Evaluation of Nutritional Value, Heavy Metals Content, and Health Risk Assessment of Wild Edible Mushrooms in Mamit District, Mizoram, North East India

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Ab s t rac t

The present study was conducted to determine the nutritional value, heavy metal content, and any potential health risks related to the consumption of wild edible mushrooms. The samples of mushrooms were collected in an area with reasonable anthropogenic disturbances linked with tourism. The collected samples were explicitly identified by analyzing their morphological and molecular characteristics. Results revealed that the identified mushrooms *Lactifluus volemus* and *Vascellum pratense* contain a substantial amount of protein and carbohydrates with low-fat content. Therefore, the selected mushrooms were enriched with biomolecules that are essential for maintaining human nutrition and health. Further, the heavy metals content in both mushrooms ranged from 0.61 to 0.75 mg/kg Cd, 7.75 to 13.14 mg/kg Mn, 0.19 to 0.44 mg/kg Ni, and 0.33 to 0.85 mg/kg Pb. Health risk assessments based on the concentration of heavy metals in *Lf. volemus* and *V. pratense* validated their suitability for safe consumption. However, long-term consumption of these mushrooms may impose human health implications through carcinogenic effects.

Keywords: Estimated daily intake, Food, Health risk assessment, Heavy metals, Nutrients, Wild edible mushrooms.

Highlights

- The phylogenetic tree was constructed using maximum likelihood (ML) and neighbor-joining (NJ) approaches.
- *• Lf. volemus* and *V. pratense* can be consumed as dietary supplements because they contain substantial amounts of protein and carbohydrates with low fat content.
- The heavy metal content in the mushrooms was found to be in the following order: for *Lf. volemus*, Mn > Cd > Pb > Ni, and for *V. pratense*, Mn > Pb > Cd > Ni.
- Environmental conditions and the proximity to pollution sources influenced the concentration of heavy metals in mushrooms.
- Health risk assessment of both mushrooms showed hazard index (HI) values below 1, indicating a low non-carcinogenic risk. However, long-term consumption may pose a carcinogenic risk, especially due to cadmium (Cd) exposure.

International Journal of Plant and Environment (2024); **ISSN:** 2454-1117 (Print), 2455-202X (Online)

INTRODUCTION

Wild edible mushrooms have fascinated and sustained humans for millennia, and their culinary value as well as possible medicinal properties, makes them a flexible and intriguing addition to our diets (Hyde *et al*., 2019; Dimopoulou *et al*., 2022). Fruit bodies of approximately 200 mushroom species are consumed worldwide as a delicacy (Kalač, 2013). Edible mushrooms are a rich source of essential nutrients, including vitamins, minerals, dietary fiber, and bioactive compounds such as polysaccharides, steroids, and polyphenols (Mridu and Atri, 2017; Kour *et al*., 2022; Khumlianlal *et al*., 2022; Thachunglura *et al*., 2023a). These compounds possess various health-promoting properties, including antioxidant and anti-cancer effects (Cheung, 2013; Paloi *et al*., 2023).

The contamination of the natural environment by heavy metals has become a pressing global issue, primarily driven by the rapid processes of industrialization and urbanization (Rai, 2009; Rai *et al*., 2019). Bioremediation has shown remarkable efficacy in addressing this problem, making it a promising alternative for tackling heavy metal pollution (Rai *et al*., 2023; Rai, 2022). Fungi, including edible macrofungi, have been proven to be ideal for the biosorption of heavy metals (Das, 2005; Jing *et al*., 2021). Their ability to absorb heavy metals makes them a valuable resource in combatting environmental threats from

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How to cite this article: Thachunglura, V. L., Chawngthu, Z., Rai, P. K., Zothanzama, J. (2024). Evaluation of Nutritional Value, Heavy Metals Content, and Health Risk Assessment of Wild Edible Mushrooms in Mamit District, Mizoram, North East India. International Journal of Plant and Environment. 10(3), 79-85.

Submitted:25/11/2023 **Accepted:**07/08/2024 **Published:**30/11/2024

toxic metals, providing an effective solution for eliminating hazardous waste and its detrimental impacts (Chen *et al*., 2022; Kapahi and Sachdeva, 2017). Ectomycorrhizal mushrooms also protect host plants by absorbing and storing heavy metals from the soil (Ediriweera *et al*., 2022). In addition to their role in food and mycoremediation of heavy metals, wild edible mushrooms containing high levels of heavy metals can pose serious health risks to consumers (Dowlati *et al*., 2021; Liu *et al*., 2022), as these metal concentrations have been found to exceed safety limits set by numerous countries.

Tropical and subtropical regions are indeed known for their rich fungal biodiversity, and they are considered potential hotspots for the discovery of new fungal species (Karunarathna *et al*., 2017). The tropical and subtropical forest in Mizoram, which is a part of the Indo-Burma biodiversity hotspot, is also known to be rich in fungi (Zothanzama *et al*., 2011; Chawngthu *et al*., 2023; Thachunglura *et al*., 2023b). However, there is insufficient research available to evaluate the nutritional content and presence of heavy metals in wild edible mushrooms of this biodiversity rich landscape. Therefore, the study aims: (i) to evaluate the nutritional value and heavy metal content of *Lf. volemus* and *V. pratense* (ii) to assess the potential health risks linked to the consumption of these wild edible mushrooms.

MATERIAL AND METHODS

Collection, Storage and Samples Preparation

The samples were collected from Reiek forest, Mamit District, Mizoram (Fig. 1). Reiek forest is a site popular for tourism, which is situated in the north-western region of Mamit District, Mizoram, covering an area of 10 sq. km and positioned between 23.678 °N 92.603 °E . The Peak of Reiek Mountain rises above 1400 m above sea level. Reiek Forest is located far away from industrial zones and other pollution sources with moderate anthropogenic disturbances. However, the samples were collected near a popular hiking site that sees frequent human traffic.

During the collection, we carefully cleaned all the samples from soil substrates, tagged and photographs of the fruiting bodies were taken and stored them in air-tight containers before transporting them to the laboratory. From the collected samples, two species were selected for assessing their nutritional value, levels of heavy metals and health risk assessment. These specimens were dried by using a hot air oven at 45°C for 2 days without division into the stipe and pileus, ground into a fine powder and placed in sterilized containers at 4°C until further analysis.

Identification of Sample

Morphological Identification and Molecular Analysis

The collected samples were identified by employing a combination of both micro and macro-morphological

Fig. 1: Map indicating study sites and Sands (1978).

characteristics (Phillips *et al*., 2010; Zothanzama, 2011). For the microscopic examination, thin sections of the dried specimens were taken carefully using a feather blade. These sections were then placed in a 3% KOH solution and stained with a 2% aqueous phloxine solution. The sections were also mounted in either Lactophenol or a solution of 60% lactic acid and cotton blue (Zohmangaiha *et al*., 2019).

Molecular methods were performed following Zothanzama *et al*. (2018) and references therein, where DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method, followed by amplification of the internal transcribed spacer region (ITS) of the rDNA and sequenced with both primers (ITS1F and ITS4B). After the identification, the samples were deposited in the National History Museum of Mizoram (NHMM), Mizoram University.

PCR Amplification

PCR reactions were set up with 25.5 µL total volume, containing GoTaq Green Mastermix, nuclease-free water, bovine serum albumin, forward and reverse primers, and fungal DNA template. PCR was performed using primers ITS1-F and ITS4-B with the following parameters; 94°C for 5 minutes, followed by 35 cycles of 94°C for 1-minute, 52 °C for 1-minute and 72°C for 1 min with a final extension step of 72°C. The PCR amplicons were confirmed through electrophoresis on a 1% agarose gel stained with SYBR green, followed by visualization using a gel documentation system. Sanger sequencing was conducted employing both primers on an ABI 3730xl DNA sequencer. Sequences were aligned using Bioedit, compared to GenBank using BLASTn and submitted. The sequences were then aligned with Clustal W (Larkin *et al*., 2007) and the phylogenetic tree was established using Maximum Likelihood in RaxML GUI software with the available sequences representing all the species.

Phylogenetic Analysis

Phylogenetic analysis of the ITS gene data was carried out using both Maximum Likelihood (ML) and Neighbor-Joining (NJ) approaches. The ML and NJ searches were conducted using RaxML GUI. Alignment gaps were considered as missing data. The NJ trees were constructed based on the total character differences, with bootstrap values calculated from 1,000 replications.

Nutritional value and heavy metal

Nutritional Value

The moisture, fat, protein, fiber and ash content of the samples were analyzed by following the standard methods of the Association of Official Analytical Chemists (AOAC, 2000). The moisture content of the sample was determined by drying at 105°C. The difference between the sample weight before the drying and after the drying was used to measure the moisture content percentage. The protein content ($N \times 6.25$) was determined by the Kjeldahl method. Fat content was determined by using the Soxhlet extraction apparatus. Crude fiber content was determined using a fiber digester. Ash content was determined by incinerating the sample at 600°C in a muffle furnace. Energy values (Kcal) and total carbohydrate content were determined by following the equation given by Crisan

Heavy Metal

A porcelain crucible containing 1 g of dried specimen was heated to 450°C for 18 to 20 hours in muffle furnace to perform the chemical analysis for the determination of heavy metals. The resulting ash was dissolved in 1-mL of concentrated HNO₃ and heated once more for four hours to enable complete combustion of the sample. Following this, 1-mL each of concentrated H_2SO_4 , $HNO₃$, and $H₂O₂$ were added to the porcelain crucible containing ash and filtered using a syringe filter (0.2 µm). Three blank samples were treated the same way. The solution was diluted with deionized water to get the final amount of 10 mL (Isildak *et al*., 2004). An Atomic Absorption Spectrometer (AAS) AA 7000 Shimadzu was used to determine heavy metal (Cd, Mn, Ni and Pb) content in the samples. The detection limits may differ according to coexisting substances in the sample. Concentration limit of detection (i) Furnace: 0.01–0.09 ppb for Ni, Pb, and Mn, and less than 0.01 ppb in Cd (ii) Flame: Less than 0.01 ppm for Cd and between 0.01–0.09 ppm for Ni, Pb and Mn.

Human Health Risk Assessment

A human health risk assessment was conducted to evaluate the potential health risks associated with mushroom consumption. In this assessment, various indicators, including the estimated daily intake (EDI), target hazard quotient (THQ), hazard index (HI), and incremental lifetime cancer risk (ILCR) were determined using the equation given by Hu *et al*. (2017), Sun *et al*. (2017) and Fu *et al*. (2020).

The EDI is determined based on daily food intake, exposure duration, and food sample concentrations. EDI was calculated as follows:

$$
EDI = \frac{MC \times IR \times EF \times ED}{ET \times BW}
$$

Where MC represents the concentration of heavy metal in food/mushrooms in mg/kg, IR is the ingestion rate (6.6 \times 10⁻³ kg/ person/day). EF stands for exposure frequency (365 days/year). The exposure duration (ED) refers to the period for an adult, specifically 70 years. ET represents the exposure time (365 days/year \times 70 year) and BW represents the body weight of the individual (60 kg).

The evaluation of non-carcinogenic health risk associated with the consumption of heavy metals in mushrooms was conducted by using THQ as follows:

$$
THQ = \frac{EDI}{RfD}
$$

The value of EDI was divided by the oral reference dose (R*f*D) for heavy metals given by USEPA (2012) and USEPA (2017). The THQ serves as an index for evaluating the potential non-carcinogenic health risks that may arise from consuming food contaminated with toxic elements. The hazard index (HI) is determined by adding together the THQ values corresponding to each food element. An HI below 1 signifies acceptable chronic systemic risk, while an HI of 1 or higher indicates potential long-term non-carcinogenic health risks. The HI was calculated as follow:

 $HI = THQ_{Cd} + THQ_{Mn} + THQ_{Ni} + THQ_{Pb}$

The ILCR determines the cancer development risk arising from exposure to carcinogens through the consumption of mushrooms. ILCR was determined through the multiplication of the EDI by the relevant oral cancer slope factor (CSF). The values of CSF for Cd, and Pb were defined by USEPA (2010). An ILCR value falling within the range of 10−4 to 10−6 is typically regarded as an acceptable level of impact on the body. Risks exceeding 10−4 are seen as potential carcinogenic risks, while an ILCR value below 10−6 is not deemed to pose health risks (Hu *et al*., 2017).

RESULTS AND DISCUSSION

Morphological and Molecular Analysis

Morphological identification of the collected specimen from Reiek, Mizoram was first carried out according to characteristics of the spores and reproductive structures if discernible using previously described methods (Zothanzama *et al*., 2018). The fruiting bodies of the identified sample *Lf. volemus* and *V. pratense* are depicted in Fig. 2, along with some highlighted morphological characteristics. *Lf. volemus* bears many resemblances with *Lf. corrugis*, and both species are generally similar in appearance and fruit at the same time in the same place. However, *Lf*. *corrugis* differs from *Lf*. *volemus* with darker cap, stem, and gills, and it is popularly consumed in Mizoram. On the other hand, *V*. *pratense* is an edible mushroom when the fruiting body is white inside, but it is not consumed in Mizoram. Moreover, *V*. *pratense* nests among core species of *Lycoperdon* (*Lycoperdon* sensu stricto), and is now being considered as a member of the *Lycoperdon*: *L*. *pratense* (= *V*. *pratense*) (Krakhmalnyi *et al*., 2023).

Lactifluus volemus (Fr.) Kuntze

Pileus 4 to 14 cm abroad; convex with an inrolled margin, becoming flat with age, with a central depression; orangish brown, smooth to minute velvety, surface dry. Gills adnate, crowded, creamy white or pale white bruising brown where injured. Stipe 5–10 x 0.7–2.2 cm; same colored as cap or paler; dry. Flesh brittle, white, staining brown slowly when cut. Latex white, sticky, discoloring brown. Spores 7.5–9.5 x 7–9 µ; subglobose, well develop amyloid reticulum. Spore print white.

Vascellum pratense (Pers.) Kreisel

Basidiomata 2 to 4.2 cm across; subglobose to pyriform, squat stem, white when young, then yellowish flesh-colored, changing to light brown at maturity, outer layer scurfy, white, composed with small spines, inner wall smooth and shiny, white. Spore mass firm, white, becoming olive brown then powdery. Sterile base 1.4 to 1.9 cm high, well developed, separated from the

Fig. 2: Fruiting body of (A, B & C) *Lf. volemus*, (D, E, & F) *V. pratense*

spore mass by a distinct membrane. Basidiospores 3–4.5 x 3–5 μ, globose, finely warted, olive-brown.

In addition to the morphological identification, the two representative fungal isolates from Reiek, Mizoram were also confirmed using molecular analysis. The ITS1-5.8S-ITS2 sequences of these isolates were compared to 19 reference fungal taxa in the database. The species, along with their voucher numbers, GenBank accession numbers, and locations used for the analysis, are given in Table 1 and the phylogenetic tree in Fig. 3.

Nutrient Composition

The nutritional profiles of the identified species are presented in Table 2. Mushrooms are rich in nutrients. However, their nutritional composition varies due to factors such as the species, growth stage, and environmental conditions in which they are grown (Bellettini *et al*., 2019). Certain wild edible species also exhibit higher nutritional value compared to other varieties. The moisture content is also a critical factor that influences the nutrient content of mushrooms (Mattila *et al*., 2002).

Fig. 3: Phylogenetic tree - The evolutionary history was inferred by using the Maximum Likelihood method based on the GTR-GAMMA model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in RaxmlGUI 2.0. In the maximum Likelihood tree generated, the specimen, *Lf. volemus* and *V. pratense* were clustered with the related species with high support value. *Ganoderma enigmaticum* was used as the Outgroup.

Table 2: Nutritional value of *Lf. volemus* and *V*. *pratense* (g/100g)

Each value is expressed in mean \pm SEM, (n = 3) dry weight basis

Table 3: Concentration of heavy metals in wild edible mushrooms (mg/kg)

Each value is expressed in mean \pm SEM, ($n = 2$) dry weight basis.

Puffballs and milk-cap are known to be good sources of proteins and essential nutrients (Rugolo *et al*., 2022; Khumlianlal *et al*., 2022). In the present study, *Lf*. *volemus* has more protein, ash and fibre content than *V*. *pratense* based on g/100 g of mushroom sample, while *V*. *pratense* was observed to be higher in fat, carbohydrate and energy value. The protein content of edible mushrooms varies depending on several factors such as the type of mushroom, nutrient nitrogen levels, and geographical location (Bellettini *et al*., 2019; Thachunglura *et al*., 2024).

Heavy Metals

The concentrations of heavy metals (Cd, Mn, Ni and Pb) are shown in Table 3. *V*. *pratense* contained higher concentrations of heavy metals than *Lf*. *volemus*. In the present study, *Lf*. *volemus* was found to contain 0.61 mg/kg of Cd, 7.74 mg/kg of Mn, 0.19 mg/kg of Ni and 0.33 mg/kg of Pb, whereas *V*. *pratense* contained 0.75, 13.14, 0.44 and 0.85 mg/kg of Cd, Mn, Ni and Pb.

Keles and Gençcelep (2023) documented 0.72 mg/kg of Cd, 6.38 mg/kg of Mn, 2.12 mg/kg of Ni, and 0.12 mg/kg of Pb in *Lf*. *volemus* from Turkey. Our study corresponds closely to their report for Cd, Mn, and Pb concentrations. However, Ni concentration of 0.19 mg/kg is notably lower than their reported value. Zsigmond *et al*. (2015) also reported 0.87 mg/kg of Cd, 22.8 mg/kg of Mn, and 2.29 mg/kg of Ni in *Lf*. *volemus* from Romania. Gençcelep *et al*. (2009) reported 29 mg/kg of Mn in *V*. *pratense,* which surpasses the 13.14 mg/kg of Mn concentration observed in our findings, Uzun *et al*. (2011) also reported 53.8 mg/kg of Mn, 4.19 mg/kg of Ni, 0.27 mg/kg of Cd and <0.01 of Pb in *L*. *pratense*. The absorption of heavy metals in mushrooms is dynamic and influenced by environmental factors and growth conditions and mushrooms have been found to contain high levels of heavy metals, particularly in areas close to pollution sources such as highways with heavy traffic, sewage sludge landfills, and urban emission zones (Das, 2005; Kalač, 2013; Kokkoris *et al*., 2019).

Health Risk Assessment

The estimated daily intake (EDI) represents the amount of each metal that individuals consume through mushroom consumption. The target hazard quotient (THQ) is the ratio of exposure to a toxic element and the reference dose, representing the highest level at which no adverse health effects are anticipated (Antoine *et al*., 2017). The Hazard Index (HI) reflects the potential risk to human health resulting from the accumulation of various toxic elements (Orywal *et al*., 2021). The values for EDI, THQ and HI in *Lf*. *volemus* and *V*. *pratense* are shown in Table 4. It was found that *Lf. volemus* has an EDI of 0.067 x 10⁻³ for Cd, 0.853 x 10⁻³ for Mn, 0.021 x 10⁻³ for Ni, and 0.036 x 10-3 for Pb. On the other hand, *V. pratense* exhibited higher EDI values, 0.083 x 10⁻³ for Cd, 1.445 x 10⁻³ for Mn, 0.048 x 10⁻³ for Ni, and 0.094 x 10⁻³ for Pb. The higher EDI in *V. pratense* can be attributed to its higher accumulation of heavy metals.

The total THQ values for heavy metal were found in the order of Ni (0.003) < Pb (0.0085) Cd < (0.150) < Mn (0.106). The HI values of *Lf. volemus* and *V*. *pratense* were 0.084 and 0.122 respectively. This implies that *V*. *pratense* contains more levels of heavy metals, which may slightly increase the potential health risk associated with its consumption compared to *Lf*. *volemus*. However, HI values for both samples were below 1, suggesting a relatively low overall health risk linked to non-carcinogenic exposure to these heavy metals through mushroom consumption (Fu *et al*., 2020). Certain species from the same location have safe profiles, while others exceed safety limits, which can pose health risks (Sarikurkcu *et al*., 2020; Liu *et al*., 2021). This variability depends on both the collection site and the species and the THQ values were found to be very low, suggesting that the dietary intake of mushrooms, in terms of non-carcinogenic exposure to heavy metals, is unlikely to pose adverse effects on human health.

The carcinogenic health concerns of Cd and Pb were assessed. The ILCR values for Pb in *Lf. volemus* and *V. pratense* are 3.06 x 10^{-7} and 7.99 x 10^{-7} . The ILCR value for Pb was found to be very low, indicating that Pb accumulation in mushrooms poses no possible carcinogenic health concern. The ILCR values for Cd in *Lf*. *volemus* and *V*. *pratense* are 4.22 x 10-4 and 5.23 x 10⁻⁴, respectively, indicating a potential risk of carcinogenic effects with regular consumption. Furthermore, Pb has no negative effects on human health in both non-carcinogenic and carcinogenic aspects, while Cd is likely to contribute to carcinogenic health issues, consistent with the findings of Pb

Table 4: Health risk assessment

and Cd (Fu *et al*., 2020; Liu *et al*., 2015). Puffballs are recognized for accumulating elevated concentrations of Mercury (Hg), Lead (Pb), Cadmium (Cd), and Arsenic (As), posing potential dangers to human health, particularly due to Hg and As contamination (Nowakowski *et al*., 2021). The ability of various mushroom species to absorb heavy metals varies, and there is an increased health risk associated with consuming mushrooms that contain higher levels of heavy metals exceeding established safety limits of carcinogenic health risk (Orywal *et al*., 2021).

The EDI, THQ, HI and ILCR are useful parameters to calculate health risk of elements associated with mushroom intake (Nowakowski *et al*., 2021). It is important to note that the heavy metals content also varies within the same species (Kalač, 2013). The value of ash content is a key to determining the minerals present in the mushrooms. However, the ash content (Table 2) of *Lf*. *volemus* was observed to be higher than *V*. *pratense*. On the other hand, the heavy metals contents were observed to be higher in *V*. *pratense*. This can be attributed to the absence of mineral analysis for essential elements such as potassium (K) and phosphorus (P), which are recognized as major components of mushroom ash (Matilla *et al.,* 2001).

CONCLUSION

Lf. *corrugis* and *V. pratense* are enriched with essential biomolecules required to secure nutritional value and safeguard human health. However, the lack of adequate identification restricted their use by the people of Mizoram, considering their possible poisonous nature. This study, based on their scientific elucidation taxonomy and bio-chemical characterization, recommends for safe consumption without any immediate health risks*.* However, regular consumption may increase the potential for carcinogenic health issues ascribed to Cd accumulation. The uptake of heavy metals in mushrooms varies across species. Therefore, in order to have a comprehensive understanding of their food safety, pertinent attributes like nutritional value, heavy metal content, and associated health risks need to be frequently monitored.

ACKNOWLEDGMENT

The authors express their sincere gratitude to the Ministry of Tribal Affairs, Government of India, for providing the National Fellowship for Scheduled Tribes (NFST) to Mr. VL Thachunglura. The authors also want to express their gratitude to the Department of Chemistry, Department of Environmental Science, and Department of Biotechnology, Mizoram University, for their crucial assistance.

AUTHOR'S CONTRIBUTION

VL Thachunglura (VLT), Zohmangaiha Chawngthu (ZC), Prabhat Kumar Rai (PKR), and John Zothanzama (JZT) conceptualized the work. VLT and ZC conducted experiments aided by JZT. VLT and PKR drafted the MS. PKR reviewed the MS and made the necessary corrections as well.

CONFLICT OF INTEREST

None

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