

Nutritional quality evaluation and proteome profile of forage species of Western Himalaya

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Abstract

Naturally occurring forage species in the high-altitude pasturelands of Western Himalaya are traditionally being utilized for feeding of livestock. However, the nutritional quality evaluation and validation of their potential as feed for livestock needs to be undertaken. Here, we evaluated proximate compositions, minerals and amino acids (AAs) of five high-altitude forage species, namely, *Festuca kashmiriana* L., *Medicago sativa* L., *Trifolium pratense* L., *Medicago falcate* L. and *Melilotus indica* L. The results revealed that proximate compositions, mineral and AAs varied significantly among the forage species. The carbohydrate, crude protein, moisture content, crude fat, crude fiber, crude ash, total phenol, oil absorption capacity and water absorption capacity were found in the range of 17.44–37.27 mg/100 mg, 3.34–14.71 mg/100 mg, 88.73%–90.72%, 0.98–2.32 mg/100 mg, 11.16–24.16%, 7.71–34.49%, 292.50–488.12 µg/100 mg, 3.91–4.67 g/g and 2.64–3.41 g/g, respectively. Elemental composition showed that calcium was the predominant element among the minerals (13.91–132.05 mg/kg DM) followed by magnesium (4.60–12.92 mg/kg DM), iron (2.04–76.13 mg/kg DM) and zinc (1.07–2.17 mg/kg DM). Furthermore, we found that these high-altitude forage species are rich in essential AAs like histidine, tryptophan, valine, leucine, phenylalanine and to some extent isoleucine and tyrosine. In addition, these five species showed distinct proteomes but shared a similar functional group. The proteome profiling of these forage species will help to understand the molecular basis of nutritional enrichment and stress tolerance potential against harsh environmental conditions of high altitudes. Overall, we conclude that traditionally used high-altitude forage species are nutritionally rich and can be recommended as part of the daily nutritive feed for livestock animals.

KEYWORDS

adaptation, feed, forage species, high altitude, proximate analysis

Robin Joshi and Raman Kumar contributed equally in this project.

[Correction added on 28 February 2023, after first online publication: The copyright line was changed.]

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1 | INTRODUCTION

Indian Himalayan region occupies nearly 35% of the geographical area and is characterized by warm temperate grasslands, sub-alpine, cool temperate grassy slopes and steppe formations of cold arid regions. Natural grasslands are extremely important for cattle raising, agro-pastoralist and nomadic herders that depend almost on these ecosystems (Dong et al., 2011). Grasses are the fourth largest group of flowering plants belonging to the *Poaceae* family, which consists of more than 10,000 species. They efficiently recycle atmospheric CO₂ to O₂ without any negative impact on the ecosystem and grow in various environmental conditions (Kipriotis et al., 2015). Grasses are a rich source of starch, protein, fiber, lignin and minerals, which are essential determinants of cattle's growth, reproduction and behavior; however, their nutritive values are modified by the climate condition of their habitats (Lee, 2018). Further, high-altitude forage species are good in taste and easily digested, enriched with higher nutritive values and have been used as feed stuff for livestock for a long time (Koutsoukis & Akrida-Demertzi, 2020). The grasslands of the Western Himalayan region support a large number of wild herbivores, domestic livestock and several agro-pastoral cultures. It is the significant pool of milching and wool-producing animals providing the high quality of milk and wool. Gaddis (rear sheep and goat) and Gujjars (rear buffaloes) are the main nomadic communities that mainly rely on alpine grasslands for rearing their livestock. However, other local tribal peoples also utilize them for fodder purposes. Most of the grasslands in the lower temperate belt of Western and Central Himalaya are grazed by domestic livestock throughout the year. Grassland in the Western Himalayan region consists of a variety of forage species that are reported to improve the production of grazing animals, which support several agro-pastoral cultures in the region (Dev et al., 2009; Misri, 2003).

Global demand for animal products would likely increase up to 60–70% by 2050 due to the increasing population worldwide. For example, global demands of meat and dairy products will likely increase to 58% and 74%, respectively, by 2050 (Makkar, 2018). Previous studies have demonstrated that in India, approximately 27 million tonnes (t) of additional feed would be required by 2030 to meet the chicken meat production (Robinson & Makkar, 2012). Feed accounts for up to 70% of the total cost of livestock operation. Currently, ~800 million t of cereals are being used as animal feed and its consumption is projected to cross 1.1 billion t in the near future (Makkar, 2018). The use of food as feed will intensify food insecurity in developing countries. Thus, food versus feed competition is one of the biggest challenges for the livestock sector. Efficient use of grasslands and selection of important forage species for commercial propagation and fodder production could offer possibilities and opportunities for feed production, which reduces pressure from food. Beside plant-based products, rendered animals, their waste to antibiotics and organ arsenicals are the most common ingredients of animal feed (Sapkota et al., 2007). The inclusion of these ingredients could affect the quality and safety of animal-based food products and poses potential risks to human health (Sapkota et al., 2007). To fulfill the livestock feed demand usage of forage plants as animal feed sources would be required. In this regard, high-altitude

forage species may offer a feed source to promote and support the emerging livestock-based industries. However, their nutritional profiling has not been reported yet, limiting their usage as feed. The nutritional composition of forage plants depends on species, tissue type, stage of maturity at harvesting, environmental condition, topographical and geographical features (Reine et al., 2020). At the early stages of development, forages have relatively higher crude protein (CP) and ash content; however, crude fiber, lignin, hemicellulose and cellulose increase in later stages of development, resulting in a significant decrease in dry matter digestibility (Mirza et al., 2002). In addition, forage plants of temperate and tundra regions have comparatively more fiber and lignin and less protein than forage plants grown in arid and equatorial regions (Lee, 2018). Besides the nutritional aspects, high-altitude forage species are tolerant to harsh environments including cold, drought and high UV conditions. Proteins are direct effector molecules that control plant growth, development and also provide tolerance to various stress conditions. With the help of mass spectrometry-based proteomic tool, it is possible to identify and quantify stress-responsive proteins, elucidate their signaling pathways and also inspect post-translational modifications (Kumar et al., 2014).

Usually, a single forage species cannot form a balanced diet. For this, livestock must get their nutrition from a broad range of plants, including grasses (annual and perennial), herbs, shrubs and forbs. The plant diversity can provide healthier nutrition for livestock as it proposes a variety of forage options (unique nutritional composition of each), due to which livestock would meet their nutritional requirements. This will help to prepare a better nutritional feed for increasing animal productivity. Therefore, in the present study, five forage species, namely, *Festuca kashmiriana*, *Medicago falcate*, *Medicago sativa*, *Trifolium pratense* and *Melilotus indica*, from Lahaul-Spiti, Himachal Pradesh, India, were screened for nutritional profiling. In addition, proteomic profiling of these forage species was performed to understand the molecular basis of different nutritional compositions and adaptation mechanisms at high altitudes.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Five different forage species, namely, *F. kashmiriana* Stapf (Vernacular Neeru), *M. falcate* L. (Vern. alfalfa), *M. sativa* L. (Vern. Bhuksukh), *T. pratense* L. (Vern. Red clover) and *M. indica* L. (Vern. Ladakhi bhuksukh), were collected from Lahaul and Spiti region of Himachal Pradesh, India. The above species were chosen on the basis of a reconnaissance survey conducted in the region, where traditional communities were consulted. The patterns of foraging livestock in pasturelands were also observed. Further, all the five species are abundantly distributed in the region. Six different sites, which are representative of the region, were selected, namely, Tabo (3283 m: 32°5'24"N, 78°23'24"E), Ribling (3450 m: 32°34'12"N, 76°58'48"E), Shego (3539 m: 32°10'48"N, 78°6'0.1"E), Kaza (3661 m: 32°13'48"N, 78°4'12"E), Kibber (4139 m: 32°19'48"N, 78°0'36"E) and Gete

(4365 m; 32°18'0"N, 78°1'48"E), which were chosen for sampling at noon in the month of August 2017. The voucher specimens of the targeted species were also collected, which were deposited in the Herbarium of CSIR-IHBT, Palampur, Himachal Pradesh, India (Acronym PLP), and later authenticated by consulting existing specimens from the region and various regional floras. The specimens were assigned Herbarium accession numbers as PLP13038, PLP3419, PLP3420, PLP3418 and PLP3421, respectively, as specimen numbers (Figure 1). All forage species were in flowering stages at the time of harvesting. Environmental data like average temperature, rainfall and relative humidity of Lahaul–Spiti area were $13.59 \pm 4.42^\circ\text{C}$, 80.26 mm and $64.6 \pm 16.8\%$, respectively (data recorded at the agro-meteorological tower of CSIR-IHBT installed at our research center, Centre for High Altitude Biology, Lahaul and Spiti), at the time of sampling. At each site, approximately 100–125 plants of each forage species (above ground part) were randomly collected in liquid nitrogen and stored at

-80°C . We pooled each sample of different forage species collected from six different sites to minimize variation. Further, 10–12 plants of each species were used for experimental work. All experiments were performed in triplicates. Fresh plants were also stored in an airtight container for the determination of moisture content.

2.2 | Proximate composition of forage species

The moisture content of the fresh tissue was determined by drying 2 g of plant samples at 110°C to a constant weight. The remaining frozen forage samples were thawed to room temperature and the excess water was removed by using blotting paper and the plant samples were oven dried at 40°C until constant weight. The average moisture of the samples was maintained at 10% on a dry weight basis for conducting proximate analysis. The dried plant samples were

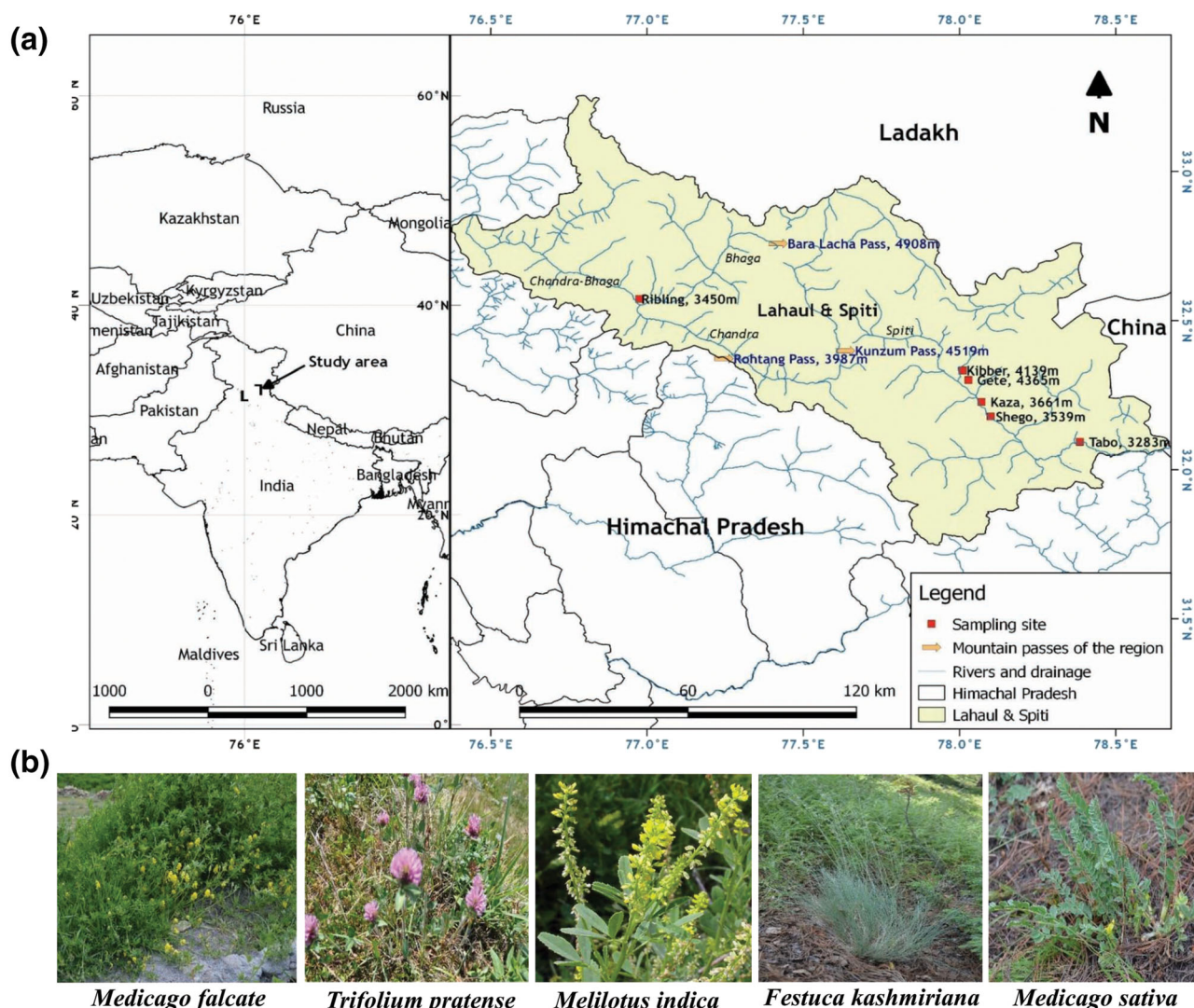


FIGURE 1 Illustration of sampling site. (a) Six different sites, namely, Tabo (3283 m), Ribling (3450 m), Shego (3539 m), Kaza (3661 m), Kibber (4139 m) and Gete (4365 m), were chosen for sampling of five forage species at Lahaul and Spiti Himachal Pradesh, India, and (b) representative image of five forage species *Medicago falcate* (PLP3419), *Trifolium pratense* (PLP3418), *Melilotus indica* (PLP3421), *Festuca kashmiriana* (PLP13038) and *Medicago sativa* (PLP3420) at the time of harvesting

pulverized to an average particle size of 400 microns, and the proximate analysis was performed with dry powders of respective plant samples. The proximate composition of forage species was characterized by standard procedures (AOAC, 2005).

The phenol-sulfuric acid method was used to estimate carbohydrate content from 200 mg of plant sample (AOAC, 2005). The CP was estimated by quantifying total nitrogen content using micro-Kjeldahl method and multiplying the total nitrogen content with a conversion factor of 6.25 according to the protocol described earlier (Magomya et al., 2014). Further, crude fat was determined using 5 g of samples by solvent extraction with *n*-hexane. Total crude fiber (acid and alkali soluble fiber) content was determined using 3 g of tissue through a series of digestion and calcination procedure (AOAC, 2005). Total ash was determined by calcination of samples (2 g) in an oven at 550°C until constant weight (AOAC, 2005). Water absorption capacity (WAC) and oil absorption capacity (OAC) of each forage species were determined by the centrifugation method (Pradeep et al., 2014). Refined groundnut oil was used for the determination of OAC. All experiments were performed in triplicates.

2.3 | Mineral and amino acid analysis

Calcium (Ca), magnesium (Mg), iron (Fe) and zinc (Zn) concentrations were determined by atomic absorption spectrometry (Shimadzu, Japan) according to the protocol described previously (Juknevičius & Sabiene, 2007). Each sample (500 mg) was placed in a 100-ml volumetric flask, and 14 ml of a mixed acid solution of nitric acid (HNO₃), sulfuric acid (H₂SO₄) and perchloric acid (HClO₄) in a ratio of 9:4:1 was added and mixed by gentle swirling of the container. The flask was placed on low heat hot plate in a digestion chamber. Then, the flask was heated at a higher temperature until the production of red NO₂ fumes ceased. The contents were further evaporated until the volume was reduced to about 3 to 5 ml but not to dryness. The complete digestion was confirmed when the liquid became colorless.

After cooling of the flask, 20 ml of deionized or distilled water was added. Then these digested samples were transferred in 100-ml volumetric flasks and the volume was made up to 100 ml with distilled water. The extracts were filtered using Whatman filter paper No. 1, and the filtrate was collected in labeled reagent bottles. Aliquots of this solution were used for the determination of K⁺, Mg⁺², Ca⁺², Na⁺, Fe⁺², Mn⁺², Cu⁺³, Cr⁺³, Zn⁺², Ni⁺³, Pb⁺⁴ and Cd⁺². The solutions were analyzed for the elements of interest utilizing the Atomic Absorption Spectrometer (Shimadzu AA-6300) with suitable hollow cathode lamps. The percentage of different elements in these samples were determined by the corresponding standard calibration curves obtained by using standard AR grade solutions traceable to standard reference material (from NIST library of the elements, that is, K⁺, Mg⁺², Ca⁺², Na⁺, Fe⁺², Mn⁺², Cu⁺³, Cr⁺³, Zn⁺², Ni⁺³, Pb⁺⁴ and Cd⁺² (Pequerul et al., 1993; Zafar et al., 2010; Zarcinas et al., 1987).

Estimation of amino acids (AAs) was performed using the protocol described previously (Kumari, Thakur et al., 2020). All experiments were performed in triplicates.

2.4 | Protein extraction and mass spectrometry

Protein extraction was performed according to the previously described protocol with slight modification (Kumari, Joshi & Kumar, 2020). Approximately 2 g of tissue was ground to fine powder in liquid nitrogen and suspended in 10 ml of pre-chilled homogenization buffer (50 mM HEPES-KOH [pH 7.5], 1 mM PMSF, 40% sucrose [w/v], 1% β-mercaptoethanol [v/v], 1 mM ascorbic acid, 1 mM EDTA and 1% Triton-X [v/v]). Next, equal volume of Tris-saturated phenol (pH 8.0) was mixed with homogenate, vortexed and placed on a shaker for 30 min at 4°C. Proteins were separated by centrifugation (5000 g, 15 min) and precipitated using 0.1 M ammonium acetate in methanol for overnight at −20°C followed by centrifugation (10,000 g, 15 min at 4°C). Protein pellet was washed with cold acetone (80%) thrice, air dried and dissolved in rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS [w/v]). Protein quantification was performed according to the Bradford assay (Bradford, 1976).

Gel-free approach was used for protein profiling according to Kumari, Joshi and Kumar (2020). For LC-MALDI, approximately 100 µg of protein from each sample was dissolved in 100mM ammonium bicarbonate followed by reduction and alkylation with DTT (10 mM) and iodoacetamide (55 mM), respectively. Trypsin digestion of protein sample was performed using trypsin (Promega, India) at 37°C for 12–16 h. Digested peptides of each sample were lyophilized (Labconco, USA) and dissolved in 10 µl of 0.1% trifluoroacetic acid in water for data acquisition using LC-MALDI-TOF-TOF MS/MS (Bruker Daltonics, Germany) coupled with fraction collector (ProteinPrep II, Bruker Daltonics, Germany) controlled with HyStar 3.2 software (Bruker Daltonics, Germany). Peptide separation was performed using trap column C18 (100 µm × 2 cm, 5 µm, 100 Å) and Zorbax 300 SB-C18 (0.075 × 150 mm, 3.5 µm) equilibrated with solution A (0.1% [v/v] formic acid in water) with flow rate of 500 nl/min having a linear gradient starting from 5% B (0.1% formic acid in acetonitrile) in 8 min, 45% B in 98 min, 98% B in 118 min and again at 5% B in 125 min. A total of 384 fractions (80 nl each) were collected and automatically mixed with 420 nl of matrix for MALDI-TOF/TOF analysis operated in a reflectron mode with Peptide Calibration Standard mixture (Bruker Daltonics, Germany). A list of precursor peaks was obtained using WARP-LC software (Bruker Daltonics, Germany) analyzed with MS/MS mode for protein identification. The following parameters were set for MS and MS/MS mode: database, NCBI (2017); search engine, Mascot 2.4.1 (Matrix Science, London, UK); taxonomy, *Viridiplantae*; enzyme, trypsin; fixed modification, carbamidomethyl (C); variable modification, Glu->pyro-Glu (N-term Q) and oxidation (M); precursor-ion mass tolerance ±50 ppm; fragment-ion mass tolerance ±0.7 Da; peptide masses, monoisotopic; and [M + H]⁺ peptide charge, +1.

2.5 | Statistical analysis

Data were summarized in the form of means ± standard deviation and further analyzed by one-way analysis of variance (ANOVA). The

significant differences between samples were analyzed by Tukey–Kramer multiple comparison test ($p < 0.05$). Principal component analysis (PCA) was performed using R software (v. 4.0.3) with “factoMine R” and “factoextra” package. Blast2GO (v.3.3) tool was used to assign functions of identified proteins.

3 | RESULTS AND DISCUSSION

3.1 | Proximate analysis of five high-altitude forage species

To develop forage plant species as a potential animal feed, one of the main characteristics that has to be taken into consideration is proximate analysis. Proximate analysis is the profiling of key nutrients like protein, starch, fat, ash and moisture content of any food/feed material. These nutrients play an important role in biological processes like growth and reproduction. Here, we have performed proximate analysis of five high-altitude forage species to determine the complete nutritional composition (Table 1). Carbohydrates is an important parameter of nutritional quality that represents carbon pool and forms the basis for energy metabolism in the cell. In the present study, carbohydrate content in all the species was found to be in the range of 17.44–37.27 mg/100 mg, which was significantly higher ($p < 0.05$) in *M. falcate* (37.27 mg/100 mg) and *T. pratense* (31.13 mg/100 mg) as compared with *F. kashmiriana* (30.97 mg/100 mg), *M. indica* (26.23 mg/100 mg) and *M. sativa* (17.44 mg/100 mg). Our data are comparable with those reported for *Brachiaria mutica* from Dhanikhola, Bangladesh, having carbohydrate content in the range of 23.40–30.91 mg/100 mg (Alam et al., 2015), suggesting that these forage species could be considered as a potential source of energy and used as stimulating factor for dairy cattle production. The CP is another important parameter of nutritional quality that represents nitrogen pool and provides raw material for AA and nucleotide synthesis. The CP content directly represents maturity of forage along with fertilization amount. Higher CP is usually associated with better quality of forage, and it is expected that any forage having CP content $> 9\%$ is considered to be a good quality forage (Van Saun, 2013). High CP content contributes high digestibility of feed and better animal performance. The CP content in the targeted species ranged between 3.34 and 14.71 mg/100 mg. Among these species, CP was significantly higher ($p < 0.05$) in *T. pratense* (14.71 mg/100 mg), followed by *M. falcate* (12.96 mg/100 mg), *F. kashmiriana* (12.59 mg/100 mg) and *M. sativa* (12.12 mg/100 mg), whereas *M. indica* had the lowest protein content (3.34 mg/100 mg). The CP content of four forage species was similar to other species reported in literature such as *Agropyron cristatum* (8–36 mg/100 mg), *Lolium perenne* (6–34 mg/100 mg), *Lolium multiflorum* (6–28 mg/100 mg), *Elymus sibiricus* (5–26 mg/100 mg), alfalfa (17 mg/100 mg), Bermudagrass hay (6–12 mg/100 mg) and Tall Fescue (8–16 mg/100 mg) (Henry & Morrison, 1917).

TABLE 1 Proximate analysis of five selected high forage species

Forage species	Moisture (%)	Crude ash (%)	Crude fiber (%)	Crude fat (mg/100 mg)	Crude protein (mg/100 mg)	Carbohydrates (mg/100 mg)	Phenol ($\mu\text{g}/100\text{ mg}$)	Oil absorption capacity (g/g)	Water absorption capacity (g/g)
<i>Medicago falcate</i>	89.79 ± 0.32^a	7.71 ± 0.42^a	13.33 ± 0.33^a	1.42 ± 0.02^b	12.96 ± 0.34^b	37.27 ± 0.27^d	471.25 ± 4.06^d	3.92 ± 0.11^a	2.64 ± 0.16^a
<i>Medicago sativa</i>	88.98 ± 0.25^a	14.12 ± 0.42^b	11.16 ± 0.50^a	0.98 ± 0.02^a	12.12 ± 0.12^b	17.44 ± 0.48^a	292.50 ± 3.30^b	3.91 ± 0.06^a	3.41 ± 0.17^b
<i>Melilotus indica</i>	90.02 ± 0.12^b	34.49 ± 0.20^c	15.00 ± 0.33^a	2.32 ± 0.01^c	3.34 ± 0.03^a	26.23 ± 1.67^b	37.56 ± 3.62^a	4.07 ± 0.08^a	2.69 ± 0.03^a
<i>Trifolium pratense</i>	90.73 ± 0.19^b	8.79 ± 0.01^d	24.16 ± 0.83^c	2.14 ± 0.01^c	14.71 ± 0.34^c	31.13 ± 3.22^c	418.75 ± 3.14^c	4.43 ± 0.04^b	3.10 ± 0.01^b
<i>Festuca kashmiriana</i>	88.73 ± 0.31^a	8.27 ± 0.20^d	14.33 ± 0.33^b	1.34 ± 0.95^b	12.59 ± 0.34^b	30.97 ± 1.68^c	488.12 ± 0.75^e	4.67 ± 0.04^b	3.08 ± 0.12^b

Note: Values are average of three replicates ($n = 3$) and represented as mean $\pm$ standard deviation. Values carrying different superscripts are significant at $p < 0.05$ based on one-way ANOVA followed by Tukey's test. The difference among values scripted with the same letter is not significant ($p < 0.05$).

The moisture content of the fresh plant samples ranged between 88% and 91% and when dried in an oven, the average moisture content of the samples was maintained at 10% on dry weight basis (Table 1). Moisture content is an important parameter that directly affects the storage, microbial growth and nutritional value of the feed. Previously, it has been reported that lesser moisture content prevents the growth of microorganisms, and thereby increasing storage capability of the forage species (Khan et al., 2018). Any forage/hay having moisture content < 15% could have higher potential to be an animal feed product (Van Saun, 2013). Thus, the data obtained in the present study revealed the suitability of the dry forage of the selected plants species for preparation of animal feed products. Further, the crude fat content was found to range between 0.98 mg/100 mg and 2.32 mg/100 mg and was significantly higher ($p < 0.05$) in *M. indica* (2.32 mg/100 mg) and lowest in *M. sativa* (0.98 mg/100 mg). Earlier reports suggest that few species with good forage potential, namely, *Andropogon gayanus*, *Cynodon dactylon*, *Panicum maximum*, *Pennisetum purpureum* and *Sorghum alatum*, were also found to be lower fat content (Onyeonagu & Eze, 2013). The crude ash content was analyzed and found to be in the range between 7.71% and 34.49% (Table 1). Significantly higher ($p < 0.05$) ash content was found in *M. indica* (34.49%), whereas the lowest was observed in *M. falcata* (7.71%). Except for *M. indica*, the crude ash content of other forage species was comparable with some weedy species such as *Alopecurus myosuroides* (15.14%), *Brachiaria ramosa* (13.21%), *Chrysopogon aucheri* (12.94%), *Phalaris minor* (18.03%), *Saccharum bengalense* (8.29%) and *Stipa capensis* (9.91%) (Khan et al., 2018). It has been reported that higher acid insoluble ash results in poor quality of feed (Ranjhan & Pathak, 1979).

The indigestible portion of plant-based feed in small intestine is known as dietary fiber (DF), which is generally composed of cellulose, hemicellulose, pectins and fructans. DF is categorized into soluble and insoluble fiber. Soluble fiber is digested slowly in small intestine, whereas the insoluble fiber is involved in fermentation process that improves the growth of colonic bacteria (Tosh & Yada, 2010). Crude fiber content is a good indicator of feed quality; lower value suggests a better feed quality and vice versa. It has been reported earlier that a good quality of forage should have fiber content < 35% (Van Saun, 2013). The crude fiber content in the five forage species was found to be in the range from 11.16% to 24.16% with the highest in *T. pratense* whereas the lowest in *M. sativa*. Previously, a high protein and low fiber content has been reported for *Festuca arundinacea*, *L. perenne* and *Phleum pratense* grasses that occur in contrasting environment (Lee, 2018). The differences in crude fiber content among the various forage species suggested that the high intensity of solar radiation and lesser amount of rainfall might be causing faster maturation during the growing season (a characteristic short season associated with high-altitude plants) (Evitayani et al., 2004). Plant secondary metabolites, particularly the phenolic compounds, possess antimicrobial activity that inhibit bacterial growth and enhance cattle health (Savadi et al., 2020). We estimated the total phenolic content for the five forage species and found that four of these had good phenol content (292.50–488.12 µg/100 mg), whereas *M. indica* had the lowest

content (37.56 µg/100 mg). Further, OAC has been defined as trapping of oil within protein through noncovalent interaction. The OAC is an important functional property that influences flavor, texture and product yield of a feed (Seena & Sridhar, 2005). The OAC of five forage species is presented in Table 1. All five forage species have comparable OAC in the range of 3.91–4.67 g/g. Furthermore, these five forage species have also comparable WAC (2.64–3.41 g/g), which suggest that these forage species could be useful for feed development. Therefore, proximate analysis revealed that these five forage species have higher carbohydrate and CP contents, and less moisture, crude fiber and crude fat content. Apart from their nutritional properties, these forage species possess acceptable functional properties such as OAC and WAC. The nutritional characteristics of grasses vary along the altitude. For example, CP content in the pasture herbage of a high hill (10.04%) was slight higher than midhills (9.54%) and lower hills (4.36%). Further, the nitrogen (N), phosphorous (P) and potassium (K) contents are usually high in comparison with the soil of midhills and lower hills (Misri, 2003). The possible reason for the variation was the soil type of the alpine pastures, which is mainly humus and rich in organic matter with sandy loam in texture. In addition, high-altitude region is often characterized by several abiotic stress that leads to increased synthesis of proteins and secondary metabolites. Thus, this study suggested that these high-altitude forage species could be utilized for animal feed development.

3.2 | Mineral content of five high-altitude forage species

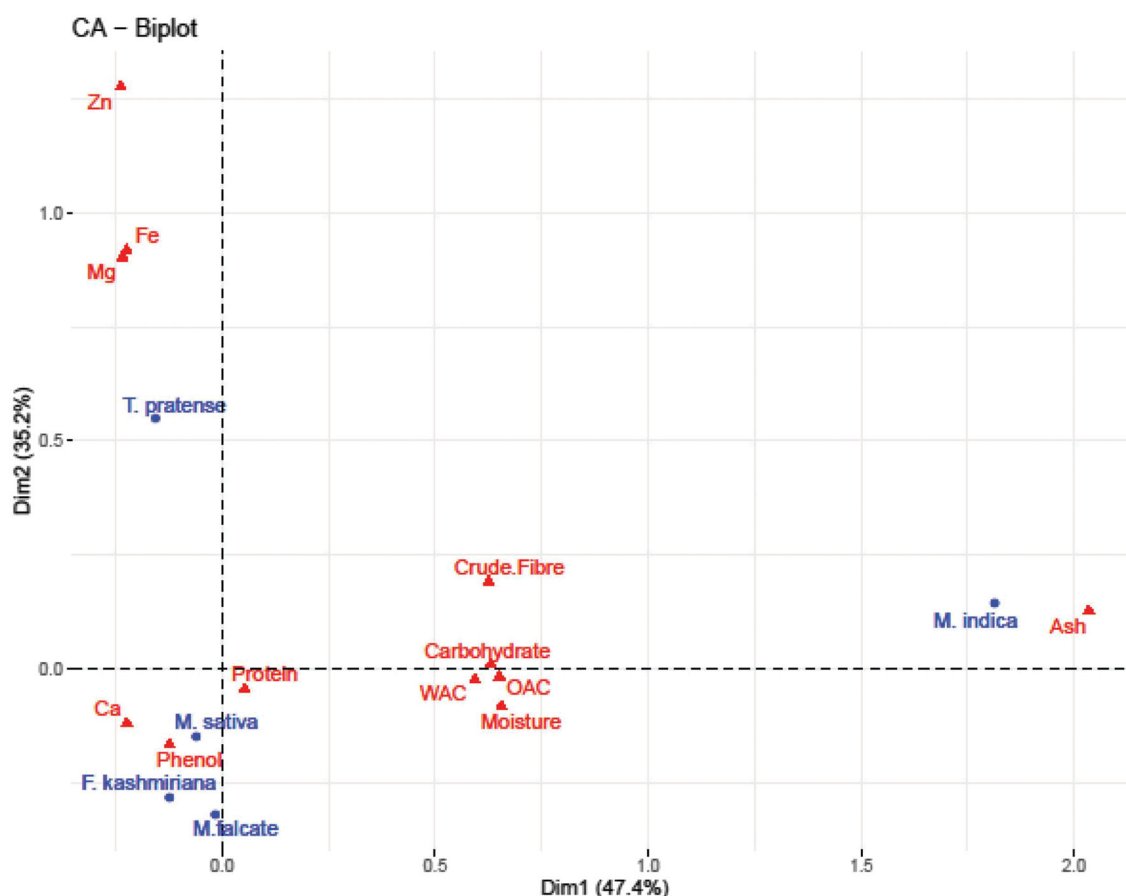
Minerals are essentials for normal metabolic activities in the cell. For example, Ca is essential for bones, teeth, muscle and neuron function; Fe is a constituent of hemoglobin, myoglobin and cytochromes; Mg is an active component of several enzymes; and Zn acts as cofactor of many enzymes. Mineral deficiency results in a variety of health problems for the grazing animals (Lopez-Alonso, 2012). The mineral content of forage species is, thus, an important consideration for developing feed for cattle; however, no information is available in the literature about mineral content of high-altitude forage species. Therefore, we have estimated the major mineral (Fe, Ca, Mg and Zn) content in five high-altitude species (Table 2). Ca level was found to be in range from 13.91 to 132.05 mg/kg dry matter (DM) with the highest in *F. kashmiriana* and the lowest in *M. indica*. Mineral analysis suggested that these high-altitude species are rich in Ca. However, a considerable variation in Ca content was found to be in these species, which might be the species-specific differences. Mg is an another important macronutrient whose concentrations ranged from 4.60 to 12.92 mg/kg DM in all the five species. Compared with the other four species, *M. indica* had the lowest Mg levels. The Fe level was found to be in the range of 2.04–76.13 mg/kg DM with the highest in *T. pratense* and the lowest in *M. indica*. The average Zn content was found to be in the range of 1.07–2.17 mg/kg DM. An appreciable amount of Fe, Cu, Zn, Ca and Mg has been reported previously in *T. pratense* and *Trifolium*

TABLE 2 Mineral content of five selected high-altitude forage species

Forage species	Essential elements (mg/kg DM)			
	Fe	Ca	Mg	Zn
<i>Medicago falcate</i>	3.23 ± 0.04 ^b	52.96 ± 0.10 ^b	12.25 ± 0.05 ^b	1.32 ± 0.03 ^b
<i>Medicago sativa</i>	20.13 ± 0.04 ^d	126.86 ± 2.01 ^d	12.87 ± 0.04 ^b	2.14 ± 0.01 ^c
<i>Melilotus indica</i>	2.04 ± 0.01 ^a	13.91 ± 0.15 ^a	4.60 ± 0.35 ^a	1.07 ± 0.05 ^a
<i>Trifolium pratense</i>	76.13 ± 0.06 ^e	56.36 ± 0.22 ^c	12.41 ± 0.06 ^b	2.17 ± 0.01 ^c
<i>Festuca kashmiriana</i>	6.22 ± 0.03 ^c	132.05 ± 0.02 ^e	12.92 ± 0.06 ^b	2.17 ± 0.02 ^c

Note: Values are average of three replicates ($n = 3$) and represented as mean ± standard deviation. Values carrying different superscripts are significant at $p < 0.05$ based on one-way ANOVA followed by Tukey's test. The difference among values scripted with the same letter is not significant ($p < 0.05$).

Abbreviation: DM, dry matter.

**FIGURE 2** Principal component analysis of proximate compositions (carbohydrate, protein, moisture content, fiber, ash, phenol, oil absorption content (OAC) and water absorption content (WAC) and four minerals (Ca, Mg, Fe and Zn). PCA was drawn using R software (v. 4.0.3)

hybridum (Stasys & Nomed, 2007). The ability of plants to store mineral elements largely depends on their root system, vegetation period, seasonal rainfall, soil pH, nitrogen content and the synergetic-antagonistic interactions of the elements (Clarkson & Marschner, 1995). Plants growing in heavy and more alkaline soils contain less microelements, especially Zn, Mn and Fe. However, these high-altitude species contained nutritionally important minerals in good amounts and, thus, could be recommended as a part of daily nutritive feed for livestock animals.

3.3 | PCA

In order to identify the characteristic variables associated with different species and correlation among the 13 variables (carbohydrate, CP, moisture content, crude fat, crude fiber, crude ash, total phenol, OAC, WAC and minerals, viz., Ca, Mg, Fe and Zn), PCA was performed (Figure 2). The first two components or the dimensions explained a total variance of 82.7% with Dim1 and Dim2 accounting for 47.8% and 34.9% variance, respectively. CP, crude fiber, carbohydrate and

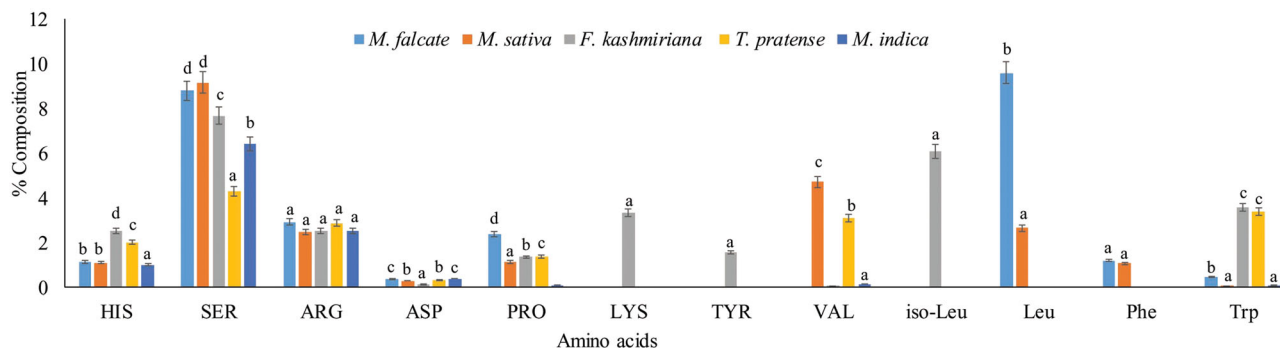


FIGURE 3 Determination of 12 amino acids in five different forage species by UPLC. All experiments were performed in triplicate. Significant differences were analyzed by one-way ANOVA ($p < 0.05$)

crude ash content were positively associated with Dim1, whereas parameters such as Zn, Fe, Mg, Ca and phenol were found to be negatively associated with Dim1. Among the minerals, Zn, Fe and Mg were more associated with *T. pratense* than other species, whereas Ca was more associated with *M. sativa*, *F. kashmiriana* and *M. falcate*. The Dim1 component, which was formed with associated parameters such as crude fiber, carbohydrate content along with WAC, OAC, moisture and crude ash, was aligned with position of *M. indica* along the axis. In Figure 2, variables such as CP, Ca and phenol are placed close to the intersection of Dim1 and Dim2, showing less variance, suggesting that these three variables are equally important for all the five forage species. The multivariate data analysis allowed easy interpretation of similarities and differences among the five forage species, based on their mineral and proximate data, which could be important for feed development.

3.4 | High-altitude forage species are enriched with essential and nonessential AAs

The AAs are building blocks of proteins that are essential for growth and development of animals and plants. The AAs are involved in storage and transport of organic nitrogen. Traditionally, AAs are classified as essential and nonessential depending upon the dietary requirements. Plants synthesize all the AAs, whereas animals are able to synthesize only the nonessential AAs. The estimation of fodder AA composition will be essential for development of feed products that meet the AAs requirements for cattle and other livestock. Earlier reports revealed that forage species growing in the open fields have 14.7% free AAs as compared with greenhouse conditions, which are reported to contain <5% of the total free AAs (Zafar et al., 2010). Therefore, we determined AAs in the sampled tissues of five high-altitude forage species (Figure 3). These high-altitude species were found to be rich in essential AAs like His, Trp, Val, Leu, Phe, to some extent, other important AAs such as iso-Leu, and Tyr. For example, His (0.98–2.53 mg/g) and Trp (0.05–3.58 mg/g) were found to be present in all the five species; Val (0.04–4.71 mg/g) was present in four species except *M. falcate*; and Leu and Phe were present in only two

species, namely, *M. falcate* (9.61 and 1.18 mg/g, respectively) and *M. sativa* (2.64 and 1.05 mg/g, respectively). On the other hand, iso-Leu was exclusively present in *F. kashmiriana* (6.09 mg/g) with a significant amount ($p < 0.05$). Further, among the nonessential AAs, Ser, Arg, Asp and Pro were reported to be present in all the five forage species with significant amounts, whereas Tyr was only present in *F. kashmiriana*. These AAs are mainly required for synthesis of protein that ultimately leads to enhanced growth of cattle and other livestock. Therefore, the development of feed products based upon all the five forage species should meet the requirement of energy as well as protein demand in animals.

3.5 | Proteomics analysis reveals high-altitude forage species to have distinct proteome but share a similar functional group

To explore the abiotic stress tolerance mechanism, we have performed proteomic profiling of five high-altitude forage species using UHPLC-QTOF-IMS. Proteomics analysis identified a total of 347, 186, 130, 88 and 86 proteins in *F. kashmiriana*, *T. pratense*, *M. falcate*, *M. indica* and *M. sativa*, respectively (Table S1). Next, PCA was performed to analyze the variability in proteome profile among these five forage species (Figure 4a). The two principal components (PCs) explained 58.5% of total variance in protein abundance with PC1 and PC2 showing 30.2% and 28.3% variance, respectively. The results suggested that these five species were significantly different in their proteome. Among these five forage species, *T. pratense* and *M. indica* shared a relatively similar proteome than the other three species. Further, we also performed a Venn diagram analysis to elucidate the overlap among the proteome of the five different species. The significant difference in proteome set was appeared to influence the degree of protein overlap as illustrated in Figure 4b. Only three proteins, namely, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, ATP synthase beta subunit and unknown protein, were common among all of the forage species, which clearly demonstrates that each of these species has a distinct proteome that could be genotype specific. Further, the identified proteins in each of these

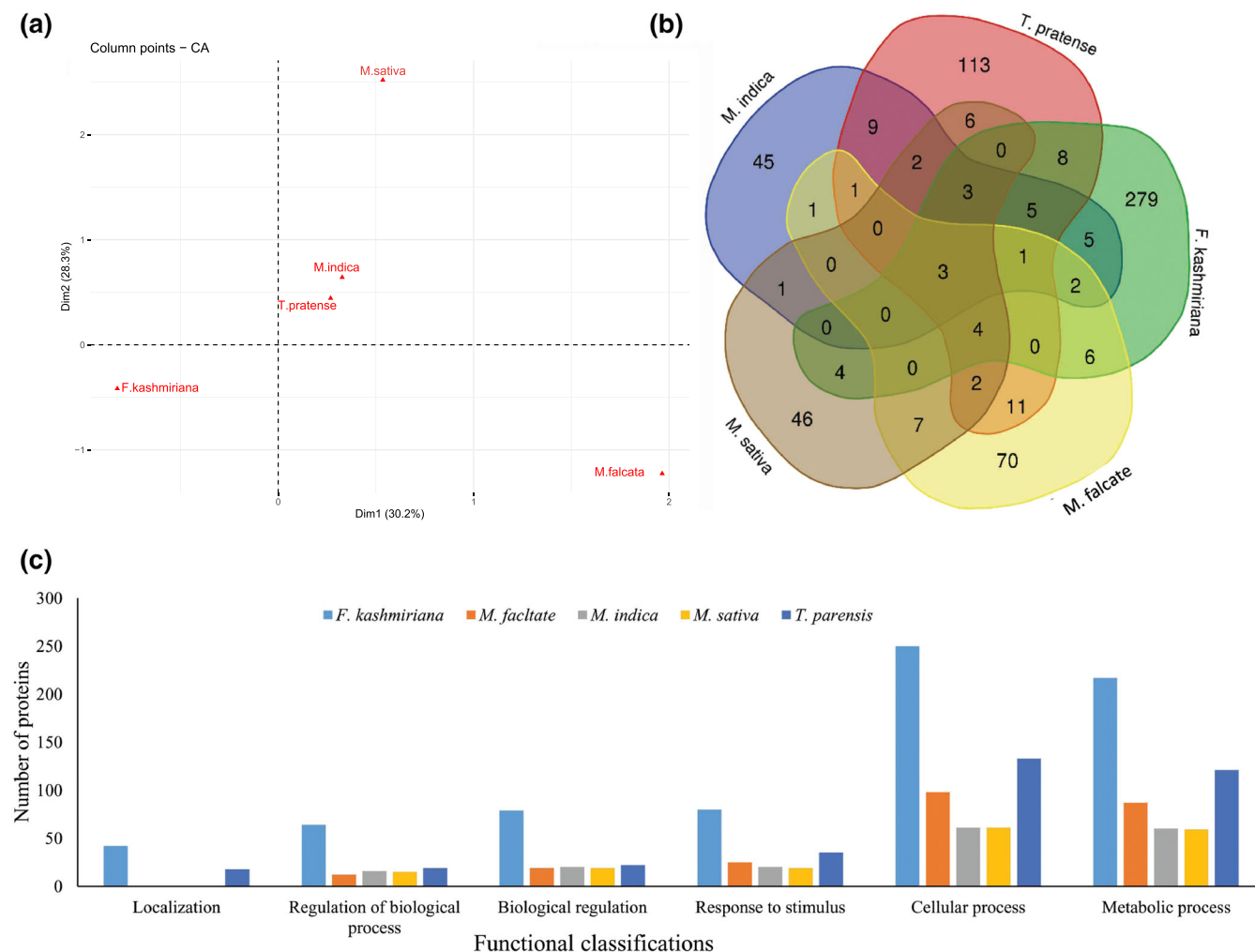


FIGURE 4 Proteomic analysis of five different forage species using gel-free techniques by UHPLC-QTOF-IMS and LC-MALDI TOF/TOF-MS/MS instrument. (a) Visualization of proteome using PCA based on expression of proteins identified by UHPLC-QTOF-IMS, (b) Venn diagram depicts overlap among five different forage species and (c) functional classification of identified proteins of five different forage species using Blast2Go program

species were classified into five Gene Ontology (GO) terms based on their putative functions (Figure 4c). Most abundant protein groups in each species are cellular process (34–41%), and the metabolic process (32–36%) followed by response to stimulus (10–11%), biological regulation (6–11%), regulation of biological processes (5–9%) and localization (5–6%). This suggests that beside cellular processes, metabolic regulation predominantly takes place at high altitudes where extreme environmental conditions prevail.

High-altitude regions of the Himalaya are characterized by extreme environmental conditions like low temperature, low partial pressure of gases, high UV and light intensity, high wind speed and so forth (Kumar & Kumari, 2018). These environmental conditions limit plant growth, development and diversity. Plants have evolved stress tolerance strategies to survive in these harsh conditions; however, the mechanism underlying plant adaptation is not fully explored for the Himalayan plants. Proteins associated with “response to stress” represent the third largest functional group in this study; however, the type

of proteins is different in five forage species. Generation of reactive oxygen species (ROS) is one of the early steps of plant response upon a stress encounter and if its accumulation exceeds the threshold level, it could be harmful for DNA, RNA and proteins. Consequently, plants activate their antioxidant defense system by increasing the expression of ROS-related proteins. In this study, proteins of antioxidant defense system like catalase, catalase isozyme 1, ascorbate peroxidase, ascorbate peroxidase 2, stromal ascorbate peroxidase, glutathione transferase, glutathione S-transferase tau 2, glutathione S-transferase F13, NADP-dependent malate dehydrogenase, peroxides and monodehydroascorbate reductase-like isoform 2 were found to be enriched in five forage species, which could be helpful to counter the harmful consequences of ROS. Further, heat shock proteins (HSPs) play critical roles in many biological processes, including various abiotic and biotic stresses (Jacob et al., 2017). HSPs are associated with protein folding, stability and intracellular distribution. It is predicted that the HSPs integrate into immune responses through post-

translational modifications, especially phosphorylation and protein turnover of signaling molecule. In our results, we found heat shock protein 70, Stromal 70-kDa heat shock-related protein, chloroplastic and heat shock cognate 70 kDa in *M. indica*; heat shock protein 90, peptidyl-prolyl cis-trans isomerase and hsp70 (AA 6–651) in *M. falcate*; peptidylprolyl isomerase-like protein, heat shock protein 70 and TCP-1/cpn60 chaperonin family protein in *F. kashmiriana*; heat shock cognate 70-kDa protein-like and heat shock protein 70, hsp70 (AA 6–651), in *T. pratense*, whereas hsp70 (AA 6–651) in *M. sativa*. We also observed the presence of proteins associated with protein degradation such as cysteine proteinase, cysteine protease 1, cysteine proteinase precursor and 26S proteasome regulatory subunit N₂ in forage species. Therefore, we conclude that each high-altitude forage species has a distinct stress tolerance mechanism; however, the type of proteins varies for every species.

3.6 | Proteomic study provides insights into the molecular basis of different nutritional composition of five high-altitude forage species

The present study revealed that five targeted forage species have different nutritional compositions. Therefore, we tried to explore these differences at the molecular level, that is, protein level. Photosynthesis is an important biological process, where solar energy is transformed into chemical energy. The enrichment of photosynthetic and Calvin cycle pathways proteins/enzymes will likely increase carbon availability in plants. Proximate analysis revealed a significant difference in carbohydrate concentration among five forage species (Table 1). Therefore, we speculate that proteins/enzymes associated with photosynthesis were enriched in forage species with significantly higher carbohydrate content. Consequently, we screened proteomic data and found 24, 19, 23, 6 and 14 nonredundant proteins/enzymes associated with photosynthetic and Calvin cycle pathways were identified in *M. falcate*, *T. pratense*, *F. kashmiriana*, *M. indica* and *M. sativa*, respectively (Table S1). Among the proteins like RuBisCo and its subunits were identified in all forage species. Strikingly, proteins involved in photosystem I and II-like photosystem I light-harvesting complex protein 5, oxygen evolving complex of photosystem II subunit PsbQ, chloroplast oxygen-evolving enhancer protein 1, oxygen-evolving enhancer protein 1, photosystem I P700 chlorophyll a apoprotein A2, photosystem I subunit II precursor, photosystem II 32-kDa protein (chloroplast), photosystem II CP43 chlorophyll apoprotein and photosystem II CP47 chlorophyll apoprotein among others were exclusively identified in *F. kashmiriana*; oxygen-evolving enhancer protein, oxygen-evolving enhancer protein 2, chloroplastic, photosystem I reaction center subunit IV, chloroplastic and oxygen-evolving complex protein 1 in *M. falcate*; and putative PSII-P protein, photosystem I subunit VII and photosystem I reaction center subunit N in *T. pratense*, which suggests that these three forage species have higher photosynthetic rate that results in higher carbohydrate accumulation compared with the other two forage species. Photosynthetically generated carbon provides source for synthesis of primary and secondary

metabolites. Consistent with higher photosynthetic rate, *M. falcate*, *T. pratense* and *F. kashmiriana* have comparatively higher phenol content than the other two forage species. The GO analysis of identified proteins revealed that proteins associated with metabolic processes were enriched in three forage species. The proteins classified in metabolic process are involved in synthesis of primary and secondary metabolite, nitrogen compound (AAs and protein), lipid, hormone and nucleotides. Of a particular interest, proteins involved in synthesis of secondary metabolites like cytochrome P450 family protein, terpene synthase/cyclase family protein, type I polyketide synthase, polyphenol oxidase, phenylcoumaran benzylic ether reductase-like protein, chorismate synthase, isopentenyl pyrophosphate isomerase and PREDICTED: violaxanthin de-epoxidase chloroplastic-like (Table S1) were identified in three forage species and are in accordance to higher accumulation of phenolics. Further, proteins involved in nitrogen compound synthesis like methionine synthase, glycine dehydrogenase P protein, serine hydroxymethyltransferase, PREDICTED: glycine cleavage system H protein, mitochondrial-like, L-asparaginase-like family protein, PREDICTED: alanine aminotransferase 2-like, glutamine synthetase leaf isozyme, 40S ribosomal protein S15-4, 40S ribosomal protein S15-2, ribosomal protein L2, 50S ribosomal protein L4 (ISS), asparagine-tRNA ligase, eukaryotic initiation factor 4A, elongation factor-1 alpha and putative tRNA synthase among others (Table S1) are identified in forage species that supports comparable CP content. However, the type of proteins is different in each of the forage species. Therefore, different nutritional composition of five high-altitude forage species was due to expression of different type of proteins in five forage species.

4 | CONCLUSION

The traditionally utilized and climatically adapted forage plants of high pasturelands have higher nutrition content, therefore would be able to improve the performance of the dairy sector and alleviate the current shortages and environmental crises. The results of this study showed that these high-altitude forage species contained appreciable amounts of nutrients as compared with widely used animal fodders like soybean meal and alfalfa hay, suggesting that these five forage species can be considered as an alternative source of nutrition fodder. Thus, it is recommended that a combination of all these forage species could be utilized to develop a feed product for livestock. Such forage species could serve as a safe and inexpensive natural source of feed for livestock. Further, the knowledge of proteins/enzymes associated with the cellular and metabolic processes like photosynthesis, Calvin cycle, primary and secondary metabolites and nitrogen compounds would help to understand the molecular basis of nutrient enrichment in five high-altitude forage species. In addition, proteomics analysis reveals that all five forage species have distinct proteomes but share a similar functional group, suggesting the different survival mechanisms opted in high-altitude climatic conditions. This study provides a research platform that will be helpful for future research in forage species and the formulation of feed products for livestock.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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