum albumin (BSA) for 60 min. Subsequently, cells were incubated overnight at 4°C with the rabbit monoclonal antibody anti-γ-H2AX (ab81299, Abcam, USA). After being maintained at room temperature for 1 h, cells were immersed in PBS for 5 min, then incubated by Goat Anti-Rabbit IgG (H+L) Fluor488-conjugated (Affinity Biosciences, USA) at room temperature for another 1 h. Last, the cells had a 10 min dark incubation at 37°C with 4’, 6-diamidino-2-phenylindole (DAPI, Service bio, China) for nuclear counterstaining. Immunofluorescent images were observed and photographed with an inverted microscope system (IX73, Olympus, Japan).

Gene expressions analyses

To further investigate the mechanisms of cell apoptosis and DNA damage, the associated regulatory gene expression levels were estimated. After each exposure, the total RNA of HGEC was extracted using the total RNA isolation kit (Yi Fei Xue, Quawell, USA). The cDNA was synthesized from total RNA by the commercial reverse transcription kit. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect related gene expression levels (Bax, Bcl-2, p53, p21, GADD45α, Caspase-3, Caspase-8, and Caspase-9) by applying SYBR Green qPCR Master Mix and Roche LightCycler 480II Real-Time PCR system (Roche, Swiss). The reaction cycle condition started at 95°C for 15 min, followed by 40 cycles for 15 s, then maintained at 60°C for 1 min, and β-Actin was utilized as an internal reference. The data were calculated by the 2−ΔΔCT method (20), with the primer sequences being listed in Table 1.

QA/QC and statistical analysis

Reagent blank, artificial saliva solution, and certified reference materials (GBW07405 and GBW10015a, Institute of Geophysical & Geochemical Exploration, China) were used to ensure quality control and assurance. The recovery rates were all above 80%. All the samples had triplicate determinations to ensure precise results. The accuracy of the measurements is assured within 10% RSD. ICP-MS detection limits of Cr, As, Cd, Pb, and Ni are 0.0002, 0.0002, 0.0003, 0.0001, and 0.002 mg/kg, respectively. XRF detection limits of Cr, As, Cd, Pb, and Ni are 5, 0.2, 0.05, 0.8, and 1 mg/kg, respectively. Statistical analysis was conducted by GraphPad Prism Version 8.0 software (GraphPad Software LLC, CA, USA). Data are presented as the mean ± standard deviation, and differences among exposure groups and the control group were analyzed by one-way ANOVA at p < 0.05.

Results and discussion

Contents of heavy metal(loid)s in wild vegetables

Total concentrations of Cr, As, Cd, Pb, and Ni (mg/kg, dry weight) in the wild vegetables were shown in Figure 2A. Generally, Ni or Pb had the highest concentrations among all heavy metal(loid)s in PAL (13.7 ± 0.83 mg/kg), TMM (3.99 ± 1.49 mg/kg), CAL (3.24 ± 1.65 mg/kg), and PAM (1.42 ± 0.51 mg/kg). Interestingly, Pearson correlation analyses indicated that Ni contents in wild vegetables were negatively correlated with those in soils (r = −0.99, p < 0.05). These results were consistent with the concentrations found in cabbages that grew up in the same areas, and atmospheric deposition of the sampling site probably increased the absorption of metal(loid)s in the overground part of wild vegetables, which affected the results (21, 22). Cr, As, and Cd revealed varied levels in different wild vegetables, with the lowest concentrations of Cr (0.19 ± 0.06 mg/kg) and As (0.13 ± 0.00 mg/kg) found in PAM, and Cd (0.31 ± 0.12 mg/kg) in CAL. The results also showed lower levels of Cr, Cd, and Pb in TMM compared with the previous study (23), which could be explained by the fact that lower contamination levels of the corresponding soils in the current study (Table 2). In addition, PAL was contaminated by the highest levels of Cr,
TABLE 1 Primers for qRT-PCR of cell apoptosis regulatory genes.

| Gene      | Forward primer (5′−3′)                                                                 | Reserve primer (5′−3′)                                                                 | Accession no. | Production size (bp) |
|-----------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------|----------------------|
| Bax       | CCGAGAGGCTCTTTTTTCGAG                                                                | CCAGCCTGTGATGTTCCTGAT                                                                | NM_138761.3   | 155                  |
| Bcl-2     | GGTGAGCAGTACGGAGGTGG                                                               | CCGTGCAGGACTCAGTGTCACCC                                                               | NM_000633.2   | 89                   |
| p53       | CAGACAGTACGGAGGTGG                                                                 | TCATCCAAATACCTCGACGGC                                                                | NM_000346.5   | 125                  |
| p21       | TTGCGGTCAGAAACCAGCC                                                                  | AAAGTGAAATGTCCTGACGGCT                                                              | NM_001291549.1| 139                  |
| GADD45α   | GAGAGGAGGAGAAGACGGAGAAGGA                                                          | CAGTGATGCGGCTGACT                                                                   | NM_001924     | 87                   |
| Caspase-3  | CATGGAAGTACGGAGATGGG                                                                | TCGTACAGAGACGGAGATGGC                                                               | NM_004346.3   | 139                  |
| Caspase-8  | CCGACTCTCCCGAAGAAACGGA                                                             | TCAAAGTCTGCGTGAAGAGCC                                                              | NM_033353.3   | 199                  |
| Caspase-9  | CGGCTCAGGAGATTGGCGAACACC                                                         | GCATTTCCTCCAACACTCTCAA                                                              | NM_032996     | 116                  |
| β-Actin   | GTACACTGGCATCGTGATGGA                                                              | CCGTCATGGCAAATGGTGAT                                                                | NM_001101.3   | 323                  |

As, Cd, Pb, and Ni among four wild vegetables, with average concentrations being 5.72 ± 0.61, 7.07 ± 0.48, 0.96 ± 0.14, 13.7 ± 0.83, and 6.20 ± 0.90 mg/kg, respectively. Cr and Cd levels were lower when compared with Abbasi et al. (6) study, but Pb was higher in our study, which may be attributed to the different industrial activities. In addition, PAL corresponding soil did not have the highest concentrations of metalloid(s) compared with the other three soils (Table 2), indicating that PAL easily accumulated the above-mentioned metalloid(s), especially for As, which is consistent with previous studies (24, 25). Although the concentrations of As, Cd, Pb, and Ni in PAM corresponding soils were higher compared to the other three vegetable soils (Table 2), the levels of heavy metalloid(s) in PAM were lower than those in the others. The differences in metalloid(s) contents of wild vegetables and their corresponding soils may be caused by the different contents of metalloid(s) in environmental matrices as well as uptake capacities and preferences of various plant species (2, 24, 26). Furthermore, bioaccumulation coefficients of metalloid(s), soil physical and chemical characteristics, and environmental parameters also play important roles in the transportation and accumulation of heavy metalloid(s) from soils to plants (1, 27). In addition, the average concentrations of heavy metalloid(s) in wild vegetables exceeded the leafy vegetables regulatory limit (GB2762–2017, China; WHO/FAO, 2019).

Bioaccessible concentrations of Cr, As, Cd, Pb, and Ni in wild vegetables were presented in Figure 2B. Among all the wild vegetables, the highest bioaccessible concentrations of Cr (0.04 ± 0.008 mg/kg), As (1.76 ± 0.13 mg/kg), Cd (0.045 ± 0.006 mg/kg), and Pb (0.03 ± 0.004 mg/kg) were found in PAL, consistent with the results of total concentrations. As a bioremediation plant, PAL can accumulate various heavy metalloid(s) (24). The highest bioaccessible Ni (1.47 ± 0.23 mg/kg) was found in TMM, which is consistent with a previous study (7). Especially, the bioaccessible Pb in PAL was reduced to 0.032 ± 0.003 mg/kg compared with its total concentration of 13.7 ± 0.83 mg/kg, similar to the other study (28). Since inorganic Pb (II) is the main lead speciation in food (29) and it is barely soluble in alkaline solutions, the soluble fraction of Pb in extracts (pH = 7.5–9.3) should be low. Although the bioaccessible concentrations of heavy metalloid(s) in wild vegetables were much lower than the total concentrations, we further conducted cytotoxicity experiments to investigate whether these bioaccessible extracts have adverse effects on HGEC.

Wild vegetable extracts induced cytotoxicity

The observations of cell morphology and viability are significant visualized indicators to evaluate cytotoxicity, which were widely employed to assay cell survival and proliferation (16, 30). Nevertheless, the impacts of bioaccessible metalloid(s) in vegetables on the morphology and viability of human gingival cells were not reported. Our previous study indicated gastric bioaccessible metals in cabbage did not cause the alteration of cell viability and morphology in human gastric adenocarcinoma cells (31). In this study, transformed cell morphologies were observed under all exposures excluding PAL, and cell viabilities were decreased across all exposures (Figures 3A–E). Specifically, HGEC exhibited varied levels of cell viability inhibition from 12 to 29% after being exposed to the extracts, especially after being exposed to TMM extracts. TMM extracts had the highest bioaccessible concentration of Ni among all vegetables (Figure 2B), and Ni was demonstrated to decrease cell viability and elicit cytotoxicity in oral mucosa cells and gingival fibroblasts (15, 32). Consistently, bioaccessible Ni was negatively correlated with the cell viability (r = −0.92, p < 0.05) in this study among all vegetables (Table 3).

The changes in cellular morphology were consistent with the results of cell viability besides the exposure to PAL extract (Figures 3A–E). As one of the most important traditional Chinese herbs, PAL is rich in beneficial nutrients...
such as PAL polysaccharides, which could alleviate the cytotoxicity caused by environmental pollutants (8). We speculated PAL polysaccharides play an important role in mediating the extract-induced cytotoxicity of PAL. Specifically, the typical cobblestone and polygonal appearances of confluent monolayers were clear in the control group (Figure 3A) and the PAL exposure group (Figure 3C). However, HGEC emerged with loose and irregular morphologies along with increased floated and round cells under the exposures of PAM, CAL, and TMM extracts (Figures 3B,D,E), revealing cell deaths. Since these results cannot firmly confirm that bioaccessible heavy metal(loid)s are the causes of cell death, further experiments are crucial to elucidate the mechanisms of the observed cytotoxicity.

Wild vegetable extracts triggered cell apoptosis

As a gene-controlled cell death under stress conditions, apoptosis plays an indispensable role in maintaining tissue homeostasis by eliminating cells, but disordered apoptosis induced by xenobiotics is a pathological phenomenon (33). Apoptosis of HGEC after being exposed to extracts for 24 h are shown in (Figure 4). In this study, Annexin V-FITC/PI double staining tests detected cell apoptosis after exposing HGEC to PAM, CAL, and TMM extracts (Figures 4C,E,F), which was consistent with the corresponding decreases in cell viability (Figure 3F) and changes in cell morphology (Figures 3B,D,E). Similarly, studies reported that

Table 2: Total concentrations (mg/kg) of heavy metal(loid)s in wild vegetables and their corresponding growing soils.

| Site | Sample | Wild vegetable | Soil |
|------|--------|----------------|------|
|      |        | Cr  | As  | Cd  | Pb  | Ni  | Cr  | As  | Cd  | Pb  | Ni  |
| S3   | TMM-1  | 0.64 ± 0.36 | 0.88 ± 0.55 | 0.59 ± 0.19 | 2.08 ± 0.08 | 2.27 ± 0.39 | 83.63 ± 5.31 | 50.3 ± 4.25 | 1.83 ± 0.09 | 221.9 ± 9.94 | 69.30 ± 1.84 |
| S3   | PAM    | 0.19 ± 0.06 | 0.13 ± 0.00 | 0.67 ± 0.22 | 0.87 ± 0.25 | 1.42 ± 0.51 | 49.45 ± 0.76 | 115.7 ± 1.23 | 4.48 ± 0.03 | 193.4 ± 0.30 | 90.17 ± 1.26 |
| S3   | CAL-1  | 2.02 ± 0.59 | 1.07 ± 0.19 | 2.49 ± 0.37 | 5.60 ± 0.36 | 1.71 ± 0.05 | 66.40 ± 1.25 | 39.7 ± 0.96 | 1.73 ± 0.04 | 149.1 ± 0.78 | 62.25 ± 0.38 |
| S2   | TMM-2  | 4.02 ± 0.81 | 2.59 ± 0.49 | 0.97 ± 0.06 | 3.97 ± 0.47 | 2.51 ± 0.43 | 34.90 ± 1.17 | 20.9 ± 0.72 | 0.96 ± 0.01 | 26.23 ± 0.49 | 26.70 ± 1.51 |
| S2   | PAL    | 5.72 ± 0.61 | 7.07 ± 0.48 | 0.96 ± 0.14 | 13.7 ± 0.83 | 6.20 ± 0.90 | 117.8 ± 5.65 | 7.30 ± 0.60 | 0.54 ± 0.01 | 34.90 ± 0.60 | 11.25 ± 0.05 |
| S1   | CAL-2  | 1.04 ± 0.61 | 2.45 ± 0.16 | 0.43 ± 0.29 | 2.10 ± 1.02 | 2.55 ± 0.15 | 59.40 ± 3.35 | 52.5 ± 3.86 | 2.11 ± 0.25 | 50.73 ± 2.78 | 88.87 ± 3.63 |
| S4   | CAL    | 0.72 ± 0.61 | 0.64 ± 0.39 | 0.31 ± 0.12 | 0.90 ± 0.60 | 3.24 ± 1.65 | 161.9 ± 5.64 | 8.31 ± 0.02 | 0.72 ± 0.03 | 12.40 ± 1.97 | 62.50 ± 1.32 |
| S3   | TMM    | 1.26 ± 0.50 | 0.37 ± 0.05 | 0.82 ± 0.16 | 1.19 ± 0.53 | 3.99 ± 1.49 | 130.2 ± 5.05 | 25.5 ± 2.48 | 1.09 ± 0.17 | 57.40 ± 2.95 | 51.15 ± 1.15 |

Regulatory limit a,b) 0.5 0.5 0.2 0.3 0.5

background value c)

0.218 0.406 0.425

a) The results were presented in mean value ± SD (standard deviations).
b) Regulatory limits of Cr, As, Cd, and Pb in vegetables recommended by the WHO/China are 0.5, 0.5, 0.2, and 0.3 mg/kg in leafy vegetables, respectively.
c) Background value: soil background value of Yunnan province, southwest China.

d) Not given.
TABLE 3 Pearson correlation coefficients between wild vegetable bioaccessible metal(loid)s and various toxicity indexes (cell viability, apoptosis, and genes) in HGEC after exposures to four extracts for 24 h.

|                  | Cr    | As    | Cd    | Pb    | Ni    |
|------------------|-------|-------|-------|-------|-------|
| Cell viability   | 0.36  | 0.03  | −0.66 | 0.22  | −0.92*|
| Cell apoptosis   | −0.03 | −0.38 | −0.62 | −0.22 | −0.42 |
| Genes            |       |       |       |       |       |
| Bax              | 0.12  | 0.23  | −0.16 | 0.24  | −0.52 |
| Bcl-2            | 0.80  | 0.90  | 0.47  | 0.89  | −0.26 |
| Caspase-8        | −0.06 | 0.03  | −0.31 | 0.04  | −0.54 |
| Caspase-9        | 0.16  | 0.25  | −0.18 | 0.27  | −0.56 |
| Caspase-3        | −0.23 | −0.17 | −0.48 | −0.15 | −0.56 |
| GADD45α         | −0.18 | −0.48 | −0.96 | −0.31 | −0.84 |
| p21              | 0.35  | 0.41  | −0.13 | 0.44  | −0.63 |
| p53              | 0.89  | 0.99  | 0.64  | 0.96  | −0.10 |

*The absolute values of coefficients >0.9 between bioaccessible metal(loid)s and various toxicity indexes are bold.

Heavy metal(loid)s in environmental matrices (e.g., dust, air particulate matter, and foods) caused cytotoxicity and induced apoptosis of human cell lines (34–36). Furthermore, the most elevated apoptotic ratio (39.7%) was observed under the exposure of CAL extract compared with the control group (1.82%), implicating CAL extract was more potent to induce cytotoxicity and apoptosis than that of other extracts (37). However, the PAL extract, which had the highest bioaccessible Cr, As, Cd, and Pb, did not decrease cell viability, change cell morphology, and induce cell apoptosis. It is possibly due to the protective effects of PAL polysaccharides, such as alleviating the cytotoxicity caused by environmental pollutants and inducing the maturation of dendritic cells (8, 38). Thus, we speculated PAL polysaccharides extracted by the artificial saliva may play pivotal roles in inhibiting PAL-extract-induced apoptosis. These results indicated the nutrients in the vegetables may also influence the contaminant-induced toxicity, thus the human health risk assessments simply based on the total concentrations of heavy metal(loid)s may not be accurate enough (39).

Wild vegetable extracts caused DNA damage

To further elucidate how extracts triggered HGEC apoptosis, a DNA damage assay was performed (40). The damage and repair of DNA play important roles in cell transformation and cell death in the human oral cavity (41), while DNA damage as a blockage in DNA replication could lead to cell apoptosis (42). Histone H2AX phosphorylation on serine 139 is an early signaling event triggered by DNA double-strand breaks. Thus, as the phosphorylated version of histone H2AX, γ-H2AX is a sensitive marker for DNA damage (43).

To further confirm our hypothesis that the observed HGEC apoptosis was caused by extract-induced DNA damage, we assessed DNA damages after the exposures to extracts by using immunofluorescence staining (Figure 5). The results showed that the exposure groups of PAM, CAL, and TMM extracts presented more H2AX phosphorylation focal points (Figures 5C,F,L,O) compared with that in the control group, while the level of DNA damages in the PAL exposure group and control group are similar (Figures 5A,C). These results indicated the extracts of PAM, CAL, and TMM induced DNA damage in HGEC, which is consistent with the results of cell apoptosis (Figure 4G), morphology (Figures 3B,D,E), and viability (Figure 3F).

Moreover, PAM and CAL extracts induced more severe DNA damage compared with TMM extract (Figures 5F,L,O), even
Apoptosis of HGEC after being exposed to extracts for 24 h. FL1 represents Annexin V-FITC staining and FL2 represents PI staining. HGEC populations (A, black circle) and apoptosis in the control group (B, CK). Apoptosis of HGEC after being exposed to PAM (C), PAL (D), CAL (E), and TMM (F) extracts: necrotic cells were shown in the Q1 quadrant, viable (late) apoptotic cells and non-viable (early) apoptotic cells were distributed in Q2 and Q3 quadrant, Q4 quadrant was living cells. The statistical graph showed cell apoptosis rate under different treatments (Q2 + Q3 quadrant) (G). Data were presented in mean ± SD. The different letters indicate significant differences (p < 0.05).

FIGURE 5
Effects of extracts (PAM: D–F, PAL: G–I, CAL: J–L, and TMM: M–O) on γ-H2AX protein in HGEC after being exposed for 24 h. Background control (CK) was artificial saliva alone (A–C). The immunofluorescent photographs of the phosphorylation of histone H2AX in γ-H2AX-stained HGEC (Green: B,E,H,K,N) and the counterstained nuclei with DAPI (Blue: A,D,G,J,M) were captured. Blue dots were cell nuclei, green dots indicated DNA damage. The magnification was 60× and the scale bar (A→O: yellow, located in the lower right corner) is 20 μm.

Wild vegetable extracts altered genes expressions

Measuring gene expression is an effective approach to evaluating the underlying mechanism of cell apoptosis (41, 48). Extrinsic (e.g., caspase-3, -8, and -9, Bcl-2 genes) and intrinsic (e.g., Bax, Bcl-2, caspase-9, p53 genes) related pathways were recognized as two primary modes of apoptosis (49, 50). Specifically, caspases play important roles in the initiation (caspase-8, -9, and -10) and effector (caspase-3, -6, and -7) phases of apoptosis. Furthermore, the Bcl-2 family of proto-oncogenes is the key factor to regulate apoptosis, involving...
The mRNA expression levels of cell apoptosis regulatory genes (A: Bax, B: Bcl-2, C: Caspase-8, D: Caspase-9, E: Caspase-3, F: GADD45α, G: p53, and H: p21) in HGEC after being exposed to four extracts compared with the control group. The control group (CK) was treated by DMEM only. Results were represented as mean ± SD of triplicate experiments. The different letters represent significant differences at p < 0.05.

To further explore the underlying molecular mechanism of HGEC apoptosis induced by extracts, we assessed the genotoxicity of these extracts by measuring the alterations of regulatory gene expressions in HGEC after each exposure. The results showed that after being exposed to the PAM extract for 24 h, the mRNA levels of Bax, Bcl-2, p21, GADD45α, Caspase-3, -8, -9 in HGEC were up-regulated from 1.6 to

the anti-apoptotic gene (Bcl-2) and pro-apoptotic gene (Bax) (49). Tumor suppressor gene p53 controls genomic integrity, cell proliferation, and cell death through complex signaling networks. Some studies also demonstrated that p53 is vital to induce cell apoptosis (51, 52). As important downstream genes of p53, GADD45α and p21 are involved in growth arrest and apoptosis (53).
6.6 folds (Figures 6A–F,H), while p53 was maintained at the same level (Figure 6G). This result explained the observations of HGEC apoptosis under PAM extract exposure (Figure 4C) since the activations of Caspase-3, -8, and -9 induce DNA damage and lead to cell apoptosis (15, 54). After being exposed to CAL extract, p21 and GADD45α were significantly up-regulated (p < 0.05), which indicated CAL-induced apoptosis was probably due to the induced DNA damage pathways. In the TMM extract exposure group, only p21 was up-regulated (Figure 6H), while Bax, Caspase-9, and p53 were significantly down-regulated (p < 0.05) (Figures 6A,D,G), and the rest of the measured genes were barely altered (Figures 6B,C,E,F). The up-regulation of the p21 gene usually causes growth arrest and promotes DNA damage, eventually leading to cell apoptosis (30).

In contrast, the expression levels of all measured genes were up-regulated in the exposure group of PAL extract (Figures 6A–H) but did not lead to HGEC apoptosis (Figure 4D). It implicates certain chemicals in PAL extracts such as PAL polysaccharides restrained extract-induced apoptosis, which is consistent with the prior cytotoxicity results and previous studies (55, 56). In addition, Pearson correlation analyses between bioaccessible metal(loid)s in wild vegetables and gene expressions of HGEC after exposures were performed. We found bioaccessible As was positively correlated with the mRNA expression of p53 (r = 0.99, p < 0.05) (Table 3), indicating As induced HGEC apoptosis may relate to the expression of p53, consistent with a previous study (57). In contrast, bioaccessible Cd was negatively correlated with the mRNA expression of p53 (r = −0.96, p < 0.05) (Table 3), our study found that a low concentration of Cd accelerated human gastric epithelial cells proliferation, which may decrease the mRNA expression of p53 (16).

In brief, our results showed the observed HGEC apoptosis induced by the extracts may mainly attribute to the DNA damage pathways. Furthermore, higher expression levels of GADD45α and p21 genes showed DNA damage is the key pathway that induced the HGEC apoptosis under exposure to PAM and CAL extracts. However, the alterations of GADD45α expressions were inconsistent with the results of DNA damage, HGEC morphology, and HGEC viability under the exposures of PAL and TMM extracts, and polygenic regulation is probably the main reason for that inconsistency (43, 58). Overall, three extracts (PAM, CAL, and TMM) induced HGEC apoptosis and DNA damage as well as altered the expressions of p53, GADD45α, and p21 genes. These results demonstrated DNA damage may play an important role in the observed HGEC apoptosis induced by the three extracts.

Conclusions

In this study, Ni or Pb was predominant in all vegetable samples, with Plantago asiatica L. (PAL), Taraxacum mongolicum (TMM), Chenopodium album L. (CAL), and Pteridium aquilinum (PAM) being 13.7, 3.99, 3.24, and 1.42 mg/kg, respectively. PAL had the highest bioaccessible Cr, As, Cd, and Pb, while the greatest bioaccessible Ni was found in TMM. The PAM, CAL, and TMM extracts decreased cell viability and triggered cellular apoptosis. The bioaccessible Ni was negatively correlated with cell viability. Increased DNA damage and abnormal related mRNA expression were also observed. Interestingly, PAL extract with the highest bioaccessible heavy metal(loid)s did not correlate with higher cytotoxicity, indicating beneficial nutrients such as PAL polysaccharides may play an important role in mediating the extract-induced-cytotoxicity of PAL. However, it is not clear which chemical composition and cytotoxicity are causal, and only eight wild vegetable species were harvested in this study due to the season limitation, more samples from the same site or different species would be collected in our follow-up study to further validate our conclusions. Taken together, consuming wild vegetables from mining areas may cause adverse health effects on the human oral cavity. Both oral bioaccessibility and cytotoxicity data should be considered for accurate human risk assessments.

Data availability statement

The original contributions presented in the study are included in the article-supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

WT: conceptualization, investigation, formal analysis, and writing—original draft. PG: investigation, conceptualization, formal analysis, and writing—review and editing. D-PZ, M-YZ, C-CW, and J-JL: investigation, validation, and formal analysis. Z-XW and J-MW: data analysis. Y-YN: supervision and conceptualization. PX: project administration, supervision, conceptualization, funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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