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ORIGINAL ARTICLE

Biochemical, nutritional, and toxicological properties of the edible species *Phlebopus beniensis* with ethnomycological notes from Paraguay

Propriedades bioquímicas, nutricionais e toxicológicas da espécie comestível Phlebopus beniensis com notas etnomicológicas do Paraguai

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Abstract

In recent decades, mushrooms have been recognized as an important resource and efforts to characterize their potential to aid nutrition and human health have increased. *Phlebopus beniensis* specimen from a semi-urban community in Paraguay were analyzed for its biochemical properties, nutritional value, and toxicity. The species was identified by morpho-anatomical and molecular tools. Analyses for antioxidants by Ultraviolet-visible (UV-VIS) and nutritional content revealed that *P. beniensis* is a favorable source of antioxidants, proteins, carbohydrates, dietary fiber, and fats. Spectrometry through Gas Chromatography-Mass Spectrometry (GC-MS) further showcased other mycochemicals such as the specific phenolic, antioxidant, and fatty acid compounds that serve important biological roles in human diets. Applying an ethnomycological framework across local Paraguayan populations, we also report accounts of histories, knowledge, and usage of *P. beniensis* in South America among settlers and Paraguayan people.

Keywords: Antioxidant compounds; DPPH radicals; Edible mushrooms; GC-MS; Nutritional characterization; Secondary metabolites.

Resumo

Nas últimas décadas, os cogumelos foram reconhecidos como um recurso importante e os esforços para caracterizar seu potencial para auxiliar a nutrição e a saúde humana aumentaram. Espécimes de *Phlebopus beniensis* de uma comunidade semiurbana no Paraguai foram analisados quanto às suas propriedades bioquímicas, ao valor

nutricional e à toxicidade. A espécie foi identificada por ferramentas morfoanatômicas e moleculares. Análises de antioxidantes por UV-VIS e conteúdo nutricional revelaram que *P. beniensis* é uma fonte favorável de antioxidantes, proteínas, carboidratos, fibras alimentares e gorduras. A espectrometria por meio de cromatografia gasosa-espectrometria de massa (GC-EM) mostrou ainda outros micoquímicos, como os compostos fenólicos, antioxidantes e ácidos graxos específicos, que desempenham importantes papéis biológicos na dieta humana. Aplicando uma estrutura etnomicológica em populações locais paraguaias, também relatamos histórias, conhecimento e uso de *P. beniensis* na América do Sul entre colonos e povos paraguaios.

Palavras-chave: Compostos antioxidantes; Radicais de DPPH; Cogumelos comestíveis; CG-EM; Caracterização nutricional; Metabólitos secundários.

Highlights

- *Phlebopus beniensis* is described as a novel edible wild mushroom for the first time for the Neotropical region.
- Wild basidiomata of *Phlebopus beniensis* showed medium antioxidant activity and the ethyl acetate fraction extracted the highest amount of phenolic and antioxidant compounds.
- Proximate nutritional analysis revealed the edible mushroom *Phlebopus beniensis* to be low in fat and carbohydrates, while also being rich in protein and dietary fiber.
- GC-MS analysis revealed the presence of B3 vitamin and essential fatty acids such as ω-6, ω-7, and ω-9.
- Preliminary toxicological analysis suggests that the mushroom is innocuous, and microelement content of wild basidiomata were within the parameters established for human consumption.

1 Introduction

For centuries, fungi have been a source of food and medicine cross-culturally around the world. Modern diets depend heavily on cultivated foods, yet foraging wild plants and mushrooms is still widespread both recreationally and out of necessity. Edible mushrooms are commonly appreciated not only for their texture and flavor but also for the potential of their biochemical properties providing important health benefits. In particular, wild mushrooms have become increasingly important for human diets due to their nutritional value (Li et al., 2021).

Nutritionally, mushrooms are low in fat, high in protein, and high in dietary fiber while also containing an abundance of vitamins, such as thiamine, riboflavin, ascorbic acid, ergosterol, niacin, and essential amino acids (Das et al., 2021; Landingin et al., 2021). In some developing countries, mushrooms are a potential way to address malnutrition of protein and some micronutrients. However, formal investigation of the nutritional and pharmacological properties of wild and edible mushrooms has only started to develop and intensify over recent decades.

Bolete mushrooms (*Boletales*, *Basidiomycota*) are among the most popular wild edible mushrooms in Europe and North America (Pedneault et al., 2006; Morel et al., 2018). Most studies of the order *Boletales* concern edible mushrooms of the genera *Boletus* and *Suillus*, which are frequently harvested for human consumption (Pedneault et al., 2006; Morel et al., 2018). Within *Boletales*, the genus *Phlebopus* (R. Heim) Singer, comprises approximately 17 species to date and is widely distributed in tropical and subtropical areas (Raghoonundon et al., 2021). In regard to the ecology, *Phlebopus* remains, so far, as a saprophytic genus and the supposed ectomycorrhizal associations have not yet been proven (Raghoonundon et al., 2021). Experimental studies, in South America, on *P. bruchii* have proved that no ectomycorrhizal (ECM) colonization is formed with the native species *Fagara coco* (Nouhra et al., 2008). However, other works suggest that *P. portentosus* might form mycorrhizal associations with *Pinus kesiya* in greenhouse experiments (Kumla et al., 2016). In Paraguay, Moreira-Rivas & Díaz-Lezcano (2022) reported an unidentified *Phlebopus*

species as mycorrhizal with native trees, however, this statement was not demonstrated with a proper experimental methodology. More studies are necessary to unveil the nutritional mode of species of *Phlebopus* for its domestication and scale production.

There are at least 7 records of *Phlebopus* species around the world considered edible as following: *P. braunii*; *P. bruchii* (Speg.) Heineman & Rammeloo; *P. colossus* (R. Heim) Singer; *P. marginatus* Watling & N.M. Greg.; *P. portentosus* (Berk. & Broome) Boedijn; *P. roseus* M. Yang, C.-Y. Liu & Y. Wang; *P. spongiosus* Pham & Har. Takah; and *P. sudanicus* (Har. & Pat.) Heinem. (Nouhra, 1999; Mei et al., 2021; Li et al., 2021; Raghoonundon et al., 2021). These species are considered palatable and recognized as delicacies in several Asian and South American countries, thus selling at a higher price than most other wild edible mushrooms (Deschamps & Moreno, 1999; Lumyong et al., 2007; Le et al., 2017; Prado-Elias et al., 2022). In South America, *P. bruchii* is an endemic species to central Argentina that has been collected and consumed by local people in the past. Nowadays, it is sliced, dried and sold by locals in villages and at city markets (Nouhra et al., 2008; Flamini et al., 2015, 2018). Two records of *P. beniensis* consumption have recently been documented in Brazil, and the species is locally known as "chapéu-de-baiano" (Bahian hat) (Prado-Elias et al., 2022). In Paraguay, our team received information about the consumption of a wild bolete mushroom by a local community, Areguá. Despite *Phlebopus* species being commonly consumed in South American communities, little is formally understood about the nutritional and medicinal benefits of these mushrooms in a local context.

The increasing awareness of mushrooms as a practical food source and of their associated nutritional value has resulted in an unprecedented shift toward the consumption of mushrooms (Li et al., 2021). Ethnomycological studies worldwide continue to aid researchers in differentiating species that have long been considered edible versus poisonous (Li et al., 2021). For *Phlebopus* species, to the best of the authors' knowledge, there are limited records of biochemical characteristics and ethnomycological histories, especially in South America. To contribute to the biochemical, nutritional, and ethnographic characterization of edible *Phlebopus* species, this research aimed to identify the species consumed in Paraguay, in order to characterize its biochemical and nutritional profiles, perform preliminary toxicity tests, and collect and share ethnomycological information of its use.

2 Materials and methods

2.1 Identification of the *Phlebopus* species

Morphological analyses: Wild samples of *Phlebopus* species were collected in the urban peripheries of Areguá, Central Department, Paraguay (S 25°20'50.7", W 57°22'16.59"). The fungi were growing in a patch of remnant forest with a 10-15 cm layer of leaf litter near native trees: *Chloroleucon tortum* (tataré); *Peltophorum dubium* (yvyra pytã); *Acrocomia aculeata* (mbokajá); and *Enterolobium contortisiliquum* (timbo). A reference specimen was kept at FACEN Herbarium N°4981. The species was identified by its macroscopic and micromorphological features and was confirmed by molecular phylogenetic analyses. For microscopic analysis, free-hand sections of basidiomata were mounted in 2% (w/v) aqueous potassium hydroxide (KOH) and 1% (w/v) aqueous phloxine and Melzer's reagent per Largent et al. (1977) and Singer (1986). Basidiospores were measured in KOH and phloxine mounts under oil immersion with 100X magnification.

Phylogenetic analyses: Internal transcribed spacer (ITS) and large subunit (LSU) maker sequences were obtained from cultures. DNA extraction, amplification, and sequencing were performed per Robledo et al. (2020). Scientific names of the species and GenBank accession numbers of sequences are listed in Table S1. Phylogenetic tree was constructed based on Robledo et al. (2021). The dataset was composed of 38 ITS and 27 LSU sequences with *Boletinellus* selected as outgroup (Xie et al., 2021). A node was considered strongly supported with a Bayesian Posterior Probability (BPP) ≥ 0.95 or Bootstrap Support (BS) ≥ 95% (Robledo et al., 2021).

2.2 Sample preparation from the wild basidiomata

Samples were prepared using solvent extractions with a polarity gradient by adapting methods detailed by Heleno et al. (2012). Five grams of wild basidiomata was macerated in 450 mL of 80:20 v/v methanol/water solution. The mixture was sonicated (Digital ultrasonic cleaner PS-50A) for 2 h and filtered through a 1-mm glass fiber filter. The filtered solution was evaporated (RC Ingennery-RE 200A) at 60 °C under reduced pressure to remove the methanol. The components of the methanolic remnant were separated by liquid-liquid extraction using petroleum ether (300 mL), n-hexane (H, 300 mL), diethyl ether (DE, 300 mL), and ethyl acetate (EA, 300 mL). The resulting fractions from liquid-liquid extraction were evaporated at 40 °C until dry. To prepare samples for Ultraviolet-visible (UV-VIS) assays, 10 mg of each of the dried fractions were dissolved in methanol measured by aliquot to make 1 mg mL^{-1} concentration stock solutions. An ethanolic extract sample was additionally prepared by macerating 500 g of dried mushroom powder in ethanol (96%). The mixture was agitated periodically over 48 h and then filtered through a 1-mm glass fiber filter. The filtered solution was evaporated (RC Ingennery-RE 200A) to remove the ethanol resulting in an ethanolic fraction.

2.3 Determining total phenolic compounds by UV-VIS

The concentration of Total Phenolic Compounds (TPC) measured by UV-VIS spectrophotometry using the Folin-Ciocalteu reagent and gallic acid as a standard per Campi et al. (2021). Gallic acid was the standard and the TPC was calculated as mg g^{-1} gallic acid equivalents (GAE).

2.4 Determining antioxidant concentrations by the radical scavenging activity assay

Antioxidant concentration and activity were also determined utilizing UV-VIS spectrophotometry per Campi et al. (2021). Tests were performed in triplicate. The percentage of activity (A) was calculated as: $A = (\lambda DPPH - \lambda Sol/\lambda DPPH) \times 100$, where AbsDPPH and AbsSol are the absorbance measurements of DPPH solution and DPPH with extract respectively, measured at 517 nm. Ascorbic acid was used as a pattern.

2.5 Determining chemical profile of compounds by GC-MS

Solutions were dissolved in n-Hexane at an approximate concentration of 1 mg mL^{-1}, Gas Chromatography-Mass Spectrometry (GC/MS) analysis was performed using the Shimadzu GC2010 Plus Gas Chromatograph equipped with a QP2010SE Electron Impact Mass Detector and Supelco SLB 5-ms (30 m X 0.25 mm, 0.25 μm) column. Helium 5.0 was the carrier gas, operated at a flow rate of 0.87 mL min^{-1} and an injection volume of 1.0 μL. The machine settings were set to a 270 °C injector temperature, an injection mode of "Splitless", a ratio of 10:1, and a 250 °C ion source temperature. The oven temperature was set to 2-min at 60 °C isothermal, with an increase of 6 °C/min to 280 °C, and ending with a 20-min isothermal. The mass detector was programmed in full-scan mode 55-550 m/z at 70 eV. The analysis was done in triplicate. Compounds were identified from NIST database library of the GC-MS instrument. All compounds identified with homology above 80% were considered positive and were reported in the results. The molecular structures were designed in ChemDraw Version 20.1.1.125.

2.6 Nutritional composition analysis

The proximate nutritive composition of *P. beniensis* was determined by the basidiomata extract samples. Crude fat content was determined using Soxhlet apparatus with hexane as the reagent. Moisture was determined using a laboratory stove at 105 °C, and ash content was determined using a furnace at 550 °C. To measure the protein and dietary fiber contents, guidelines from the AOAC – Association of Official Analytical Chemists (2000). The total carbohydrate content was determined by modifying the anthrone test presented by Fernandes et al. (2012) using sulfuric acid (98%), perchloric acid (50% v/v), and Anthrone reagent (9,10 dihydro-9-oxoanthracene). Glucose anhydrous was used as a standard, and the total

carbohydrate content was calculated as mg g^{-1} of glucose equivalent. Trace elements in the fruiting bodies of *P. beniensis* were calculated following the guidelines of physicochemical methods for food analysis (Zenebon & Pascuet, 2005). Finally, the energetic value was calculated following the Mercosur Technical Regulations (Grupo Mercado Común, 2003).

2.7 Determination of median lethal dose (LD50)

Drosophila melanogaster larvae were prepared following Graf et al. (1984). The larvae were exposed to the five prepared concentrations (30, 50, 70, 90, 120 mg mL^{-1}) of ethanolic extract for 96 h. For each concentration, 100 third-instar larvae were isolated. The lethality of the extract was estimated as a percentage of dead flies in each concentration. A concentration was considered toxic if it killed at least 50% of the population. Results were analyzed, and the median lethal dose (LD50) was calculated using "Probit Analysis" based on a mortality (%)/Log_{10}-concentration curve (Finney 1952).

2.8 Ethnomycological notes

Members of our research team from the Mycological Investigation Laboratory at the National University of Asunción (*Universidad Nacional de Asunción*) founded the non-profit organization FungiParaguay, which shares local mycological information through social media (Instagram and Facebook). FungiParaguay acts as a network between academia and communities with the primary objective to communicate scientific findings about Paraguayan Funga to the general population. Through this resource, individuals can inquire about native mushrooms, their edibility, and their safety, while sharing knowledge about histories and traditional uses. Our research team conducted several interviews with several people who contacted FungiParaguay. The primary topics covered in these interviews were within the framework of Prado-Elias et al., regarding taxonomy (folk names), uses/applications (*i.e.*, medicinal, gastronomic, recreational, and toxic species), storage methods, knowledge transmission, and folklore related to the fungi (Prado-Elias et al., 2022).

2.9 Statistical analyses

One-way Analysis of Variance (ANOVA) (95% confidence interval; with previous verification of assumptions) and Tukey's test were used to compare the variable evaluated (*i.e.* polarity of solvent) between the different fractions studied. These analyses were performed using the statistical program Past v. 4.03b (Hammer et al., 2001). The results were expressed as the mean of the analysis performed in triplicate in milligrams of standard per g of crude extract (mg g^{-1}) ± Standard Deviation (SD).

3 Results and discussion

3.1 Identification and taxonomy - *Phlebopus beniensis*

Morphological analyses: The observed macro- and microscopical characteristics of the specimen (Figure 1) agreed with the reference descriptions of *P. beniensis* (Heinemann & Rammeloo, 1982; Barbosa, 2016; Palacio et al., 2015; Calaça et al., 2018). Macroscopically, the specimen identified as *P. beniensis* was characterized by a pileus up to 200 mm in diameter, robust stipe, and small pores. Microscopically, it was unique by its shortly ellipsoid, yellow-brown, inamyloid basidiospores measuring 6 to 7 × 4.4-5.7 µm and the presence of cheilocystidia and pleurocystidia (Calaça et al., 2018).

Phylogenetic analyses: The molecular analyses supported the morphological identification. The dataset included 51 terminals and 1,735 characters, of which 473 were parsimony informative, 62 variables, and 1,200 constants. The partitions and evolutionary models selected were: K2P+I+G4 (ITS1 and ITS2); K2P+I (5.8S); and SYM+I (LSU). Bayesian and ML analyses resulted in similar topologies, and the ML tree is presented in Figure 2. The resulting topology was consistent with previous works (Xie et al., 2021). The specimen under study grouped within *P. beniensis,* conforming a lineage with strong statistical support.

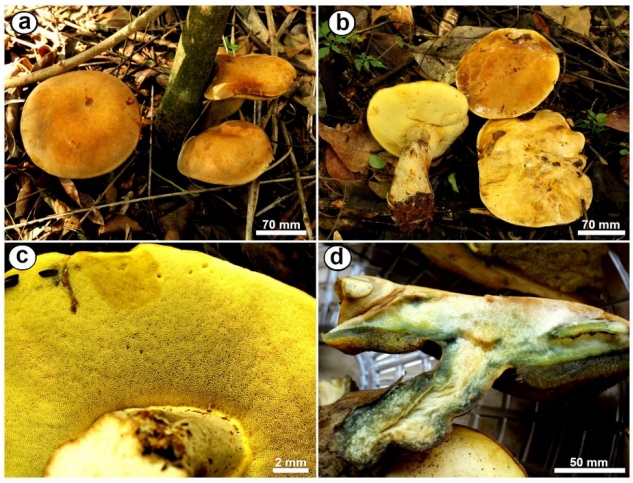


Figure 1. *Phlebopus beniensis* (a, b) Fresh basidiomata *in situ*; (c) Pore surface; (d) Longitudinally sectioned basidiome showing a blue reaction.

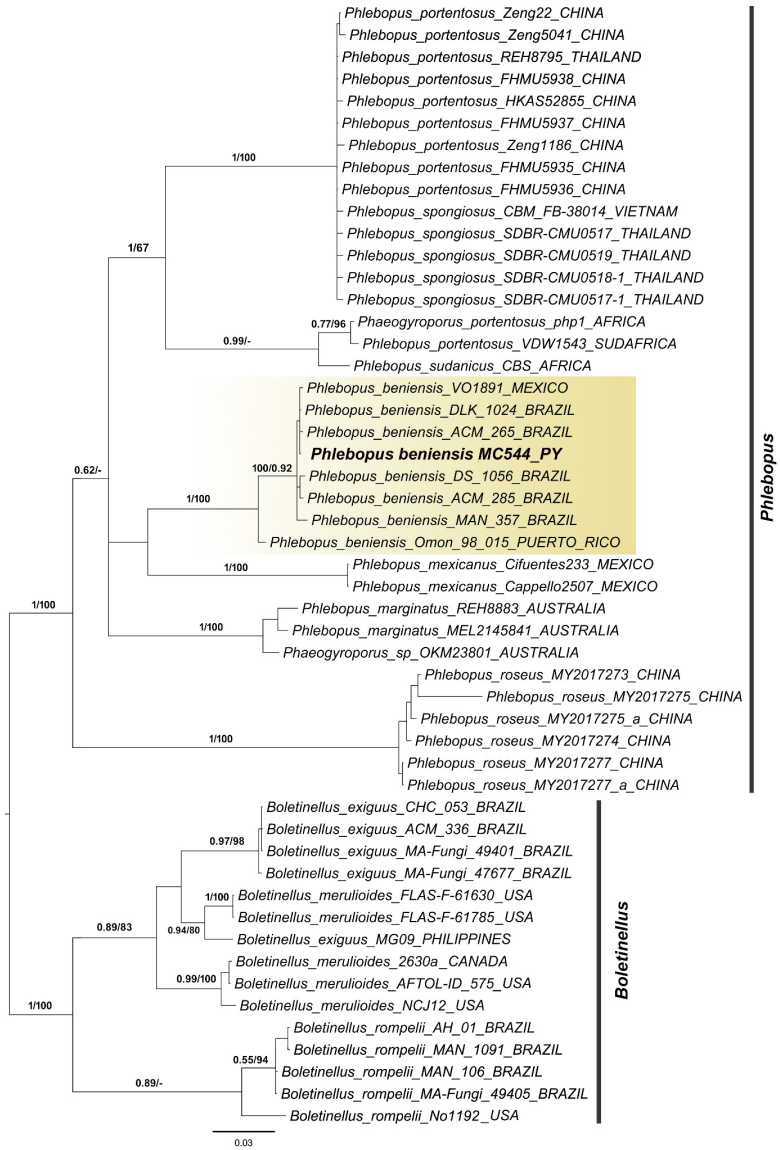


Figure 2. ML consensus tree positioning *P. beniensis* (MC544) within *P. beniensis* clade based on concatenated ITS and LSU sequence data. Bayesian posterior probability above 0.75 and Bootstrap values above 75% are shown.

3.2 Phenolic compounds

Our results for phenolic content of *P. beniensis* ranged from 17 to 87 mg g^{-1} GAE (Table 1), being significantly ($p < 0.05$) higher with ethyl acetate. For example, for *P. portentosus* from Thailand, values of 27 mg g^{-1} GAE for the methanolic extract (Kumla et al., 2021) and less than 5 mg g^{-1} GAE for the ethanolic extract (Kaewnarin et al., 2016) have been reported. In another instance, *P. colossus* was documented as exhibiting a phenolic content of 0.64 mg g^{-1} GAE in acetone extracts (Liaotrakoon & Liaotrakoon, 2018). Prior studies have suggested that different extraction methods can affect the content of phenolic and antioxidant compounds (Kaewnarin et al., 2016). Likewise, high drying temperatures and long exposure times can also change the properties of dried edible mushrooms, such as *Phlebopus*, *Pleurotus* and *Lentinula* (Liaotrakoon & Liaotrakoon, 2018).

Table 1. Comparison of phenolic compounds, antioxidant concentration, and antioxidant activity values from wild fruiting body extracts of *P. beniensis*.

	Fractions	Phenolic compounds (mg g^{-1} GAE)	Antioxidants (mg g^{-1} AAE)	Antioxidant Activity (%)
Wild Fruiting bodies	Ethanolic extract (EE)	26 ± 3^a	26 ± 3^a	11 ± 0^a
	Diethyl ether (DE)	17 ± 2^a	26 ± 3^a	24 ± 2^b
	Ethyl acetate (EA)	87 ± 9^b	53 ± 2^b	41 ± 1^c

GAE: Gallic Acid Equivalents. AAE: Ascorbic Acid Equivalent. Different letters mean significant differences ($p < 0.05$).

3.3 Antioxidant concentration and activity

From our antioxidant assays, *P. beniensis* showcased antioxidant concentrations ranging between 26-53 mg g^{-1} AAE (Table 1), which were highest in the ethyl acetate fraction. The percentage of antioxidant activity ranged from approximately 11% to 41%, being significantly different ($p < 0.05$) for all fractions, higher in diethyl ether and the highest for ethyl acetate. In studies of other *Phlebopus* species, such as *P. portentosus,* antioxidant concentrations of 5.5 mg g^{-1} GAE and 0.37 mg g^{-1} GAE in the DPPH assay using Gallic Acid as the standard, they have been reported utilizing FRAP and DPPH assays, respectively (Liaotrakoon & Liaotrakoon, 2018; Kumla et al., 2021). Compared to prior literature, our results suggest that the basidiomata of *P. beniensis* have a medium antioxidant capacity and phenolic content compared to other *Phlebopus* species. The results suggest that mushroom extracts of this edible bolete can be a natural source of antioxidants.

3.4 GC-MS chemical profile

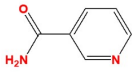
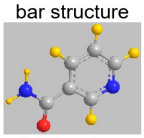
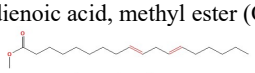
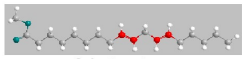
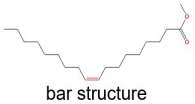
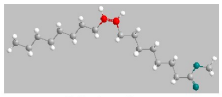
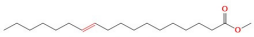
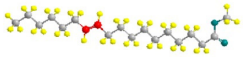
A summary of GC-MS results is shown in Table 2, which includes a list of the identified compounds with their name, structure, molecular weight, retention time, and abundance. The GC-MS chromatogram is shown in Figure S1, Figure S2. Various compounds were identified in the wild basidiomata and corresponded primarily to the DE and EA fractions. Our analysis revealed the presence of fatty acids (saturated, monounsaturated, and polyunsaturated), fatty acid esters, sterols, waxes, phenolic compounds, and vitamins. Several of the identified compounds were especially significant for their role in human health and nutrition (Table 3).

Fatty acids are of particular interest for edible fungi, because of their role as an as anti-inflammatory, antioxidant, hypocholesterolemia and nematicide (Kumar et al., 2010; Aparna et al., 2012). In these results, we reported the presence of two saturated fatty acids, n-hexadecenoic acid (palmitic acid) and octadecanoic acid (stearic acid). Palmitic acid (16:0) is the fatty acid mainly found in *Basidiomycota* species (Pedneault et al., 2006; Kalač, 2009; Sande et al., 2019). Nutritionally the palmitic and stearic acids are the most commonly consumed saturated fatty acids in the Western diet, each having different effects on fasting serum lipoproteins (van Rooijen et al., 2021).

Table 2. Compounds (names, molecular weight and formula) identified by GC-MS in the diethyl ether (DE) and ethyl acetate (EA) fractions obtained from *Phlebopus beniensis*.

Nº	Names	Molecular weight	Molecular formula	Fraction
1	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	278	$C_{16}H_{22}O_4$	DE
2	1-Dodecene	168	$C_{12}H_{24}$	EA
3	1-Eicosanol	298	$C_{20}H_{42}O$	EA
4	1-Hexadecene	224	$C_{16}H_{32}$	EA
5	1-Nonadecene	266	$C_{19}H_{38}$	EA
6	1-Octacosanol	410	$C_{28}H_{58}O$	EA
7	1-Tetradecene	196	$C_{14}H_{28}$	DE
8	5-Octadecene, (E)-	252	$C_{18}H_{36}$	EA
9	9,12-Octadecadienoic acid (Z,Z)-	280	$C_{18}H_{32}O_2$	DE
10	9,12-Octadecadienoic acid, methyl ester	294	$C_{19}H_{34}O_2$	EA, DE
11	9-Hexadecenoic acid, methyl ester, (Z)-	268	$C_{17}H_{32}O_2$	DE
12	9-Octadecenoic acid (Z)-, methyl ester	296	$C_{19}H_{36}O_2$	EA
13	Acetamide, N-(2-phenylethyl)-	163	$C_{10}H_{13}NO$	DE
14	*Cis*-Vaccenic acid	282	$C_{18}H_{34}O_2$	DE
15	Dodecane	170	$C_{12}H_{26}$	EA
16	E-14-Hexadecenal	238	$C_{16}H_{30}O$	DE
17	E-15-Heptadecenal	252	$C_{17}H_{32}O$	EA
18	Ethanol, 1-methoxy-, benzoate	180	$C_{10}H_{12}O_3$	EA
19	Formamide, N-(2-phenylethyl)-	149	$C_9H_{11}NO$	DE
20	Hexadecane	226	$C_{16}H_{34}$	DE
21	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	EA
22	Hexanedioic acid, bis(2-ethylhexyl) ester	370	$C_{22}H_{42}O_4$	DE
23	Indole	117	C_8H_7N	DE
24	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	DE
25	Niacinamide	122	$C_6H_6N_2O$	EA
26	n-Nonadecanol-1	284	$C_{19}H_{40}O$	DE
27	n-Propyl 9,12-octadecadienoate	322	$C_{21}H_{38}O_2$	EA
28	Octadecanoic acid	284	$C_{18}H_{36}O_2$	DE
29	Octadecanoic acid, methyl ester	298	$C_{19}H_{38}O_2$	DE
30	Phenol, 3,5-bis(1,1-dimethylethyl)-	206	$C_{14}H_{22}O$	DE
31	Propanedioic acid, phenyl-	180	$C_9H_8O_4$	DE
32	Squalene	410	$C_{30}H_{50}$	DE
33	*trans*-Cinnamic acid	148	$C_9H_8O_2$	DE

Table 3. Main compounds of nutritional interest from the basidiomata.

Name (MW) and structure	Retention time (min)	Abundance (%)
Niacin (B3 vitamin) (122)  bar structure  3d structure	19.842	3.36
9,12-Octadecadienoic acid, methyl ester (Omega-6) (294)  bar structure  3d structure	32.206	2.11
9-Octadecenoic acid (Z)-, methyl ester (Omega-9) (296)  bar structure  3d structure	32.304	5.4
cis-Vaccenic acid, methyl ester (Omega-7) (282)  bar structure  3d structure	33.069	10.95

Unsaturated fatty acid levels are generally higher than saturated ones in mushrooms (Sande et al., 2015). A stereoisomer of vaccenic acid (*cis*-VA), which is an omega-7 fatty acid (ω-7), showed an 11% abundance in our results. The *cis*-VA has been reported for several mushroom species from the orders *Agaricales*, *Boletales* and *Polyporales* (Sabri et al., 2020; Bhat et al., 2020). Regarding the nutritional value of ω-7, emerging studies suggest that the consumption of this fat may offer numerous health benefits (Field et al., 2009).

Additionally, two unsaturated omega fatty acids, the methyl esters 9,12-octadecadienoic acid (omega-6, ω-6) and 9-octadecenoic acid (omega-9, ω-9) were reported as being present. Omega-6 is an essential fatty acid present in most mushroom species, playing a vital role in many physiological functions, such as maintaining bone health, regulating metabolism, and stimulating skin and hair growth (Sande et al., 2015; Al-Khudairy et al., 2015). Omega-9 is a monounsaturated fatty acid typical in vegetable oils, known for its effectiveness in reducing cholesterol levels (Puiggros et al., 2002). The abundance of ω-9 has been reported for the *Agaricacea* family across various species, such as *Agaricus bisporus, Pluteus atricapillus, Lentinula edodes, Flamulina velutipes* and *Ramaria aurea* (Pereira et al., 2012; Stojković et al., 2014). One final fatty acid of potential medicinal interest, hexadecenoic acid methyl ester, was found to be present in both DE and EA fractions. This acid has shown biochemical potential as antioxidant and as a hypocholesterolemia agent

(Kumar et al., 2010). It has also been recorded in *Hohenbuehelia serotonina*, *Cordyceps militaris,* and *Cordyceps sinensis* species (Cao et al., 1996).

In addition to fatty acids, several biologically promising alcohols were detected from our characterization, including 1-octacosanol, 1-eicosanol and n-Nonadecanol-1. The compound 1-octacosanol has been studied to provide several health benefits, such as for being a stimulant, anti-hypoxic therapeutic, antioxidant, anti-inflammatory, and antitumor substance (Zhou et al., 2022). Another precursor of sterols, squalene, was also identified in our DE fraction. This polyunsaturated triterpene is a hydrophilic natural antioxidant widely found in nature (Amarowicz 2009). Finally, niacinamide (vitamin B3) was also present in the specimen and is often abundant in edible mushrooms, both fresh and processed (Bernaś et al., 2006).

Several phenolic compounds, which aid antioxidant activity, were also detected in our assay results: *trans*-cinnamic acid; phenol, 3,5-bis(1,1-dimethylethyl)-; and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester. Cinnamic Acid Derivatives (CAD) are medically important compounds that exhibit a wide range of biological activities, such as having antibacterial, antifungal, antioxidant, and antitumoral effects (Hu et al., 2016). Previous studies have found CAD in *Agaricus bispous*, *Collybia dryophyla*, *Maramius oreades*, *Lepista nuda* and *Tapinella panuoides* (Çayan et al., 2020). The 1,2-benzenedicarboxylic acid has also been shown to be present in the genus *Pleurotus,* specifically *P. ostreatus* (Wekesa et al., 2016).

Organonitrogen compounds, including indole, formamide, N-(2-phenylethyl)- and acetamide, N-(2-phenylethyl)- were found in the DE fraction of the extracts. Out of these compounds, the indole heterocycle is found in over 140 natural products produced by mushrooms (Homer & Sperry, 2017). For example, acetamide, N-(2-phenylethyl)- was reported in the methanol extract of *Volvariella volvacea*, which is used for cosmetic-related products (Punitha & Rajasekaran, 2015).

3.5 Nutritional composition analysis

The proximate nutritional composition of *P. beniensis*, shown in Table 4, was generally consistent with literature on edible fungi, in which wild mushrooms are recognized for their nutritional value, such as being low in fat while also rich sources of protein and carbohydrates (Breene, 1990; Barros et al., 2007). Our analyses revealed that *P. beniensis* is low in fat (4 ± 0.14%), high in protein (39.2%), and high in dietary fiber (20.05%). Its protein concentration was similar to *P. colossus* and higher than *P. portentosus* and *P. spongiosus,* as documented for the edible species from Thailand. In contrast, the energy value is the lowest compared to the other species.

The values obtained for ash content (13%) fell below previously reported ranges for dried fruiting bodies of wild mushrooms, which have been estimated at approximately 20% (Kalač, 2016). Nevertheless, this value was still similar to those reported for other edible *Phlebopus* species (Table 4). For fat content, our results fell within the general range for wild mushrooms, reported as ranging from 0.1% to 16% (Sande et al., 2019). Compared to *Phlebopus* species in particular, our sample resulted in slightly higher values than those reported in Asia.

As for the carbohydrate content, it varies according to the genus (Boa, 2005; Cheung, 2010). In the boletes *Suillus luteus* and *Boletus edulis*, the range is 57-71% (Boa 2005; Heleno et al., 2011). Our result for *P. beniensis,* obtained with the anthrone method (specific for carbohydrates) was 13%, much lower than other edible *Phlebopus* species (45% to 55%). However, those percentages were calculated with the difference method (Liaotrakoon & Liaotrakoon, 2018; Kumla et al., 2021, 2022) which could be inaccurate for the correct determination of proximate composition.

Finally, edible mushrooms are considered a novel source of dietary fiber due to the presence of non-starch polysaccharides, however the previous reported values for *Phlebopus* species correspond to crude fiber (Table 4), data that underestimates the dietary fiber content and should not be used for nutritional information. *Phlebopus beniensis* is a remarkable source of dietary fiber with 20%.

Table 4. Comparison of proximate composition on a dry basis (% dry weight) of dried edible *Phlebopus* species.

Dried Fruiting bodies	*P. beniensis* Paraguay	*P. colossus* Thailand	*P. portentosus* Thailand	*P. spongiosus* Thailand
References	This work	Liaotrakoon & Liaotrakoon (2018)	Kumla et al. (2021)	Kumla et al. (2022)
Moisture	91.66 ± 0.3	93.1 ± 0.0	82.7 ± 2	–
Ash	13 ± 2.06	10.1 ± 0.0	9.6 ± 0.2	9.59 ± 0.30
Crude Protein	39.2	38 ± 0.2	19.6 ± 0.4	28.03 ± 0.93
Fat	4 ± 0.14	1.4 ± 0.0	1.0 ± 0.1	2.15 ± 0.09
Dietary fiber	20.05 ± 0.0	–	–	–
Crude fiber	–	5.8 ± 0.1	6.3 ± 1	6.67 ± 0.3
Carbohydrates	13.5 ± 0.5*	44.9 ± 0.1**	54.8 ± 0.7**	50.86 ± 1.19**
Energetic value kcal/100 g	245	344.2***	306.6***	334.9***

*Anthrone method. **Different method. ***Calculated from data of the references mentioned. *Micronutrient content (Cu, Cd, Ni, Zn).*

Mushrooms have a high ability to accumulate trace elements, both non-essential (Cd, Pb, Hg) as well as essential (Cu, Mn, Se, and Zn) (Mleczek et al., 2016; Mirończuk-Chodakowska et al., 2019). Wild edible mushrooms, especially from sites affected by anthropogenic contamination, could represent a health risk due to their high bio-accumulative capacity (Árvay et al., 2015). *Phlebopus beniensis* was collected in an anthropogenic area. Therefore, we assessed the sample for microelements, and our results were within the parameters established by Mercosur Technical Regulations and the European Union, as shown in Table 5 (Grupo Mercado Común, 2003; European Union, 2011).

Table 5. Content of trace metals in the *P. beniensis* specimen.

Metals	Concentration in dry sample (mg kg^{-1} DW)	Acceptable daily intake	References
Cu	52 ± 0.02	0.9 (mg day^{-1})	Mercado Común del Sur (2003)
Zn	53 ± 0.06	7 (mg day^{-1})	Mercado Común del Sur (2003)
Ni	4.5 ± 0.02	0.34 (mg kg^{-1})	Fernández et al. (2007)
Cd	ND	0.05 (mg kg^{-1})	European Union (2011)

ND: not detected. DW: dry weight.

The concentration of copper for *P. beniensis* (52 ± 0.02 mg kg^{-1} DW) was in the acceptable range described for wild-growing edible mushrooms (20 to 100 mg kg^{-1} DW) (Árvay et al., 2015). For the zinc content, the concentrations in our sample (53 ± 0.06 mg kg^{-1} DW) were also within accordance to the ranges found in mushrooms from uncontaminated areas (25 to 200 mg kg^{-1} DW) (Kalač (2010). Our results for Nickel content (4.5 ± 0.02 mg kg^{-1} DW) were in the higher end of previous studies for wild mushrooms while still in agreement with safe limits (1 to 5 mg kg^{-1} DW) (Tüzen, 2003). Lastly, cadmium has a very high persistence in wild mushrooms and is one of the most toxic elements in the environment (Árvay et al., 2015; Vollmann et al., 2015), and fortunately, traces of this metal were not detected in our sample.

3.6 Toxicity and the median lethal dose (LD50)

None of the ethanolic extract samples that underwent toxicity testing reached 50% of mortality. Even for the highest concentration of sample analyzed (120 mg mL^{-1}), only 28% of the flies did not survive after 96 h. The Probit analysis, which was based on a best-fit curve and $\chi^2 > 0.05$, determined the LD_{50} for the ethanolic

extract of *P. beniensis* as 311.3 mg mL^{-1}. Since LD_{50} values higher than 1 mg mL^{-1} are not considered toxic, our results suggest that a regulated consumption of *P. beniensis* possesses no immediate threat to human health (McLaughlin et al., 1998; Alhadi et al., 2015). However, further research of its genotoxicity and cytotoxicity is recommended since LD_{50} values only account for acute toxicity and serve only as a primary test to follow-up toxicity tests (Schlede et al., 1992).

3.7 Ethnomycological notes

The city Areguá is located in the Central Department of Paraguay, 22 km from the nation's capital Asunción. Decades ago, European immigrants, especially German settlers, arrived in the city due to migration after World War II. A German descendant reported to our research team through the FungiParaguay social media platform about a mushroom they were collecting and eating. We interviewed two women from the area who told us that they consume this mushroom, which they call "*Boletus*": "*We have consumed Boletus since we arrived in Paraguay 30 years ago. Every time they come out in quantity we collect and dry them to store them*."

Regarding processing, they detailed that when collecting the mushrooms, they are halved and dried under the sun and stored in closed containers for future use. In another case reported to FungiParaguay, a Paraguayan woman from downtown Asunción mentioned that the mushrooms grew in her backyard. Her house was previously a country house with fruit trees and grasslands, and to date, remains that way despite being in an urban area. The woman commented that she processed the mushrooms the same as the German women. When the interviewees were asked how they decided to consume the mushroom, the Germans responded that in Germany the consumption of wild mushrooms is frequent and that these fungi (*P. beniensis*) are very palatable. The Paraguayan woman commented that her grandmother was who passed down this knowledge of the consumption of *P. beniensis*.

Tropical *Phlebopus* species, such as *P. portentosum*, *P. roseus,* and *P. spongiosus* from Asia, *P. colossus* and *P. sudanicus* from Africa, and *P. marginatus* from Australia, are documented as being edible and nutritious (Wu et al., 2019; Mei et al., 2021; Li et al., 2021). For South America, only *P. bruchii* has been previously reported in Argentina as edible and consumed by native people (Nouhra et al., 2008; Li et al., 2021). *Phlebopus bruchii* was synonymized under *P. braunii,* a species described originally from Africa (Heinemann, 1951; Singer & Digilio, 1960) where its edibility has not been reported, therefore whether it is edible or toxic has never been formally documented. The presence of *P. braunii* is unlikely in South America, although it has been recorded in Brazil (Putzke et al., 1994). To resolve the taxonomic status of *Phlebopus* in South America, phylogenetic studies are necessary (Sulzbacher et al., 2012).

Even though *P. beniensis* is well distributed in Neotropical area, this is the first formal documentation in literature of its consumption by local people. It was recently reported that some people in rural areas of São Paulo, Brazil, refer to this species as "chapeu do baiano" (Bahian hat) but did not consume it (Prado-Elias et al., 2022). Despite not being consumed by the locals, the Brazilian researchers tested its edibility by themselves (Prado-Elias et al., 2022) confirming the safe consumption of the species registered in Paraguay.

3.8 Recommendations for gathering *P. beniensis*

The high moisture content (92%) in *P. beniensis* and the season in which they appear (wet summer) suggest that the mushroom favors the growth of microorganisms along with invertebrates, such as *Diptera* larvae, which feed on the basidiomata. For these reasons, we suggest collecting younger samples and cooking them thoroughly. It is recommended that the foraging mushrooms for consumption is primarily done in non-urban areas due to the risk of pollutants being present in the fruiting bodies. When collected in large quantities, dehydrating the mushrooms is the best method to preserve the flavor for long storage.

4 Conclusions

In addition to a morphological approach, we confirmed the identification of *P. beniensis* in South America through phylogenetic analyses. Two genetic markers (ITS and LSU) formed a strongly supported clade with other specimens from Brazil and Mexico. *Phlebopus beniensis* is attractive to consume due to its robust basidiomata and palatable flavor, as well as for its nutritional value. The mushroom is low in fat, high in protein, and rich in dietary fiber content, while also being a source of phenolic and antioxidant compounds. The results of microelements of the collected samples from semi-urban areas showed values within the parameters established for human consumption. *Phlebopus beniensis* is a healthy source of lipids, containing essential ω-6, ω-7, and ω-9 fatty acids and niacinamide (vitamin B3), which are all of nutritional importance.

This paper presents for the first time the chemical and nutritional profile of an endemic species of the Neotropical region in combination with the ethnomycological importance of the Paraguayan population. Preliminary toxicological studies suggest that the mushroom is innocuous, especially when coupled with previous accounts of its safe consumption by local communities. With the growth of interest in healthy and alternative diets, more people are interested to learn more regarding wild edible Paraguayan mushrooms. The consumption of wild or cultivated mushrooms as a cholesterol-free protein-fibers-fat-substitution of meat-based products while still maintaining nutritional characteristics are especially important for diets such as vegetarianism and veganism. *Phlebopus beniensis* might be considered an excellent source of protein, fats, and carbohydrates. This endogenous resource has a high potential to significantly open up new markets and opportunity for food security for vulnerable populations in South America through its exploitation as a culinary resource.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. *Phlebopus* species: specimens: origin,ITS and LSU Gen Bank accession numbers for sequences used in the phylogenetic analyses.. New sequence is highlighted in boldface. "---" = no sequence

Figure S1. GC/MS Chromatogram of the ethyl acetate fraction. **1.** 1-Dodecene, **2.** Dodecane, **3.** Ethanol, 1-methoxy-, benzoate, **4.** 5-Octadecene, (E)-, **5.** Niacinamide, **6.** 1-Hexadecene, **7.** E-15-Heptadecenal, **8.** Hexadecanoic acid, methyl ester, **9.** n-Propyl 9,12-octadecadienoate, **10.** 1-Eicosanol, **11.** 9,12-Octadecadienoic acid, methyl ester, **12.** 9-Octadecenoic acid (Z)-, methyl ester, **13.** 1-Nonadecene, **14.** 1-Octacosanol.

Figure S2. GC/MS Chromatogram of the diethyl ether fraction. **1.** Propanedioic acid, phenyl-, **2.** Indole, **3.** 1-Tetradecene, **4.** trans-Cinnamic acid, **5.** Formamide, N-(2-phenylethyl)-, **6.** Phenol, 3,5-bis(1,1-dimethylethyl)-, **7.** Acetamide, N-(2-phenylethyl)-, **8.** E-14-Hexadecenal, **9.** Hexadecane, **10.** E-15-Heptadecenal, **11.** 9-Hexadecenoic acid, methyl ester, (Z)-, **12.** Hexadecanoic acid, methyl ester, **13.** n-Hexadecanoic acid, **14.** 1-Nonadecene, **15.** 9,12-Octadecadienoic acid, methyl ester, **16.** 11-Octadecenoic acid, methyl ester, **17.** Octadecanoic acid, methyl ester, **18.** 9,12-Octadecadienoic acid (Z,Z)-, **19.** cis-Vaccenic acid, **20.** Octadecanoic acid, **21.** n-Nonadecanol-1, **22.** Hexanedioic acid, bis(2-ethylhexyl) ester, **23.** 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester, **24.** Unknown, **25.** Squalene, **26.** Unknown.

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