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QUALITATIVE AND QUANTITATIVE ANALYSIS OF PROTEINS CONTENTS OF FOUR WILD MEDICINAL MUSHROOMS

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ABSTRACT

Background: Mushrooms are renowned for their protein richness, yet research literature on large-scale protein analysis of mushrooms remains limited. This study represents a pioneering endeavor in the proteomic analysis of aqueous extracts from four wild mushroom species including *Phallus impudicus (P. impudicus), Trametesversicolor (T. versicolor), Pisolithusarhizus (P. arhizus)* and *Tyromycescheoneus (T.cheoneus).*

Methodology: Bradford method was used to quantify the total protein contents. Subsequent analysis involved one-dimensional gel electrophoresis (1-DGE) for proteins separation. PEAKS X was used for identification of peptides and proteins in the selected mushrooms species.

Results: Proteomic quantitative analysis results indicated that the maximum total protein contents were quantified in *T. chioneus*(330.26µg/10mg), while the minimum in*P. impudicus*(76.06µg/ 10mg). In proteomic qualitative analysis 462 proteins and 469 peptide sequences were identified in *P. impudicus* which was categorized into 55 functional protein groups. In *T. versicolor*, 161 proteins and 290 peptides sequences were identified, distributed in 16 diverse functional groups. Similarly in *P.arhizus*, 129 proteins and 192 peptides belonging to 18 functional groups and in *T. cheoneus*, 76 proteins and 160 peptides were identified and categorized in 14 diverse functional groups. Interestingly, there were 18 proteins common in all selected mushrooms. In the present study a lot of PTMs (Post translational modifications) were detected in selected four mushrooms.

Conclusions: The outcomes of this study has the potential to serve as a foundation for the industrial production of food and health-related products derived from these mushroom species.

Keywords: Mushrooms, proteins contents, SDS-PAGE, Qualitative and quantitative analysis**,** Post translational modifications

INTRODUCTION

Fungi represent an incredibly diverse array of organisms, spanning a vast spectrum of life forms, from the minuscule single-celled varieties to intricately structured multicellular organisms (Seifert, 2022). Within this vast diversity, mushrooms emerge prominently, boasting approximately 25,000

identified species, around 2,000 of which are deemed edible, presenting a plethora of culinary options (Bierend, 2021; Kozarski et al., 2021). Commercially, about 35 edible mushroom varieties are cultivated worldwide to meet the demands of global cuisine (Panda et al., 2024; Muhammad & Suleiman, 2015). However, fungi extend their utility beyond the culinary realm, holding promising potential in medicine (Imdad et al., 2022; Khattak et al., 2022). Roughly 200 wild mushroom species are recognized for their medicinal properties, serving as valuable contributors to both traditional and modern medical practices (Imdad et al., 2022; Anusiya et al., 2021). This enduring legacy underscores the manifold benefits fungi offer to humanity(Lu, 2023).

Numerous bioactive compounds, including polysaccharopeptide (PSP), amino acids, and proteins, are derived from the Turkey Tail (*Trametesversicolor*) mushroom, along with various other compounds (Bulam et al., 2022). Turkey Tail and Shiitake (*Lentinulaedodes*) mushrooms are emerging as new-generation foods due to their rich content of biologically active compounds such as (1,3) (1,6)-β-D-glucans, triterpenes, phenolic compounds, and sterols (Deo et al., 2019).

The *P. arhizus*, known for its high polyphenol content, exhibits significant reducing power. Mushrooms hold an important position in medical science for instance, the H. *radicata* exhibits selectivity towards the MDA-MB-231 breast cancer cell line, whereas *C. cornucopioides* targets the MCF-7 breast cancer cell line (Amanat et al., 2024; Farrukh et al., 2023). This suggests that different mushroom species may have specific effects on different types of breast cancer cells (Yuan et al., 2024; Farrukh et al., 2022). Furthermore, extracts from *P. arhizus* display notable anti-tumoral, antioxidant, and antifungal activities (Donadio et al., 2022). *P. impudicus* demonstrates anti-tumor properties, reduces metastases, and lowers thrombosis risk associated with cancer (Dinçer et al., 2023).

Additionally, *Phallus impudicus* exhibits significant antiviral activity against the influenza virus (Hamdan and Righetti, 2005). *T. cheoneus* has been studied for its antimicrobial, antioxidant, anti-diabetic, and anticancer properties. Previous studies synthesized the latest research on the health benefits of mushroom nutraceuticals, suggesting their potential as natural adjuncts in preventing and treating various health conditions (Jamil et al., 2022; Khattak et al., 2022; Rehman et al., 2020; Siddiqui et al., 2017). Furthermore, various studies evaluated the total protein content and identifies proteins and peptides in *P. impudicus, T. versicolor, T. cheoneus, and P. arhizus* using proteomics approaches, aiming to understand their functional mechanisms comprehensively (Irmer et al., 2009).
The current study aimed to assess mushroom extracts using proteomic analysis. It sought to elucidate the protein composition and potential functional mechanisms of these extracts. This approach will also offer valuable insights into the bioactive properties of mushrooms.

MATERIALS AND METHODS

Plant Collection

The Mushrooms species were collected from the western region of Khyber Pakhtunkhwa District Kurum(Para Chinar). Shalozanand Spinghar mountains in June 2020.

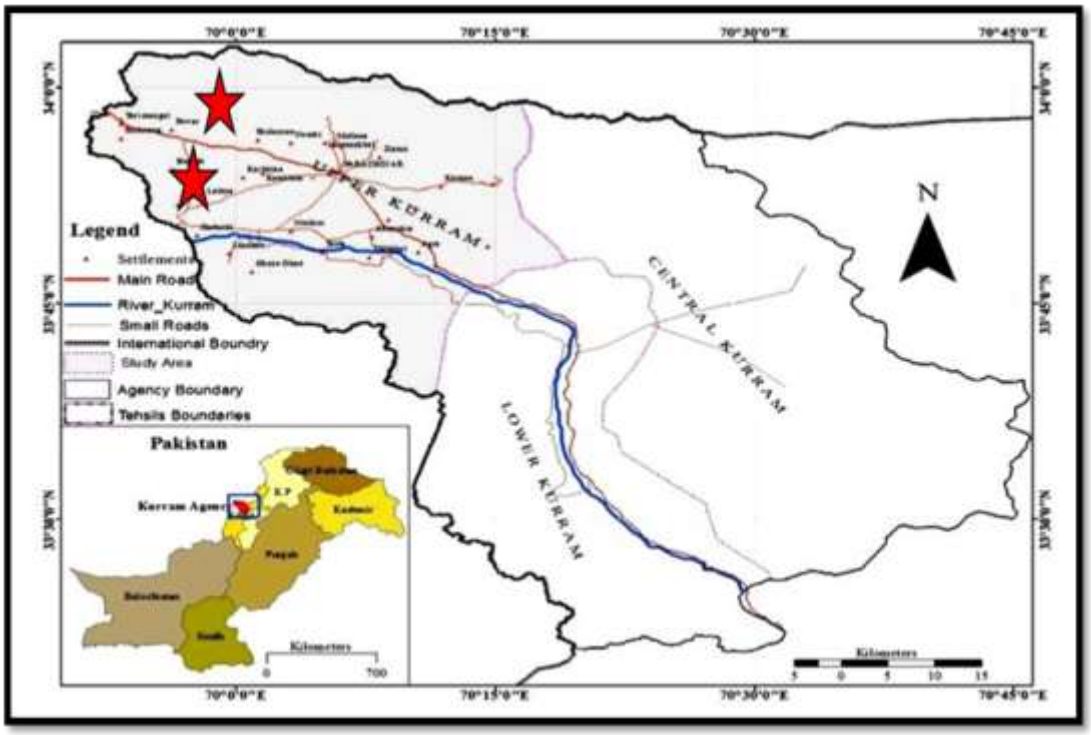

Figure1. MapofAreaof study site (Parachinar KP, Pakistan). Red Star sign indicate mushrooms collection area

Characterization

Morphological and anatomical characteristics of the mushroom samples were examined, followed by species authentication at the Kunming Institute of Botany, China. The identified mushrooms included Phallus impudicus (Stinkhorn egg), Trametesversicolor (formerly known as Coriolusversicolor, Turkey Tail), Tyromycescheoneus (White cheese polypore), and Pisolithusarhizus (Dead man's foot).

Methodology

Precipitationof proteins from sample Mushrooms

To extract proteins from the mushroom samples, a method described by Li et al. (2018) involving TCA precipitation was employed. Initially, 3g of finely powdered fruiting body was homogenized in an extraction buffer containing 0.1 M TrisHCl, 0.1M KCl, 10mM EDTA, 1% PVPP, and 0.4% mercaptoethanol for 30 minutes. Subsequently, the mixture underwent centrifugation at 20000xg for 20 minutes at 4°C. The resulting supernatant was then subjected to precipitation by adding four volumes of 20% TCA and incubating for 20 minutes at -20°C, followed by another round of centrifugation. The resulting precipitate was transferred to a fresh tube and stored at -80°C. Protein quantification was performed using the Bradford assay (1976), wherein various dilutions of a protein standard (BSA) were prepared, and unknown protein samples were treated similarly. Protein concentration was determined by measuring absorbance at 595nm.(Figure2).

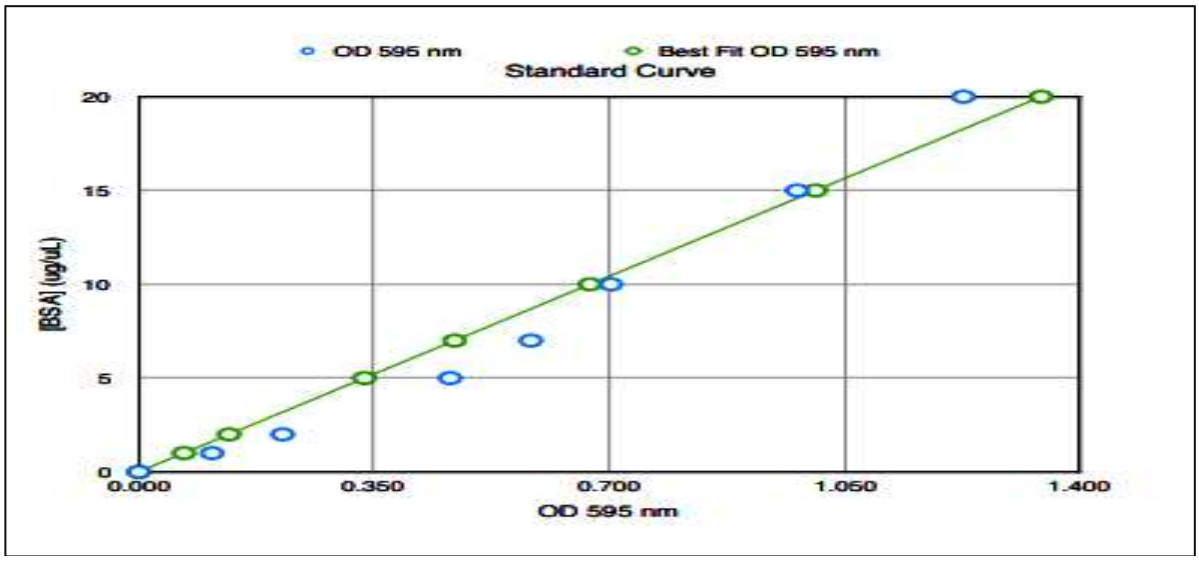

Fig.2 Standard curve for best fit OD for different dilutions at 595nm

SDS-PAGE Analysis

A modified version of the technique outlined by Laemmli (1970) was employed, with adjustments made to accommodate proteins with molecular weights below 10 kDa. This adaptation was necessary to ensure the isolation of proteins up to 10 kDa while maintaining high resolution and reproducible results, as previously demonstrated by Rivera et al. (2018).

Electrophoresis**:**

A total of 100 µg of protein extracts were mixed with 100 µL of SDS Sample Buffer comprising 0.5 M Tris-HCl (pH 6.8), 85% Glycerol, 10% (w/v) SDS, 0.1% bromophenol blue (dye), and 3 µL of β-mercaptoethanol. The protein samples were then boiled at 95°C for 5-6 minutes to denature the protein. It took around 4-5 hours at 70-80 V to migrate the proteins through the stacking gel (4% polyacrylamide), followed by 100 V to resolve the proteins in the 18% separating gel (30% acrylamide, 6ml; dH2O, 3.4ml; 1.5M Tris-HCL, pH 8.8, 100µL; 10% SDS, 100µL; 10% APS, 100µL; TEMED, 10 µL). To determine Molecular weights of alienated polypeptides, standard Unstained Protein Molecular Weight Marker ranging from 10 to 220 kDa (Cat. No.10747-012; Invitrogen Life Technologies) was co-electrophoresed. The gels stained with 2% coomassie blue solution for 30 to 40 minutes. Gels were washed with a solution containing 5% (v/v) acetic acid, 20% (v/v) methanol and distilled water in the ratio of 5:20:75 (v/v) to destain it. It was washed till such time till the color of the background disappeared and bands could be observed (Figure3).

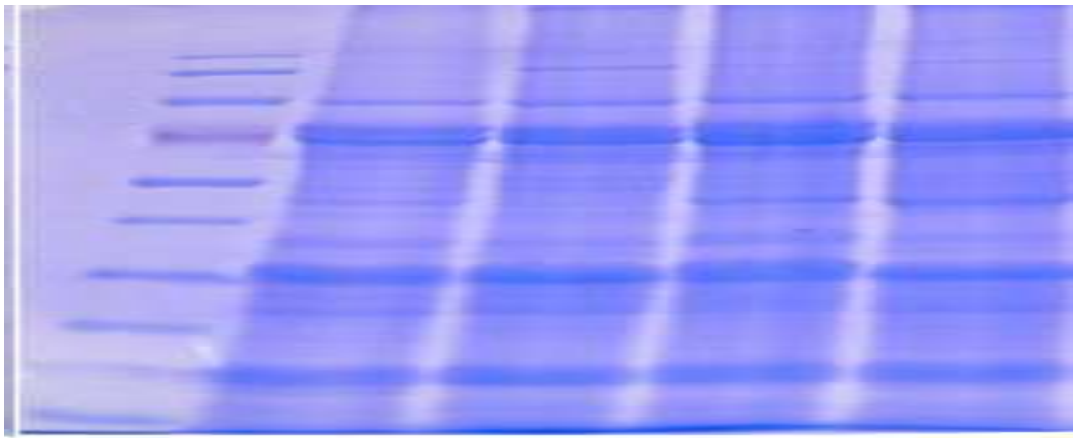

Figure 3. Protein bands of *P. impudicus*(PI), *T. versicolor*(TV), *P. arhizus*(PA) and *T.cheoneus* (TC) along with standard.

Extraction of Peptides

Zip tip protocol for peptide extraction and MCX stage protocol were adapted from for desalting of peptides were performed (Moreda-Piñeiro et al., 2014). Briefly, after spinning down the tubes in a centrifuge with low speed and incubated overnight, the supernatant was recovered in1.5 ml tubes. Then acetonitrile (50%), along with formic acid (0.3%) were used in adequate quantity, till complete coverage of gel pieces and kept warm in automatic incubator for about 15 minutes. The recovered supernatant was placed in the corresponding tubes and the procedure was repeated again and finally the extract was kept at -80°C for 30 minutes and dried in a speed vacuum. Freeze at -80C0 for 30 min. Speedvacdown just to dryness(Run-on LTQ Orbitrap XL^{TM} ETD Hybrid Ion Orbitrap Mass Spectrometer).

Statistical Analysis:

The PEAK X Thermo Fisher (V.10) (a complete solution for proteomics) was used for peaks detection, identification and analysis of proteins along with their peptides that were obtained from *P. impudicus P.arhizus, T. cheoneus, T.versicolor*and categorized by KEEG and NCBI databases. Furthermore, the results were expressed as mean ± standard deviation (SD) using SPSS Statistics (V.17.0) with one-way ANOVA and multiple comparisons was used to compare the mean values.

RESULTS AND DISCUSSION

Evaluation of protein

The analysis revealed variation in protein content among different samples, i.e P. impudicus (76.06 µg/10 mg), *T. versicolor* (161 µg/10 mg), *T. chioneus* (330.26 µg/10 mg), and *P. arhizus* (326.34 µg/10 mg). The *T. chioneus* exhibited the highest protein content, followed by P. arhizus (Table 1). The protein content of *T. versicolor* observed in this study is consistent with previous findings (Łysakowska et al., 2023). Similarly, protein content reported in a study from Turkey (2.07−2.94 g/100 g) aligns with our results (Reis et al., 2012). Notably, there is limited literature available on the proteomics of P. impudicus, T. chioneus, and P. arhizus, making this data valuable for future research endeavors (Ariaeenejad et al., 2023; Zhang et al., 2023).

Protein analysis of selected mushroom species

The obtained data were searched against the genome database of *P. impudicus* and *T.versicolor T. cheoneus*and *P. arhizus*according (Yap *et al* ., 2014) using search engine PEAKS X at NCBI and KEEG databases (Database: rs_Agaricomycetes155619_20190430_cRAP123, all taxon). The subsequent search filtered and parameters were implemented for protein and peptide identification: General conditions were MH+ scan range from 600 to 4000 Da, Parent Mass Error Tolerance: 50.0 ppm, Fragment Mass Error Tolerance: 0.1Da, while instrument parameters Ion Source: ESI (nano-spray). Fragmentation Mode: high energy CID (y and b ions) MS Scan Mode: FT-ICR/Orbitrap MS/MS Scan Mode: FT-ICR/Orbitrap. Identifications with “Distinct peptide” of 2 or greater than 2 were considered significant.

Table1. Total protein estimation in mushrooms sample

Sample fractions	Average OD at 595nm	Concentration (µg/ µL)	Amount of Protein (µg)	Volume for 10 µg (µL)
‘P.impudicus’	0.0315±0.03	0.47±0.09	76.06±0.45	21.276±1.33
‘T.Versicolar’	0.111±0.07	1.65 98±0.06	161.7±1.08	6.060±2.05
‘P. arhizus’	0.2235±0.03	3.33±0.00	326.34±2.03	3.00±0.99
‘T. chioneus’	0.226±0.002	3.37±0.5	330.26±0.056	2.967±3.02

Data was expressed as mean± SD (n=2), where $p \leq 0.05$

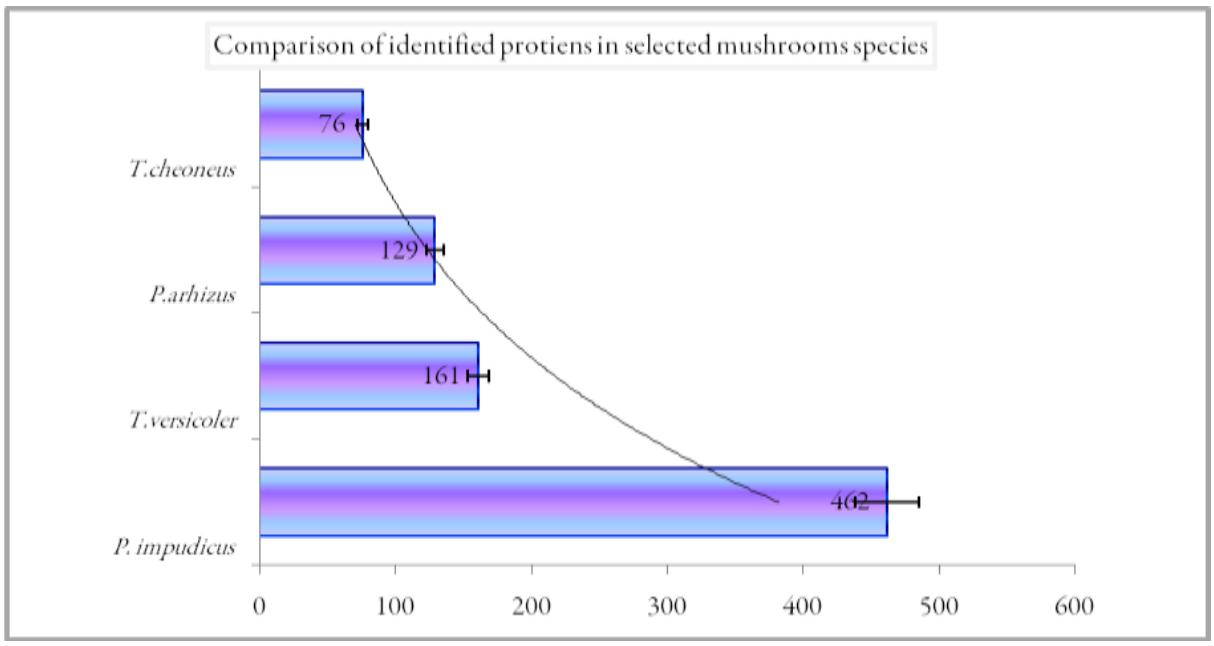

Figure 4. (%) Comparison of identified Proteins in all four mushrooms *P.impudicus* (PI) *T. versicolor*(TV) *T. cheioneus*(TC) and *P.arhizus*(PA).

A total of 462 proteins were identified in *P. impudicus*, 161 proteins in *T. versicolor*,76 proteins in *T. cheoneus*and 129 *proteins* in *P. arhizus* (Figures 4).The mushrooms species studied in present study have basic role in improving human health and are easily available in Japan, China and European countries and Pakistan at any major super markets. *P. impudicus* is also known as stinkhorn eggs which are used to treat rheumatism, epilepsy, gout, and skin cancer, anti-coagulant in platelets(Ries*et al* ., 2012).In *P. impudicus* most prominent proteins identified were 14-3-3 proteins that promotes brain function, neural signaling, neuronal development and neuroprotection (Park *et al* ., 2007). Xeming and Chieo (2018)reported that methionine adenosyltransferase obtained from various mushrooms can be used for treatment of Gaucher's disease (GD), alpha-galactosidase Promotes gastrointestinal health, 6-phosphogluconate dehydrogenase, arginino-succinate synthase enzyme is helpful Citrullinemia type I (CTLN1) treatment, Xylitol dehydrogenase (XDH) Threonyl-tRNA synthetase (TRS) is helpful in the cure of autoimmune diseases. Similarly DEAD domain proteins are involved in signaling pathways of cell (Zhou et al., 2020). Similarly in *T. cheoneus* identified proteins Heat shock proteins, cognate 70 (HSC70) are used in the pathological progression of Alzhimer's Disease (Liao et al., 2014). Cysteine peptidases an enzyme identified in *T. versicolor* are considered to play a major role for the pathogenicity of *E. histolytica* as suggested by a large number of *in vitro* and *in vivo* studies(Ziwei*et al* ., 2018).

Peptides Analysis

The obtained peptides of each sample mushroom (4µL) were loaded on LTQ Orbitrap XL^{TM} ETD Hybrid Ion Orbitrap Mass Spectrometer Thermo Scientific ^{TM}. The obtained data were analyzed by PEAKS X (version 10). According to PEAKS X studio analysis, 469 peptides sequences form 55 protein groups were detected in *P. impudicus*, where the ratio of the unique peptide was 87 (>2); 99 (=2); 276 (=1). While in *T. versicolor* 290 peptide sequences were detected the ratio of unique peptides was 25 (>2); 13 (=2); 65 (=1). Similarly in *T. cheoneus*156 peptides (unique peptide ratio was 6 (>2); 17 (=2); 16 (=1)) and in *P. arhizus*185 Peptides (unique peptide ratio is 6 (>2); 22 (=2); 100 (=1). Maximum peptides were detected in *P.impudicus* followed by *T.versicolor, P. arhizus*and *T. cheoneus*respectively (Figure8)

Post Translational Modifications (PTM)

PTMs play vital roles in regulating protein function and control many biological processes and phenotypes-changes in both prokaryotes and eukaryotes (Liet al., 2022). Several recent studies illustrate how fungal and bacterial pathogens use these PTMs to facilitate development, stress response, and host infection (Retanal et al., 2021). Additionally, post-translational modifications

(PTMs) have substantially broadened the functional repertoire of proteins. The PTMs identified in P. arhizus, P. impudicus, T. chioneus, and T. versicolor primarily included acetylation (N-term), deamidation (NQ), oxidation (M), and pyroglu from Q (Tables 2,3; figure 5,6).

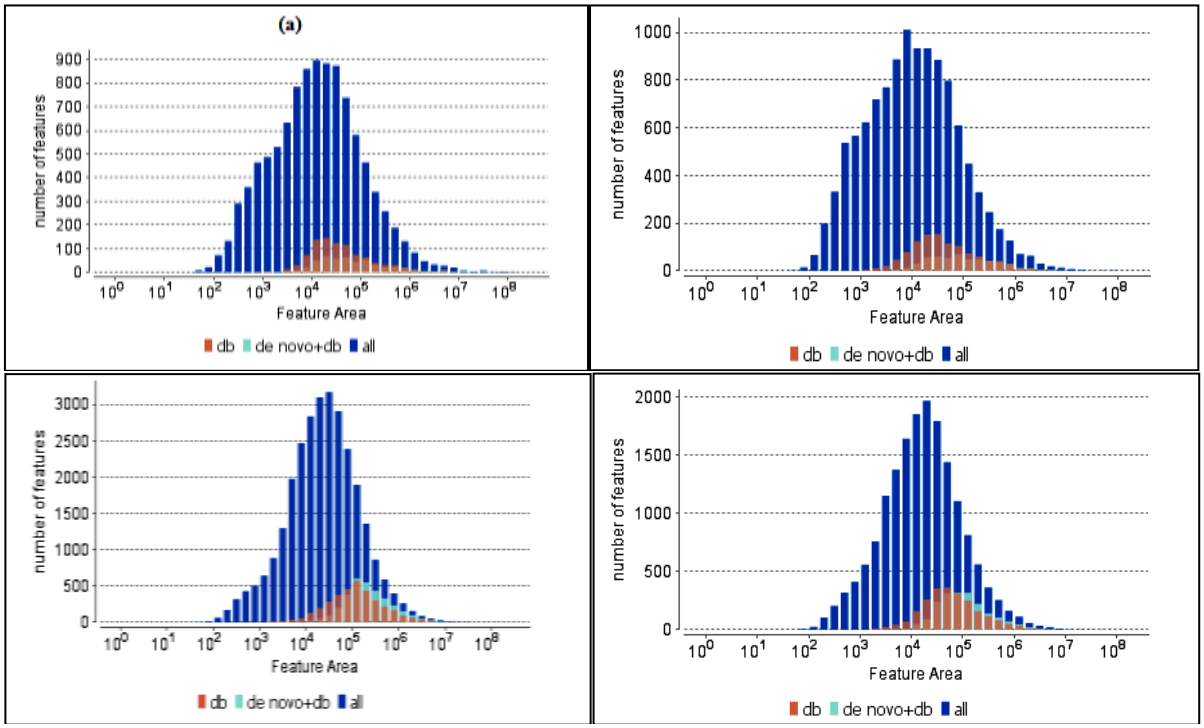

Figure 5. Distribution of identified Peptides Spectrum (A) *P.arhizus*, (B) *P.impudicus*, (C) *T. cheoneus* and (D) *T. versicolor*

Table2. Post Translational Modification (PTM) Profile of *T.cheoneus*						**PTM Profile of *P. impudicus***				
Name	**Average Mass**	**Position**	**PSM**	**-10lgP**	**Abundance**	**Average Mass**	**Position**	**PSM**	**-10lgP**	**Abundance**
Carbamidomethyl	57.02	C	13	49.02	3.59	15.99	M	17	55.21	5.79
Oxidation	15.99	M	10	32.28	3.6	0.98	NQ	10	70.98	3.82
Deamidation	0.98	NQ	9	55.22	2.05	57.02	C	10	39.39	6.13
Acetylation	42.01	N-term	4	44.31	2.68	42.01	N-term	4	47.73	6.96

PTMs are chemical modifications; N Q, C and N-term are sites where modification modifications in proteins structure occur

Table3. Post Translational Modification (PTM) Profile of *P.arhizzus*	**Post Translational Modification (PTM) Profile of *T. versicolor***

Name	Average Mass	Position	PSM	Average Mass	Position	PSM	-10lgP	Abundance
Carbamidomethyl	0.98	NQ	26	15.99	M	27	45.01	6.80
Oxidation	15.99	M	21	0.98	NQ	11	60.90	4.22
Deamidation	57.02	C	10	57.02	C	22	29.40	5.33
Acetylation	17.03	N-term	7	42.01	N-term	14	56.13	7.99

PTMs are chemical modifications; N Q, C and N-term are sites where modifications in proteins structure occur

Figure 6. Coverage of identified Post Translational Modifications (PTMs) in selected mushrooms species

CONCLUSIONS

In this study, we conducted a comprehensive proteomic analysis of four wild mushrooms: P. impudicus, T. versicolor, T. cheoneus, and P. arhizus. A total of 462 proteins were identified in P. impudicus, 161 in T. versicolor, 76 in T. cheoneus, and 129 in P. arhizus using LC-MS/MS. Our findings lay a solid foundation for the industrial-scale production of health-related products derived from these mushrooms, including P. impudicus, T. versicolor, T. cheoneus, and P. arhizus.

Authors Contributions

Abdur Rahim Khan: Performed Research Work, Technical input in every step, Amtul Sami: Analyzed Data, TahiraBatool: Helped in Data Collection, Muhammad Fiaz: Conceived the Idea and Supervised, Rahmat Ali Khan: Helped to provide the materials, GulnazParveen: Helped in research & Overall Management of the article. All authors read and approved the final manuscript.

Conflict of Interest:

The authors declare that there are no conflicts of interest related to this article. Furthermore, this research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

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