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ORIGINAL PAPER

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DETECTION OF BIOACTIVE COMPOUNDS AND AMINO ACIDS FROM FRUITING BODIES OF *Morchella tridentina*

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ABSTRACT

Morels are well known due to their nutritional and food value since ancient human history. In this study, biochemical and proteomic analyses were carried out on the ascocarp of *Morchella tridentina* Bres. For this, several ascocarp of *M. tridentina* were collected from different sites of Neelum Valley Azad Jammu and Kashmir, Pakistan. Identification was confirmed by phylogenetic sequencing using nuclear ribosomal DNA bar-coding technique along with morph-anatomical analysis. During the biochemical analysis, different bioactive compounds used in drugs to treat cancer, heart diseases, edema (veprisinium, visnagin, and bumetanide), and breast cancer (petunidin) were identified. Cerulinin, daidzein, guanthidin and okanin (imperative compounds) were also detected. Furthermore, protein analysis by FTICR/MS/Orbitrap revealed the presence of 921 proteins belonging to 171 protein groups having 165 unique peptide sequences. The study shows that this morel could be used as a source of bioactive substances to develop anticancer, antifungal, and antiviral drugs in the future. This fruitful addition of *M. tridentina* in Mycota of Pakistan increases the number of morels to three.

Key words: liquid chromatography, mass spectrophotometry metabolites Neelum Valley, proteins, *Morchella*

INTRODUCTION

Members of genus *Morchella* has been collected and consumed worldwide due to nutritional and medicinal importance. They contain vitamins, minerals, and various bioactive compounds used as antioxidant, anticancer, antimicrobial, and anti-diabetic substances and are considered a popular dietary source [Kalaras et al. 2017]. They are also used for taste, flavor, anti-inflammatory, immunostimulatory and antitumor properties [Tietel and Masaphy 2018].

The wild mushrooms contain organic and aromatic volatile compounds including antioxidant and antihyperglycemic compounds including food contents, dietary fiber, metal elements, free sugars, essential amino acids, organic acids, and fatty acids [Xu et al. 2019]. Morels contain important organic acids, sugars, amino acids, and proteins that make them more suitable for nutrition intake. The presence of organic acids, sugars, amino acids, and proteins has been re-

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ported from Morels [Wang et al. 2019]. Morels have been cultivated and collected worldwide. They contain vitamins, minerals, and various bioactive compounds used as antioxidant, anticancer, antimicrobial, and anti-diabetic substances and are considered a popular dietary source [Kalaras et al. 2017]. Some of the reported species of *Morchella* known as nutritional and medicinal mushrooms are *M. conica* Pers., *M. esculenta* (L.) Pers., and *M. elata* Fr. [Richard et al. 2015]. These reputed species have been screened out for their bioactive and nutritional components, *Morchella sextelata* M. Kuo, contains polysaccharides, important vitamins, and amino acids with immune-modulatory properties used in the preparation of drugs [Meng et al. 2019]. *Morchella conica* contains polyphenols, proteins, peptides, and amino acids while unsaturated fatty acids and organic acids are also present [Vieira et al. 2016]. Different compounds from the *M. esculenta* with antitumor activity against the carcinoma lungs cell lines were also identified [Lee et al. 2018]. Besides these three *Morchella* species, *Morchella tridentina* is a poorly known morel worldwide [Loizides et al. 2015] and up to our knowledge, there is no study about the biochemical or nutritional aspect of this morel up to date. We are reporting for the first time this medicinal morel from the Kashmir Region of Pakistan with many important bioactive compounds that could be helpful in the development of drugs in the future. In this background, this study was aimed to identify different medicinal and nutritional compounds from the *M. tridentina* and to characterize them through molecular tools.

MATERIAL AND METHODS

Collection and identification

The specimens of *M. tridentina* were collected from Neelum Valley, the western part of Himalayas, Azad Jammu, and Kashmir Pakistan at an altitude of 1816 m (Fig. 1). Morph-anatomical characters were noted in the field and the laboratory under a compound microscope (MX4300H, Japan). Specimens studied during this research work have been deposited after freezing treatment at –80°C for 15 days in the Her-

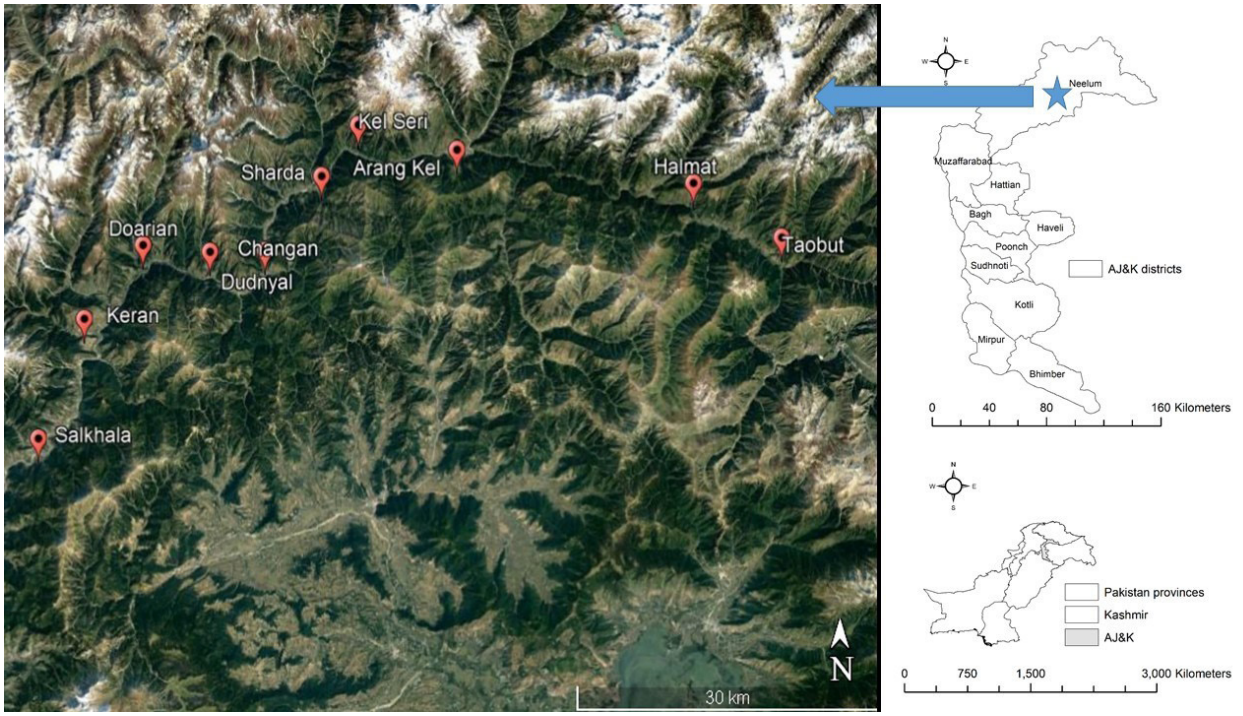

Fig. 1. Map of study area and sites (Neelum Valley, AJK). Source: Google Maps

Fig. 2. A digital photographs of fruiting bodies of *Morchella tridentina.* Scale bars: A = 4.3 cm, B = 4.5 cm (phot. T.S. Ullah)

barium of University of Azad Jammu and Kashmir (Muzaffarabad, Pakistan) and LAH Herbarium Department of Botany, University of the Punjab (Lahore, Pakistan). The digital photographs of fruiting bodies of *M. tridentina* are provided in Figure 2.

DNA sequencing and phylogenetic tree

Extraction of DNA from ascocarps was carried out by a modified 2% CTAB method [Bruns et al. 1991]. Gel Electrophoresis by using 1% agarose gel for 30 min. at 70 V for confirmation of successful extraction of total DNA was carried. Amplification of internal transcribed spacer region (ITS) was performed by polymerase chain reaction using primer combination ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') proposed by Gardes et al. [1991]. Forward and reverse sequences were obtained and final sequences of both samples T05 (849 base pairs) and T06 (878 bp) were developed with the help of BioEdit ver. 725. Nucleotide sequences were initially compared with most similar sequences at NCBI (https://www.ncbi.nlm.nih.gov/guide/) [Sherry et al. 2001] by using BLAST and were included in phylogenetic studies along with sequences of *Morchella* spp. reported from high altitude regions in Asia, Europe and South and North America which were close to our species (71318-21). Multiple sequences were aligned online using the MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle) [Edgar 2004]. The final phylogram was constructed in RAxML-HPC2 by using the XSEDE tool (8210) at 1000 bootstrap to get the best bipartition results. Figtree ver. 142 software was used for tree visualization and the initial setting of phylogram and additional tree annotation was added by using Adobe Illustrator CS10. DNA (ITS sequences) were deposited to GenBank and accession numbers were obtained.

Biochemical analysis

Preparation of extracts. A methanol and saline extracts of *M. tridentina* were used in this study for biochemical and proteomic analysis respectively. The extracts were prepared according to methods already described [Wang et al. 2018] with slight modifications. The extract was leftover in a desiccator until a fine powder was obtained from each sample. 1 mg of fine extracted powder was used for ultimate high performance liquid chromatography (UHPLC) and liquid chromatography – mass spectrometery (LC-MS) analysis that was carried out in Centre for

Mass Spectrometry and Proteomics University of Minnesota, USA.

LC-MS conditions. Samples (saline and water extracts) of *M. tridentina* were processed by following the methods as described by Cavalieri et al. [2010] and Wang et al. [2018] with slight modifications using a solution of and 50% methanol 50% water. Enough solution was added to bring all the samples to a concentration of 10 mg/mL. Samples were extensively vortexed up to no solid residue remained visible. Before analysis, the samples were diluted 10-fold with 25% methanol/water solution. Fifteen microlitres of the final sample were injected onto the column Thermo Fisher Ultimate 3000 platform UHPLC^{+} focused 3000 RS pump, 3000 RS column compartment with 3000 RS sample compartment. 25 µL syringe and 40 µL loop reverse phase for positive mode was carried out by using Waters Acquity BEH C-18 column 17 µm particle size at 40°C. Buffers used for the reaction were distilled water and 1% formic acid and acetonitrile with 1% formic acid at a flow rate of 4 mL/min. with an injection volume of 15 µL. The reverse phase for negative mode was carried with the same set of parameters with change in buffer A as Water with 10 mM ammonium acetate, pH 9.0, and buffer B as acetonitrile.

Mass spectrometry. Mass spectrometry was carried by using Thermo Scientific Q Exactive Quadrupole Orbitrap Heated-Electro-Spray Ionization probe source (HESI-II) with a spray voltage: 3400 V positive; 3000 V negative having sheath gas flow rate: 50 µL and capillary temperature: 320°C with aux gas heater: 400 S-lens and an RF level of 55%. For positive mode full MS scan was carried with a method duration of 10 min having a width of the chromatographic peak: 4 s, with positive polarity resolution, was sat at 70 000 with AGC target: 1e6 having maximum injection time: 200 ms with a scan range of 70 to 1050 m/z while for negative mode a full MS scan duration: 10 min peak width of chromatogram: 4 s with negative polarity was carried for full scan resolution was set at 70 000 RS with AGC target: 1e6 having maximum injection time: 200 ms at a scan range: 70 to 1050 m/z.

Bioinformation analysis

The acquired MS/MS spectra were searched by using MZmin-253 software (m/z and retention time) and proteomic data was analyzed by using peaks studio X (complete solution for proteomics).

RESULTS AND DISCUSSION

Molecular characterization

A phylogenetic tree was developed consisting of 52 sequences as ingroup of genus *Morchella*, while one sequence was as an outgroup of *Gyromitra slonevskii* VP Heluta [JQ691490] from Ukraine (Fig. 3). The final dataset of the phylogram consisted of 1676 characters of which 506 were conserved, 870 were variable, 491 were parsimony informative and 290 were singleton. The two *M. tridentina* sequences, T05 and T06 were identical to each other. In initial BLAST through GenBank, our *Morchella* sequences, T05 and T06 showed 100% identity to a sequence of *M. tridentina* from Spain [KM587967]. Final phylogenetic tree criteria for inclusion of sequences included all of the *M. tridentina* sequences reported worldwide and other *Morchella* spp. from Pakistan, India, China, Armenia, Cyprus, France and Spain.

In Figure 3 clade I shows a total of 21 sequences of *M. tridentina* from different parts of the world. In this clade, *M. elatoides* Jacquet., *M. frustrata* and *M. quercus-ilicis* are the synonyms of *M. tridentina* accepted by "Index fungorum" (http://www.indexfungorum.org/names/names.asp). Sequence JQ691439 is mistakenly named *M. vulgaris* (Pers.) Gray rather than *M. tridentina* because *M. vulgaris* is the synonym of *M. esculenta* and its own position is confirmed in a separate clade IV *Morchella* spp. with accession number MK758081 that seems to be different from *M. tridentina* by forming a separate branch with a bootstrap value of 99 from Turkey. This sequence was submitted in GenBank directly and has no description so more DNA sequences and its morpho-anatomical description will be helpful for its true identity. *Morchella tomentosa* M. Kuo (JQ723016) from China made a separate branch with a bootstrap value of 100 and this is the first closest species to *M. tridentina.*

Clade II separated from clade I with a supporting bootstrap value of 99 and consist of 5 sequences belonging to 4 species of *Morchella* i.e. *M. crassipes* (Vent.) Pers. from China (KR809597) and India (KF135218) *M. esculentoides* (JQ723066) from China *M. spongiola* Boud. (GQ228476) from India and *M. rufobrunnea*

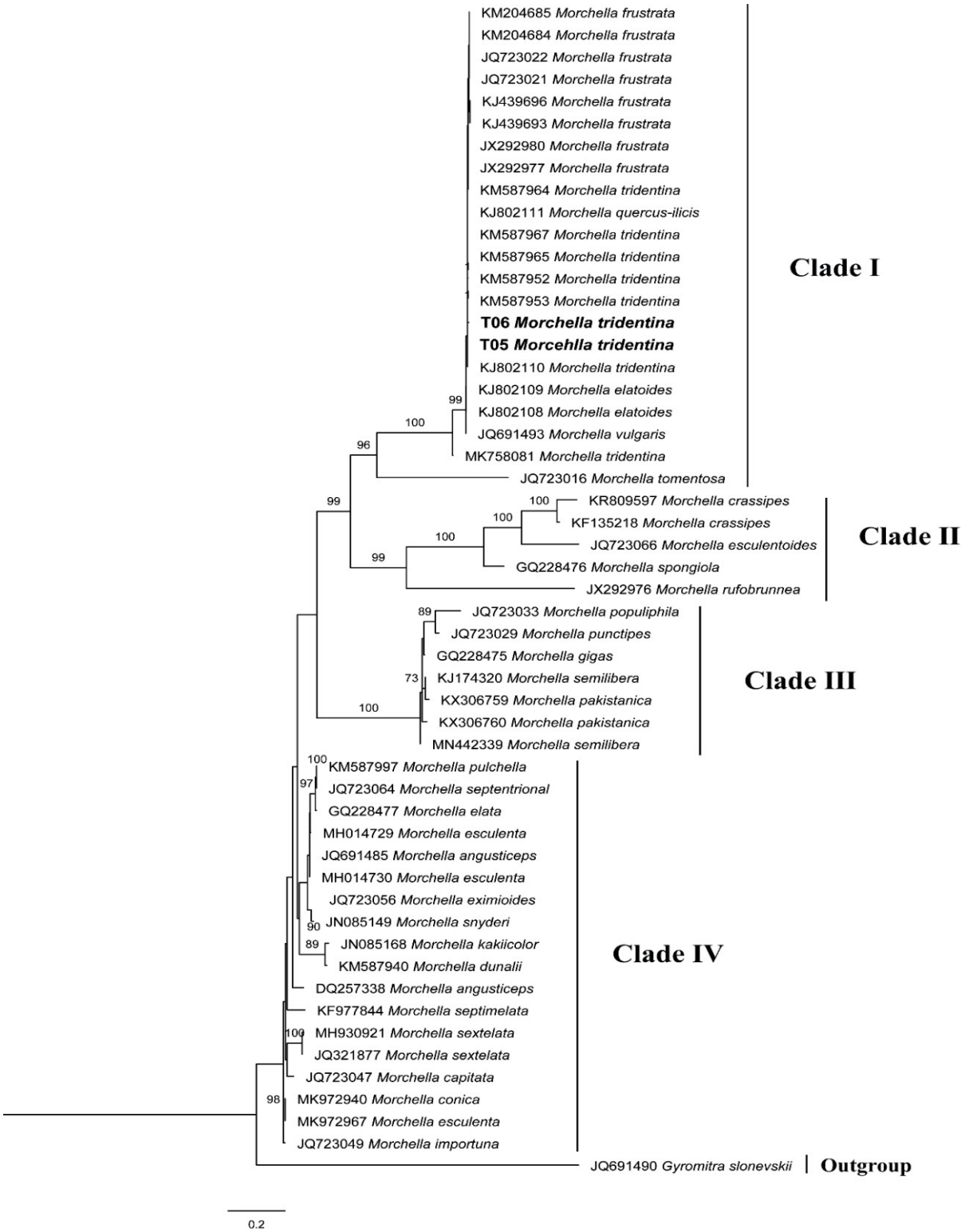

Fig. 3. Phylogenetic tree T05 [MT584841] and T06 *[*MT957957] for *Morchella tridentina* based on ITS sequence analysis

Guzman & F. Tapia (JX292976) from Cyprus. Clade III consist of 7 sequences of 5 *Morchella* species including *M. populiphila* (JQ723033) and *M. punctipes* Peck (JQ723029) from China *M. gigas* (GQ228475) from India *M. semilibera* D.C. (MN442339) from Italy and France (KJ174320) and *M. pakistanica* Jabeen & Khalid (KX306760 and KX306759) from Pakistan.

Clade IV consists of 18 sequences of *Morchella* species from different parts of the world. In this clade, taxonomic revision is required by further study of their type specimens and more than one collection will confirm the position of each species on molecular bases. *Morchella* samples from Pakistan (T05 and T06) confirmed their position after comparison with sequences of *M. tridentina* from all over the world. Morpho-anatomical characters also support its position as present in phylogenetic tree, i.e. our samples are *M. tridentina.*

The phylogenetic analysis of samples collected from Surgon and Dawarian Neelum Valley, Azad Jammu, and Kashmir confirm the specimens as *M. tridentina*, the first time recorded in Pakistan. As the previous studies by Richard et al. [2015] affirmed the study by O'Donnell et al. [2011] reported the presence of *M. tridentina* only from North West of America as a Post Fire Morel, samples assigned as T05 and T06 were crossed checked with reported *M. tridentina* samples throughout the world for morphological and phylogenetic characters. It has a medium-sized ascoma with small and deep pits. Stipe is dark to brown and swollen at the base. It can be compared with *M. tridentina* studied by Loizides et al. [2015] and a closely related taxon, *M. semilibera* D.C. common to Europe and less common in Asia. *Morchella semilibera* can be characterized by a long hollow stalk with campanulate half-free apothecial margins [Moreau et al. 2014]. Another morel specimen identified from Pakistan *M. pakistanica* also differs from *M. tridentina* by having wide and long and gradually narrow hymenium [Jabeen 2016]. It also differs from *M. pulchella* Clowez & Franc new recorded species to Pakistan and is characterized by having conical brown hymenophore with irregular to regular ridges with hollow convex and short stipe [Badshah et al. 2018]. Our samples T-05 [GenBank MT584841] and T-06 [GenBank 957957] diverged from all the taxa placed together and made a group with *M. tridentina* [KM587953] with a strong bootstrap value. Morphological features and phylogenetic study of the specimen suggest that it is *M. tridentina* as a new record to Mycota of Pakistan.

Biochemical analysis. Biochemical characterization of *M. tridentina* revealed the presence of organic acids bioactive and different nutraceutical compounds (Tab. 1). These compounds are malic acid, succinic acid, oxalic acid, xylobiose, tolmetin sodium, tetrahydropteridine, tenofovir, panamine, oxethazaine, fumaric acid, N-formyl-4-amino-5-aminomethyl-2--methylpyrimidine, N-methylhistamine, nitrilacarb, N[pi]-methyl-L-histidine, N'-nitrosoanabasine, mycolactone D, mirtazapine, L-histidinol, L-capreomycidine, lathyrine, laninamivir, L-arginine, guanadrel, iproniazid, ethambutol, galegine, and dalapon. Essential amino acids including L-arginine L-histidine and L-cysteine are found to present in the morel fruiting bodies. Bioactive compounds such as daidzein, guanthidine, petunidin, veprisinium, visnagin, psoralen, oxamniquine, okanin, bumetanide, L-arginine, and L-histidine were also identified (Fig. 4).

Proteomic analysis. Protein analysis of morel fruiting bodies of *M. tridentina* was carried out through MS/MS Scan FTICR/Orbitrap. A total of 921 proteins belonging to 171 protein groups were identified. Out of these 921 protein sequences, 165 unique peptide sequences were also identified. The major amino acids of these unique peptides were alanine, lysine, arginine, glycine, and glutamic acid. The results of the proteomic analysis are shown in Figure 5 and Figure 6.

Morels are a popular source of food due to their unique flavor and taste. Biochemical analysis of *M. tridentina* showed the presence of organic acids, vitamins, and bioactive contents. The presence of organic acids including oxalic acid, malic acid, fumaric acid and succinic acid has been reported in morel *M. importuna* by Bruns et al. [1991]. In the present study, we also identified these organic acids from *M. tridentina.* Concentration (µg/g) of detected bioactive compounds is given in Figure 5. The highest contents of succinic acid as 398 ±72 ug/g were found while the lowest contents of oxalic acids as 285 ±78 ug/g were found. Monosaccharaides such as glucose, galactose, and D-fructose were also identified.

Table 1. List of compounds detected from the fruiting bodies of *Morchella tridentina*

Compounds	Retention time	Contents (ug/g)
Acetyl phosphate	1.41	2312 ±31
Aclatonium	4.16	4113 ±52
Acrinol	5.22	995 ±24
Bumetanide	4.17	43 ±15
Canavanine	5.19	627 ±22
Candicine	1.21	312 ±14
Capecitabine	1.09	925 ±26
Cyenopyrafen	4.16	416 ±23
Cyromazine	7.32	213 ±62
Dacarbazine	4.34	3976 ±19
Dalapon	1.08	1123 ±33
Damascenine hydrochloride	2.26	821 ±29
delta-Guanidinovaleric acid	4.53	4589 ±22
Deoxycytosine	1.46	2345 ±34
Dicrotophos	3.24	1124 ±23
Diethylcarbamazine	4.91	474 ±47
Dihydropteridine	5.56	158 ±24
Dimethirimol	4.16	159 ±29
Dinotefuran	4.17	416 ±41
Dolichotheline	1.25	3623 ±17
D-Ornithine hydrochloride	1.94	228 ±13
Elaeocarpidine	9.82	1621 ±23
Ergothioneine	1.17	712 ±62
Ethambutol	2.12	2011 ±33
Galegine	5.40	556 ±35
Hercynine	1.22	432 ±52
Homoarginine	8.92	2645 ±23
Iproniazid	1.47	1416 ±37
L-Arginine	3.18	293 ±23
Methylguanidine	2.45	224 ±23
Metribuzin	2.17	213 ±18
Mirtazapine	1.8	1870 ±36
Tetrahydropteridine	8.41	1337 ±21
Tolmetin sodium	2.76	923 ±32
Veprisinium	4.66	237 ±53
Visnagin	7.32	112 ±14
Xylobiose	3.80	3532 ±31

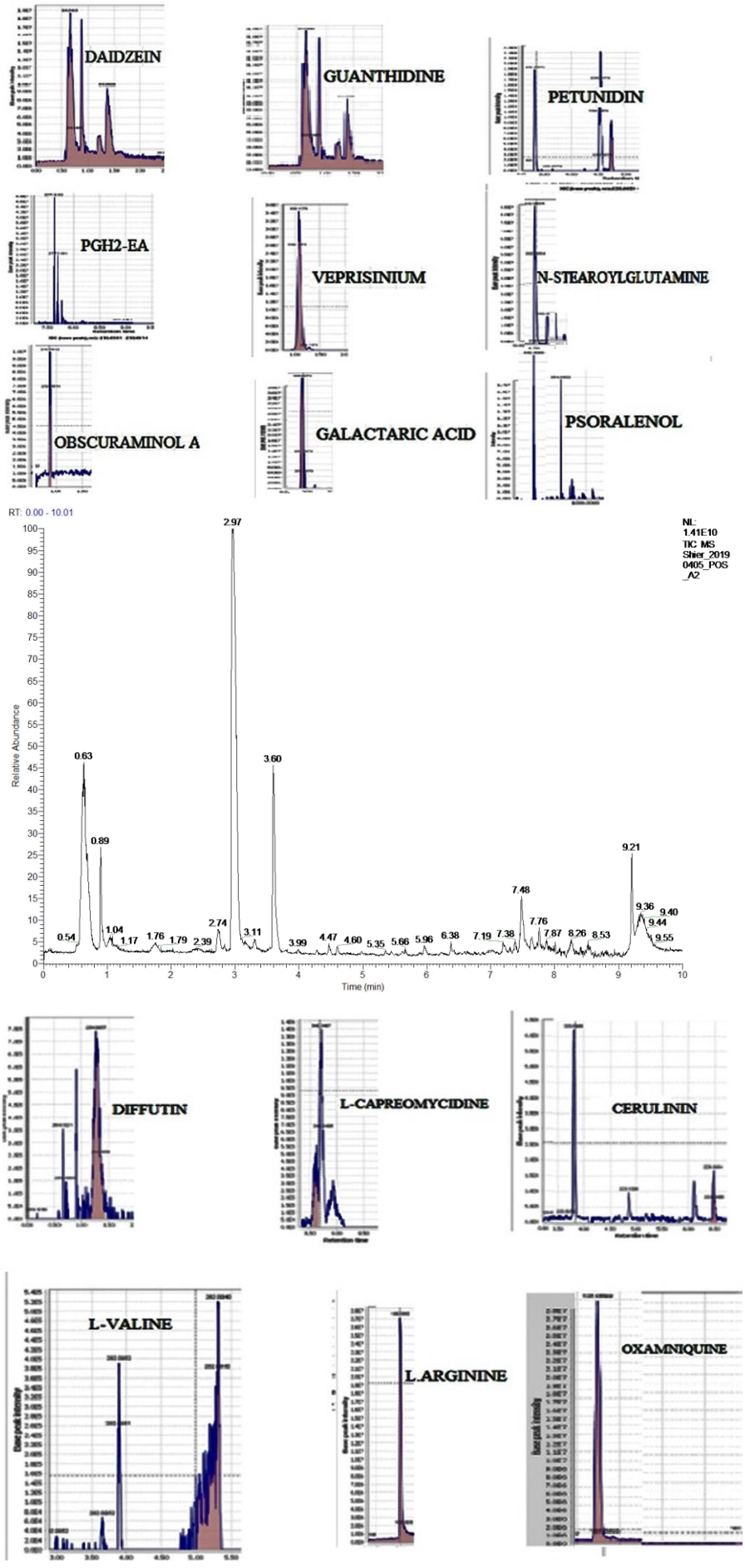

Fig. 4. Chromatogram with base peaks of detected bioactive compounds (m/z) in *Morchella tridentina*

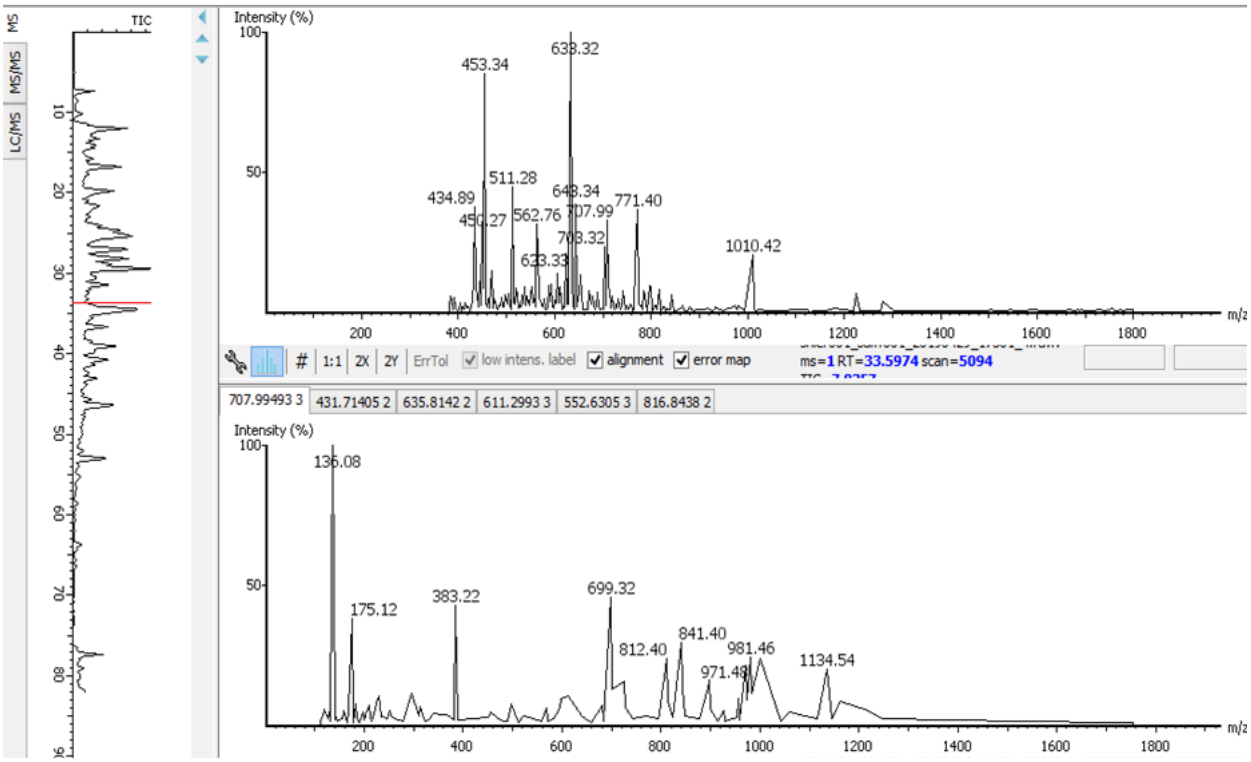

Fig. 5. Chromatogram with base peaks of detected amino acids (through MS/MS scan FTICR/Orbitrap; m/z) in *Morchella tridentina*

Glucose was the major monosaccharide present in *M. tridentina* as 386 ±27 ug/g. Studies carried by Kalaras et al. [2017] through HPLC of *M. sextelata* revealed the presence of glucose and galactose as a major component of fruiting bodies while rhamnose (Rha), arabinose (Ara) and fucose were also present. *Morchella esculenta* was recommended as a good source of food and pharmaceutics by Wagay et al. [2019]. *Morchella tridentina* is a popular source of food due to the presence of essential amino acids such as L-arginine, L-histidine and L-cysteine. Previously, amino acids like malic acid and aminobutyric acid were identified from the *Morchella* fruiting body by Rotzoll et al. [2005]. Bioactive compounds used in antibiotics, i.e. daidzein, bumetanide and petunidin were also identified. In a recent study, daidzein and its analogs have been identified as a potential immunostimulatory drug that can be used as a significant component for HIV therapy [Liu et al. 2019]. Bumetanide is found to present in *M. tridentina*. It is a pharmaceutically important compound and used in the treatment of autism spectrum disorder [Kassem and Oroszi 2019]. Extract of mushrooms can be used due to cosmeceutical and nutricosmetic ingredients to treat inflammatory skin disease and hyperpigmentation [Taofiq et al. 2020]. Petunidin having great antioxidant activity is used against oxidative stress [Chen et al. 2019]. Another bioactive compound, psoralen found in morel fruiting bodies is used as a phyto-chemotherapeutic agent for the treatment of skin diseases [Shivasaraun et al. 2018]. Visnagin induces intracellular oxidative stress and hence can be used to treat carcinoma [Aydoğmuş-Öztürk et al. 2019]. Bioactive substances, β-N-acetyl hexosaminidase and α-12-mannosidase were reported to an important role in the degradation of glycans by Kumakura et al. [2019]. Another antifungal compound, cerulenin [Hittalmani et al. 2016] is also identified from the morel *M. tridentina*.

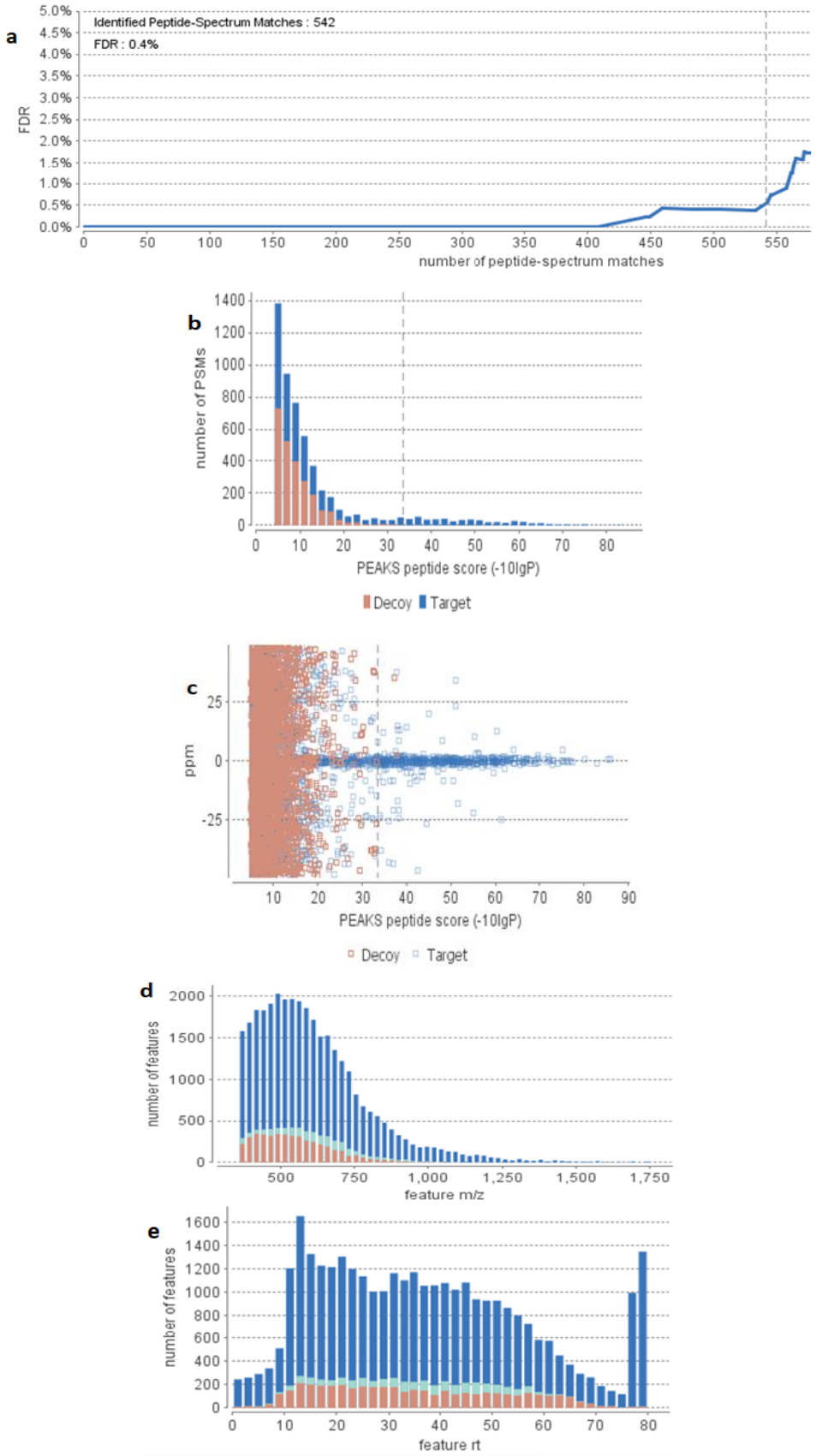

Fig. 6. Peptide score and distribution of different proteins (peptide) sequences detected in *Morchella tridentina*: a) false discovery rate (FDR) curve; b) distribution of PEAKS peptide score; c) scatterplot of PEAKS peptide score versus precursor mass error; d) peptide feature m/z distribution; e) peptide features retention time (RT). PSM – peptide spectrum match

CONCLUSION

This is the first report of *M. tridentina* from Azad Kashmir, Pakistan identified through molecular, ITS sequence analysis. The biochemical analysis shows that fruiting bodies of *M. tridentina* contain different pharmaceutical and nutritionally important compounds that can be used as a source of food along with other morel species. In the future, it can be proven as a potential source of imperative bioactive compounds and a source of drugs.

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