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RESEARCH

Optimization of protein extraction and functional proteome profiling of *Trichanthera gigantea* reveals metabolic adaptations for tropical resilience

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Abstract

Trichanthera gigantea is a high-protein content tropical shrub with strong potential as a local source of livestock feed. However, the absence of proteomic information limits assessment of its protein stability and functional reliability. Robust proteome profiling of woody, metabolite-rich species requires optimized protein extraction strategies. Here, the study presents the first comparative evaluation of protein extraction workflows across *T. gigantea* organs (leaf, stem, and root) to establish a proteomics-compatible pipeline. Protein recovery using TCA/acetone, phenol-based, and a modified extraction method was quantitatively compared and further optimized by sonication. TCA/acetone extraction produced the highest protein yield in leaves (67.38 mg g^{-1}), while the modified method was most effective for roots (83.92 mg g^{-1}) and stems (65.58 mg g^{-1}) after normalization to tissue input. Despite its widespread use in plant proteomics, the phenol method yielded comparatively low total protein per gram of tissue in *T. gigantea*. As leaves represent the primary organ for feed applications, optimization focused on TCA/acetone-extracted leaf tissue. Short-duration sonication (10–20 min) significantly enhanced protein recovery, whereas prolonged sonication (≥ 30 min) reduced yields across all organs, consistent with protein degradation. The highest total protein yield was obtained after 10 min of sonication, reaching 77.14 mg/g of protein extracted from 1 g of leaf tissue. Using the optimized workflow, the first LC–MS/MS-based proteomics study of *T. gigantea* was performed, identifying 134 proteins predominantly associated with photosynthesis, redox homeostasis, and amino acid metabolism from its leaf tissue. These proteomic signatures reveal key metabolic nodes that may be targeted to enhance stress tolerance and protein productivity, establishing a foundational resource for future functional and applied proteomics in *T. gigantea*.

Keywords Protein extraction optimization · *Trichanthera gigantea* · Plant proteomics · LC–MS/MS

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1 Introduction

The continuous increase in feed ingredient prices has substantially raised the cost of poultry production, consequently affecting the market price of chicken in Malaysia (Malaysia Gazette, 2021). This situation highlights the urgent need to identify alternative, cost-effective, and locally available feed sources to support small-scale and organically farmed poultry systems. Research into the functional components of plant-based products, such as its antioxidant capacity, is key to validating these resources for both feed and health applications (Mohamad et al. 2015). Adopting an efficient feeding strategy is crucial to maximize livestock weight gain while minimizing reliance on conventional feed ingredients (Hwa et al. 2023). Additionally, in response to the rising food cost and Malaysia's growing dependency on imported feed components, there is a pressing need to explore underutilized plant-based resources that can contribute to national food security and sustainable agricultural practices. Hence, plant-based proteins are increasingly recognized for their potential to improve average daily gain (ADG) and feed conversion ratio (FCR) in broiler chickens across different growth stages (Akter et al. 2024).

Among plants investigated is *Trichanthera gigantea. T. gigantea* (commonly known as "nacedero" or madre de agua) has gained attention as a potential local feed ingredient due to its high protein content and favorable digestibility (Heuzé et al. 2016) (refer Fig. 3e). The leaves contain crude protein at 13–22% on a dry matter basis, with a well-balanced essential amino acid profile (Nasarudin et al. 2024). They also comprise approximately 2.8% crude fat, 4.4% crude fiber, 19.7% ash, and 37% nitrogen-free extract. In addition, the leaves are rich in bioactive compounds, including flavonoids, phenolics, saponins, tannins, and antioxidant constituents. Notably, DPPH radical scavenging activity is enhanced under sunny growth conditions, indicating improved oxidative stability and potential health-promoting effects when used in animal feeds (Phúc et al. 2022). Dietary inclusion of 10–15% *T. gigantea* leaf meal (TGLM) in broiler diets was shown to improve body weight gain, feed conversion ratio, and overall production efficiency without any observable toxic effects on organ weights or blood parameters (Libatique 2020). Despite these promising results, the biochemical and proteomic characterization of *T. gigantea* remains limited. A deeper understanding of its protein composition and extractability is essential not only for nutritional assessment but also for future biotechnological applications such as protein enrichment, feed optimization, and gene-based metabolic studies. These efforts are part of a growing movement that recognizes the potential of plant-derived compounds, including proteins and secondary metabolites, as critical starting materials for various biotechnological and health-related interventions (Mahmoudieh et al. 2024). Protein extraction represents the most critical and challenging step in plant proteomic workflows. The efficiency and reproducibility of proteomic analyses are strongly influenced by the extraction method used, as plants contain abundant proteases, polyphenols, polysaccharides, and secondary metabolites that interfere with protein recovery and detection (Komatsu et al. 2014). Such compounds can lead to protein degradation, streaking on gels, and charge heterogeneity, thereby complicating downstream analyses. Previous studies have emphasized that extraction efficiency depends on cell wall composition and metabolite profiles, but somehow protein extraction on *T. gigantea* was never documented (Alima & Randa 2024). Therefore, identifying a suitable extraction strategy for *T. gigantea*, a woody and hairy plant with no prior proteomic report, is necessary to obtain high-quality protein extracts suitable for nutritional and proteomic evaluation.

In this study, three widely used extraction methods which are phenol, trichloroacetic acid/acetone (TCA/acetone), and a modified buffer-based were used. These methods were evaluated across three different organs (leaf, stem, and root) of *T. gigantea* to determine their relative efficiency in protein yield and stability. Optimization was further carried out through sonication-assisted extraction to maximize yield while preserving protein stability. Optimization and subsequent liquid chromatography-tandem mass spectrophotometry (LC–MS/MS) profiling were further conducted for protein analysis. Additionally, troubleshooting steps encountered during the extraction process were documented to provide practical insights for future studies involving complex plant matrices.

This study is the first to report on systematic evaluation of protein extraction methods for *T. gigantea*, aiming to establish an optimized workflow for proteomic and feed-related studies while contributing to the development of locally sourced, plant-based protein alternatives for sustainable poultry feed production.

2 Methodology

2.1 Sample preparation

T. gigantea plants cultivated at the Vertical Hydroponic Nursery of the Agro-Biotechnology Institute, Malaysia (ABI), under the National Institutes of Biotechnology Malaysia (NIBM), were utilized in this study. The original plant source was collected from Penang, Malaysia, and subsequently propagated under controlled hydroponic conditions to achieve uniform plant development. Five

independent plants were used as biological replicates (n = 5), with each replicate consisting of tissue samples comprising leaves, stems, flowers, and roots. The samples from each replicate were then collected from its fully developed plants at approximately four months of growth. Following harvest, tissues were rapidly frozen in liquid nitrogen to preserve protein stability and to minimize biomolecular degradation. The frozen samples were then transferred to the ABI Transformation Laboratory and stored at − 80 °C until further use. These samples were employed for extraction and proteomic investigation. Implementation of this standardized sampling and preservation procedure is essential to ensure high-quality and intact protein from *T. gigantea* tissues.

2.2 Protein extraction

Three extraction methods were used to compare the extraction efficiency in three different plant organs. Following precipitation, protein pellets obtained from each extraction method were resolubilized in buffers appropriate for the intended downstream application to ensure compatibility and minimize analytical interference.

2.2.1 Protein extraction using TCA/Acetone

Protein extraction was conducted according to the protocol prepared by Ucar et al., (2025) (Uçar et al. 2025). One gram of frozen plant organs (stem, root and leaves respectively) from *T. gigantea* (4-month-old plants) were ground in a pre-cooled mortar using liquid nitrogen. The resulting fine powder was suspended in 10% TCA/acetone at a 1:3 ratio and incubated overnight at −20 °C for protein precipitation. The mixture was then centrifuged at 10,000 × g for 30 min at 4 °C. The resulting pellet was washed multiple times with 80% acetone and centrifuged for 5 min per wash until the powder appeared whitish (refer Fig. 3d). Protein pellets obtained after TCA/acetone precipitation were initially solubilized in 8 M urea for denaturation and protein resolubilization. No additional buffering component was included at this stage. For downstream LC–MS/MS analysis, protein extracts were subsequently diluted into 50 mM ammonium bicarbonate (pH 8.0) prior to enzymatic digestion to ensure appropriate buffering conditions for trypsin activity.

2.2.2 Protein extraction using modified SDS-TCA/Acetone

The modified SDS-TCA/acetone extraction was carried out with a slight modification to the protocol from Niu et al., (2018) (Niu et al. 2018). One gram of *T. gigantea* from the plant organs (stem, root and leaves respectively) were homogenized in a pre-cooled mortar. An extraction buffer (1:3 ratio) containing 15.0 mL of 1% Sodium dodecyl sulfate (SDS), 0.1 M Tris-Hydrochloric acid (Tris–HCl) (pH 6.8), 2 mM disodium ethylenediaminetetraacetic acid (EDTA-Na_2), 20 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonyl fluoride (PMSF) was added to the homogenate, which was then heated at 50 °C for 5 min (refer Fig. 3a). The homogenate was centrifuged at 15,000 × g for 5 min, and the resulting supernatant, containing the protein extract, was transferred to fresh tubes. To precipitate proteins, an equal volume (1:1, v/v) of 10% cold TCA/acetone was added, and the mixture was incubated on ice for 30 min before centrifugation at 15,000 × g for 5 min. The supernatant was discarded, and the protein pellet was washed with 80% acetone, followed by centrifugation under the same conditions. The washing step was repeated as needed until the pellet was sufficiently purified. Finally, the protein precipitates were air-dried for 1–3 min and dissolved in 50 mM ammonium bicarbonate (pH 8.0). This procedure is referred to as "modified extraction" in the remainder of the text.

2.2.3 Protein extraction using phenol

Phenol extraction protocol was carried out based on (Isaacson et al. 2006). Five grams of frozen plant tissue (stem, root and leaves respectively) were ground into a fine powder and suspended in cold phenol extraction buffer containing 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl (pH 7.5), 50 mM EDTA, 1 mM PMSF (added fresh), and 2% β-mercaptoethanol (added fresh). An equal volume of Tris–HCl (pH 7.5)-saturated phenol was added, and the mixture was shaken at 4 °C for 30 min. Following centrifugation at 5,000 × g for 30 min at 4 °C, the upper phenolic phase containing proteins was carefully collected. The high sucrose concentration increases the density of the aqueous phase, resulting in the phenolic phase forming the upper layer. To remove contaminants, the phenolic phase was re-extracted with an equal volume of extraction buffer, followed by repetition centrifugation and collection.

Proteins were precipitated by adding five volumes of cold 0.1 M ammonium acetate in methanol and incubating at –20 °C overnight. The precipitated proteins were pelleted by centrifugation (5,000 × g, 30 min, 4 °C), washed twice with cold methanol, followed by two washes with cold acetone, and briefly air-dried on ice. The final protein pellet was dissolved in 50 mM ammonium bicarbonate (pH 8.0). Comparison on extraction methods were summarized in Fig. 1.

2.3 Optimization by sonication

To evaluate the effect of sonication on protein recovery, the entire protein pellet obtained from 1.0 g of fresh plant tissue (as described in the TCA/acetone extraction protocol in Sect. 2.2.1) was resuspended in 8 M urea. The entire resuspended extract from each biological replicate was homogenized and subjected to sonication (Qsonica, Q55A system,

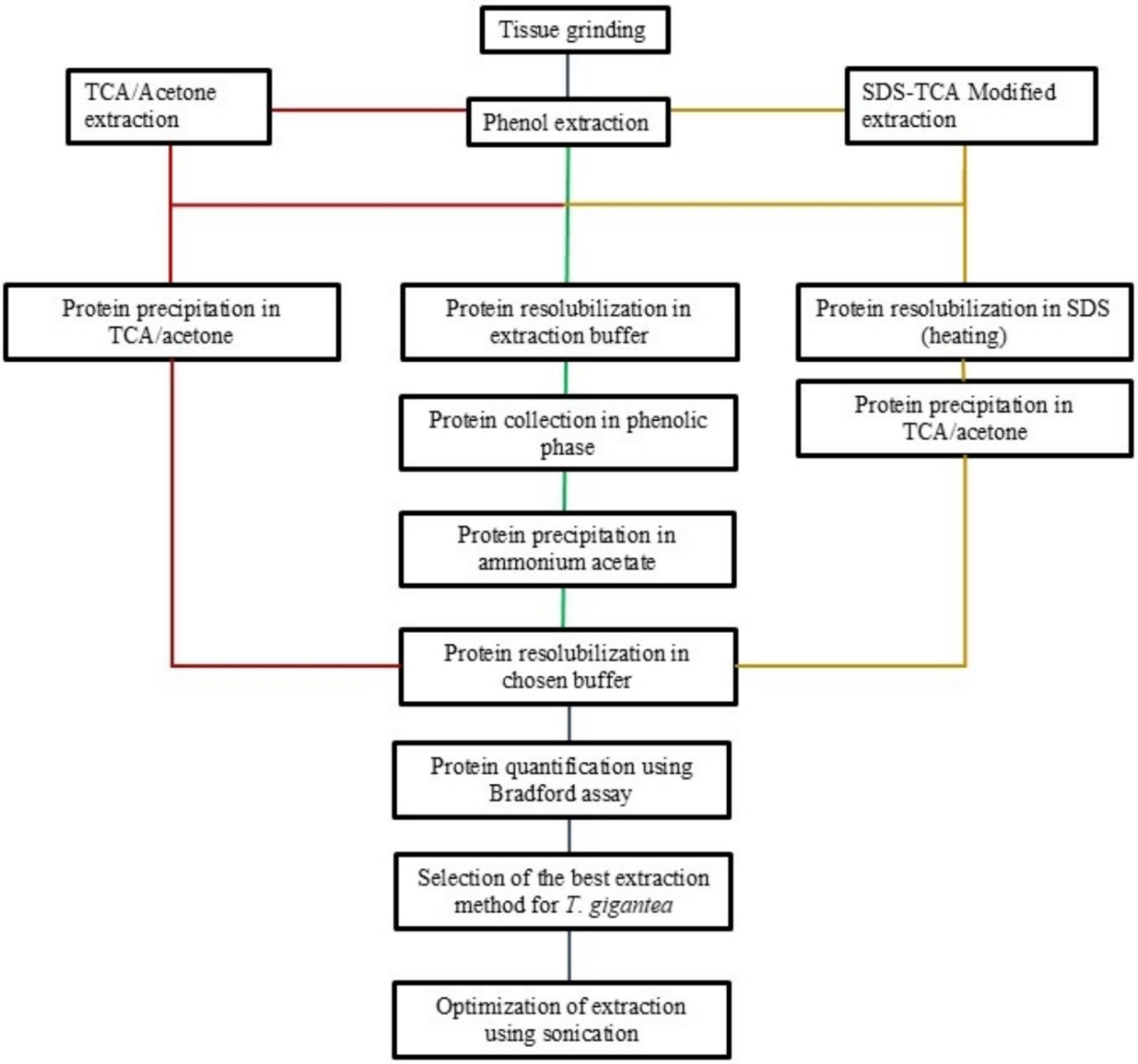


Fig. 1 Schematic overview of extraction methods conducted on *T. gigantea*

Newtown) treatment (control, 10, 20, 30, and 40 min) at 60 Hz with 30 s intervals. This approach ensured that all optimization data remained normalized to the starting tissue mass, allowing for the calculation of total protein yield per gram of tissue. To minimize heat generation and protein degradation, samples were maintained on ice throughout and intervals were incorporated between sonication cycles. Control samples were used for each organ (not sonicated). Following treatment, samples were centrifuged at 4 °C for 5 min, and the resulting supernatant was collected for protein quantification. The protein was then diluted into 50 mM ammonium bicarbonate (pH 8.0) prior to LC–MS/MS analysis.

2.4 Protein assay

2.4.1 Bradford assay

Protein quantification was done following the protocol from (Kruger 2009). Bovine serum albumin (BSA) standards (20–100 µg/µl) were prepared from a 1 mg/mL stock solution. Prior to quantification, protein samples were diluted fivefold (1:5, v/v) with distilled water to minimize interference and ensure absorbance readings remained within the linear range of the Bradford assay. Final protein concentrations were calculated by multiplying the measured values by the dilution factor. For protein quantification, 20 µL of

each diluted protein sample or BSA standard was pipetted into a 96-well microplate in duplicate. Distilled water (140 µL) was added to each well, followed by 40 µL of Bradford reagent, resulting in a final reaction volume of 200 µL per well. After mixing, the plate was incubated for 5 min at room temperature, and absorbance was measured at 595 nm. All experiments were conducted in triplicates. Experiment was conducted in the dark since the Bradford reagent is light sensitive.

2.5 LC–MS/MS analysis

2.5.1 In-solution protein digestion

A total of 50 µg of extracted protein was resuspended in 50 µL of 50 mM ammonium bicarbonate (Sigma-Aldrich). Subsequently, 50 µL of 0.1% RapiGest™ SF (Waters) was added, and the sample was vortex-mixed. The mixture was concentrated to approximately half of its initial volume using a speed vacuum concentrator for 20–30 min. The sample was then incubated at 80 °C for 15 min in a thermomixer. Reduction was performed by adding 2.5 µL of 100 mM dithiothreitol (DTT; Sigma-Aldrich), followed by incubation at 60 °C for 30 min. For alkylation, 2.5 µL of 200 mM iodoacetamide (Sigma-Aldrich) was added, and the reaction was incubated at room temperature for 45 min in the dark (microcentrifuge tubes wrapped in aluminum foil). Proteolytic digestion was initiated by adding 2.0 µL of Trypsin/Lys-C enzyme (Mass Spectrometry Grade, Promega; 1.0 µg/µL), followed by overnight incubation at 37 °C. Digestion was terminated by adding 1.0 µL of concentrated trifluoroacetic acid (TFA; Sigma-Aldrich) and incubating at 37 °C for 20 min. The samples were centrifuged at 14,000 rpm for 20 min, and the resulting supernatant was collected and stored at − 80 °C until further LC–MS/MS analysis.

2.5.2 LC–MS/MS analysis

LC–MS/MS was performed using a Dionex Ultimate 3000 RSLC nano system (Thermo Fisher Scientific, Canada) coupled with a Thermo Scientific mass spectrometer utilizing Xcalibur software. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient was programmed as follows: 5% to 40% B over 91 min, ramped to 85% B in 2 min, held at 85% B for 3 min, returned to 5% B in 1 min, and re-equilibrated at 5% B for 4 min. The flow rate was maintained at 250 nL/min. Peptide separation was carried out using an EASY-Spray Acclaim PepMap™ C18 column (100 Å, 2 µm particle size, 50 µm inner diameter × 15 cm length), maintained at 35 °C. An injection volume of 1 µL was used, and all samples were analyzed in technical triplicates. The mass spectrometer operated in positive ion mode with an Orbitrap detector featuring a resolution of 120,000. The mass range was set from 310 to 1800 m/z, with a data-dependent cycle time of 3 s and a maximum injection time set to custom conditions.

For MS analysis, a quadrupole isolation mode with an isolation width of 1.6 m/z was employed, using fixed collision energy settings of 28% for high-energy collision dissociation (HCD) and 30% for CID. This comprehensive setup ensured accurate peptide identification and quantification throughout the analysis.

2.6 Protein identification

Raw LC–MS/MS data were acquired using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 RSLCnano system. Data analysis was performed using Proteome Discoverer™ software (version 2.5, Thermo Fisher Scientific). Spectra were searched against the *Arabidopsis thaliana* reference proteome (Taxon ID: 3702) together with a UniProt contaminant database. Trypsin/Lys-C was specified as the digestion enzyme, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while oxidation (M) and protein N-terminal acetylation were defined as variable modifications. Mass tolerances were set to 10 ppm for precursor ions (MS1) and 0.6 Da for fragment ions (MS2). Peptide validation was performed using the Percolator® algorithm, and identifications were filtered at a false discovery rate (FDR) of less than 5%. Only proteins identified with at least two unique peptides were considered for further analysis. Label-free quantification was performed based on MS1 precursor ion intensities ("Abundance" values) generated by Proteome Discoverer. Protein abundance values from biological replicates (F1–F3) were used for functional weighting and gene ontology classification analyses.

The label-free proteomics data of *T. gigantea*, were analyzed by searching against several public protein databases, including the NCBI non-redundant database, UniProt, Gene Ontology (GO). For GO classification, identified proteins were assigned to functional categories using the UniProt database. The x-axis values in Fig. 4 represent the cumulative protein abundance per category, calculated by summing MS1 precursor ion intensity ("Abundance") values of all proteins assigned to each GO term across biological replicates.

2.7 Statistical analysis

All experiments were performed in triplicate and the results were expressed as the means ± standard deviation (SD). The data were analyzed using two-way ANOVA with Sidak's

multiple comparisons test, performed in Excel (Windows 11) and GraphPad Prism 10.4.2 (64-bit).

3 Result and discussion

T. gigantea has not been previously studied for proteomics, resulting in limited information on its protein characteristics. This study therefore aimed to determine the most effective protein extraction method for its different organs (leaf, stem, and root).

3.1 Comparative evaluation of protein extraction methods

Three extraction methods comprising phenol, TCA/acetone, and modified SDS–TCA/acetone were compared to determine their relative efficiency across *T. gigantea* organs. While initial protein concentration was estimated using the Bradford assay (Table 2), these values are not directly comparable because different amounts of starting plant material were used for each method (1 g for TCA/acetone and modified methods, versus 5 g for the phenol method). To accurately assess and compare extraction efficiency, the total protein yield was normalized to the starting tissue mass (mg/g), as presented in Table 3.

The results demonstrate that the optimal extraction method is highly dependent on the specific tissue type. In leaf tissue, the TCA/acetone method was clearly superior, providing the highest total yield of 67.38 mg/g, which significantly outperformed the modified method (36.42 mg/g) and phenol extraction (10.78 mg/g). This high efficiency is attributed to the ability of TCA/acetone to effectively remove pigments, phenolic compounds, and secondary metabolites that are typically abundant in green tissues (Gandhi et al. 2018).

For stem and root tissues, the modified SDS-TCA/acetone method proved to be the most effective. In stems, the modified method achieved the highest yield of 65.58 mg/g, compared to 34.88 mg/g for TCA/acetone and a low 16.12 mg/g for the phenol method. The phenol method's poor performance in stems suggests that significant protein loss may occur during its extensive precipitation and washing steps when processing lignified tissues (Pulikkottil Rajan 2024). In root tissues, the modified method was the only successful protocol, yielding 83.92 mg/g, while both phenol and TCA/acetone failed to recover any detectable protein. The success of the modified protocol in roots is likely due to its detergent-based buffer, which is necessary to disrupt complex root matrices and overcome interference from high levels of polysaccharides and secondary metabolites (Danko et al. 2022).

Table 3 Comparison of total protein yield from three extraction methods

Sample	Phenol extraction	Modified buffer extraction	TCA/acetone extraction
Leaf	10.78	36.42	**67.38**
Stem	16.12	**65.58**	34.88
Root	-	**83.92**	-

Protein yields are expressed as mg/g fresh weight. Highlighted represent the highest value for the plant organ

In terms of practicality, proteins extracted using the TCA/acetone method were challenging to re-dissolve, indicating the need for further optimization, such as incorporating sonication to improve solubilization. However, its simplicity and effectiveness in removing pigments and phenolic compounds made it ideal for green tissues. However, incomplete pellet dissolution in the rehydration buffer was observed, suggesting the need for further optimization. Similar findings were reported where yellowish or brownish pellets indicated contamination that could affect downstream resolution on SDS-gels or LC–MS (Niu et al. 2018) (refer Fig. 3c). In contrast, both the phenol and modified extraction methods produced protein pellets that dissolved readily; however, once extraction was completed, the protein yield from these methods could not be further enhanced. To obtain higher yields, the phenol method required a larger amount of starting plant material, whereas the modified extraction method was most effective for sample quantities below 1 g (Niu et al. 2018). Therefore, repeated extraction cycles were necessary when working with smaller sample amounts to maximize protein recovery.

In summary, the comparison of total yields in Table 3 confirms that TCA/acetone extraction is most effective for leaf tissue, whereas the modified method performs best for roots and stems. The performance of the phenol method in *T. gigantea*, while widely regarded as a gold standard for

Table 2 Comparison of protein content in three extraction methods from Bradford assay

Sample	Phenol extraction	Modified buffer extraction	TCA/acetone extraction
Leaf	269.58 ± 0.011	182.08 ± 0.056	**336.88** ± 0.007
Stem	**402.92** ± 0.013	327.92 ± 0.012	174.38 ± 0.011
Root	-	**419.58** ± 0.037	-

Protein contents are expressed as µg/µl of tested sample. All readings were taken in triplicates

proteomics in recalcitrant plant tissues (Kopeć et al. 2024), was hindered by its low total protein yield per gram of tissue, despite high concentrations of protein extracts. This distinction is important, as it underscores that the phenol method's apparent effectiveness is partly due to the use of larger starting material and smaller final extract volumes. Given that leaves are the primary organ of interest for livestock feed applications, and the clear efficiency of TCA/acetone in leaf protein recovery, TCA/acetone was selected for further optimization in this study.

3.2 Optimization of TCA/acetone extraction by sonication

Since the TCA/acetone method produced the highest protein yield in leaves, and leaves are the primary organ of interest for livestock feed applications, further optimization was focused on this tissue (Santamaría-Fernández & Lübeck 2020). Leaves are preferred in animal feed as they can be harvested easily with minimal inputs, contributing to sustainable and circular farming systems (Santamaría-Fernández & Lübeck 2020). Utilizing leaf material also promotes the use of agricultural byproducts and underused biomass, contributing to more sustainable and circular farming systems (Patil et al. 2021). To provide a comparable assessment of extraction efficiency across different durations, the results in Table 4 are expressed as total protein yield per gram of tissue (mg/g).

Sonication was employed to enhance cell disruption and improve the release of proteins, particularly those that were initially challenging to re-dissolve following TCA/acetone precipitation. Referring to the Table 4, there is significant difference in protein yield between the treated *T. gigantea* protein sample with the control (without sonication).

Statistical analysis revealed that the optimal sonication duration for all *T. gigantea* organs is 10 min. In leaf tissue, 10 min of sonication produced a peak yield of 77.14 mg/g, which was significantly higher than the non-sonicated control of 14.64 mg/g ($p < 0.0001$). This represents a five-fold increase in recovery, confirming that mechanical assistance is vital to overcome the solubility challenges of the TCA/acetone method. However, extending sonication beyond 20 min led to a significant decline in leaf protein yield ($p < 0.0005$), likely due to localized heating and subsequent protein degradation.

A similar optimization trend was observed in the stem and root tissues. In stems, 10 min of sonication achieved the highest yield of 64.64 mg/g ($p = 0.0048$ compared to control), while roots showed a dramatic improvement from a baseline of 3.81 mg/g to a maximum of 63.81 mg/g ($p < 0.001$). In both cases, durations of 30 min or longer caused yields to return toward baseline levels, with the 40-min stem treatment yielding only 1.31 mg/g. These findings highlight that while short-duration sonication (10–20 min) is necessary to disrupt the complex woody and metabolite-rich matrix of *T. gigantea*, excessive exposure is counterproductive (Chittapalo & Noomhorm 2009).

Based on Fig. 2, Šídák's multiple comparisons test revealed a consistent, time-dependent response across all tissue types, where shorter durations (10 and 20 min) produced the most significant effects relative to the control. Specifically, in leaf tissue, the 10-min ($P < 0.0001$) and 20-min ($P = 0.0005$) treatments were significantly different from the control, while longer durations (30 and 40 min) showed no significant variation ($P > 0.05$). This pattern was mirrored in both stem and root tissues, with the 10-min treatment yielding the highest level of significance ($P = 0.0048$ and $P < 0.0001$, respectively). Notably, in all tissues, the 30 and 40-min treatments were statistically indistinguishable from the control, suggesting a potential "rebound" effect or stabilization where the impact of sonication diminishes at longer intervals. Collectively, these results indicate that sonication enhances protein extraction at short durations (10–20 min), but extended sonication (≥ 30 min) reduces yield across all organs, consistent with possible protein degradation at prolonged exposure (Hussain et al. 2023; Jambrak et al. 2008). Consequently, these findings indicate that a sonication time of 10 to 20 min is optimal for achieving significant results across leaf, stem, and root tissues.

Table 4 Total protein yield of leaf, stem, and root extracts after sonication at different time intervals

Tissue	Control	10 min	20 min	30 min	40 min
Leaves	14.64	**77.14**	64.64	26.31	30.48
Stem	26.31	**64.64**	57.98	12.14	1.31
Root	3.81	**63.81**	56.31	25.48	11.13

Footnotes: Protein extracts from leaves, stems, and roots were subjected to sonication at 200 Hz for varying durations (control, 10, 20, 30, and 40 min) with 30−s intervals on ice. Optical density (OD) was measured at 595 nm, and protein yield was calculated. Values represent protein yield mg/g from the obtained extract

3.3 Troubleshooting and practical considerations during protein extraction

Several challenges were encountered throughout the extraction process, primarily related to tissue grinding, pellet washing, and protein solubilization. Table 5 summarizes the troubleshooting steps implemented to overcome these issues. Proper grinding, cold handling, and efficient washing were found to be critical for obtaining clean, whitish protein pellets, preventing degradation, and ensuring reproducibility.

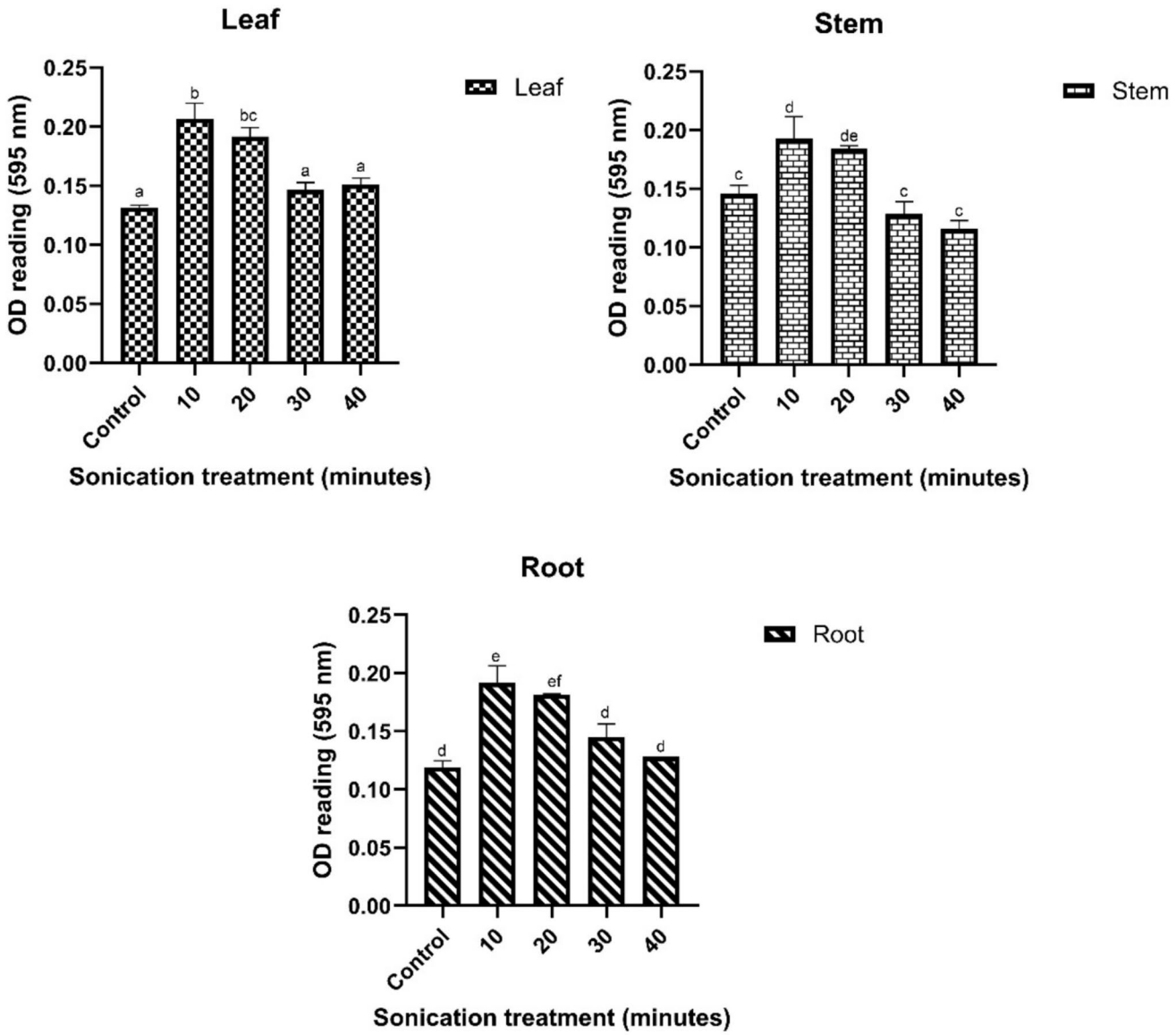


Fig. 2 Comparison of the effect of sonication treatment on protein yield in different tissue organ. Different letters above the bars indicate statistically significant differences between treatments within each organ ($p < 0.05$, Šídák's multiple comparisons test). Bars sharing the same letter are not significantly different. Error bars represent mean ± standard deviation

3.4 Validation of optimized protein extract via LC–MS/MS

To evaluate the compatibility of the optimized TCA/acetone extraction method with downstream high-throughput proteomic workflows, the optimized TCA/acetone leaf extract sonicated at 10 min was analyzed by LC–MS/MS. A total of 134 proteins were successfully identified by searching against the *Arabidopsis thaliana* database. Given that *T. gigantea* is a non-model organism without a sequenced reference genome, the identification count was expectedly constrained by the reliance on cross-species homology. The identified proteins represent a baseline profile of the most abundant components within the *T. gigantea* leaf proteome. Functional analysis, mirroring that of the upstream methodology, confirmed that the protein mass is overwhelmingly distributed across three main Gene Ontology (GO) categories characterized into Molecular Function (MF) (dominated by metal ion and ATP binding), Cellular Component (CC) (primarily focused on chloroplast and thylakoid structures), and Biological Process (BP) (showing high abundance in responses to abiotic stress response) (refer Fig. 4). The quantitative proteomic analysis, weighted by cumulative MS1 precursor ion intensities, revealed the most significant functional commitments of the organism's proteome are centered on energy transduction, ion homeostasis, and stress

Table 5 Troubleshoot conducted during experiment

Step	Common Issue	Observed Effect	Recommended Action
Sample preparation	Tissue not fully ground (residual unbroken material)	Low protein yield; incomplete extraction	Grind thoroughly into a fine, talcum-like powder before extraction (Malik & Bradford 2005)
Sample resuspension	Incomplete mixing with extraction buffer	Reduced protein recovery	Resuspend by repeated pipetting or mixing with a sterile spatula
Precipitation (TCA/acetone)	Insufficient washing of pellet	Contaminants remain (pellet appears yellow/brown) (refer Fig. 3b)	Wash repeatedly with cold 80% acetone until pellet is white
	Inadequate solvent volume (sample-to-buffer ratio too low)	Inefficient precipitation; pellet remains discolored	Maintain at least 1:3 ratio of sample to solvent
Pellet handling	Pellet overdried	Difficult to dissolve in buffer	Air-dry for only 1–3 min to remove excess acetone; invert tube for faster drying (Hao et al. 2015)
Phenol extraction	Low amount of starting material (< 5 g tissue)	Very low protein yield	Use at least 5 g tissue as starting material for phenol extraction (Nasir et al. 2018)
Quantification	Highly concentrated sample or buffer interference	Unstable absorbance; inaccurate Bradford readings	Dilute samples fivefold and/or add small volume of 0.1 N HCl before assay (Faurobert et al. 2007)

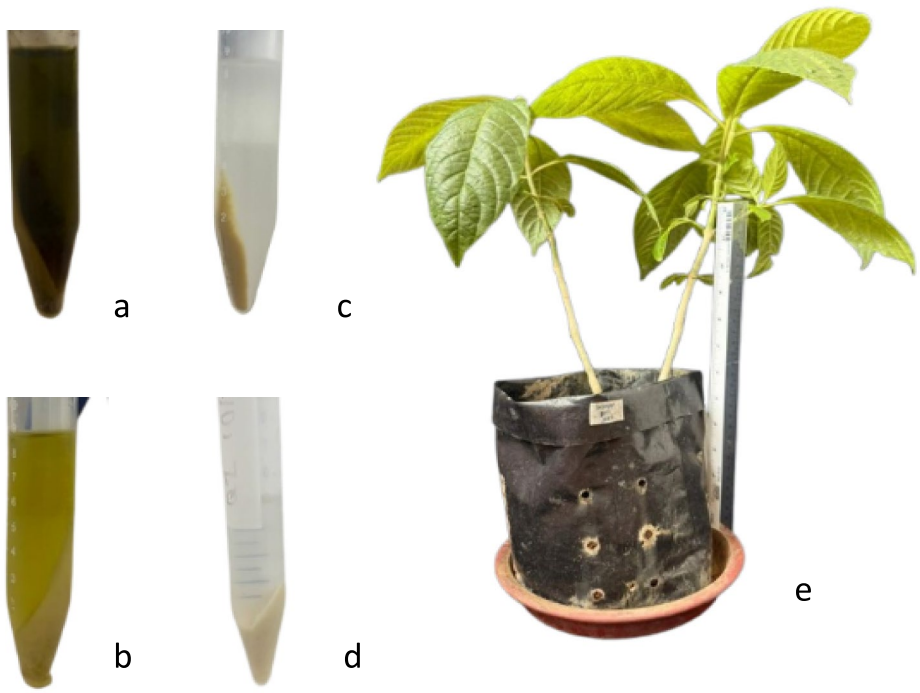
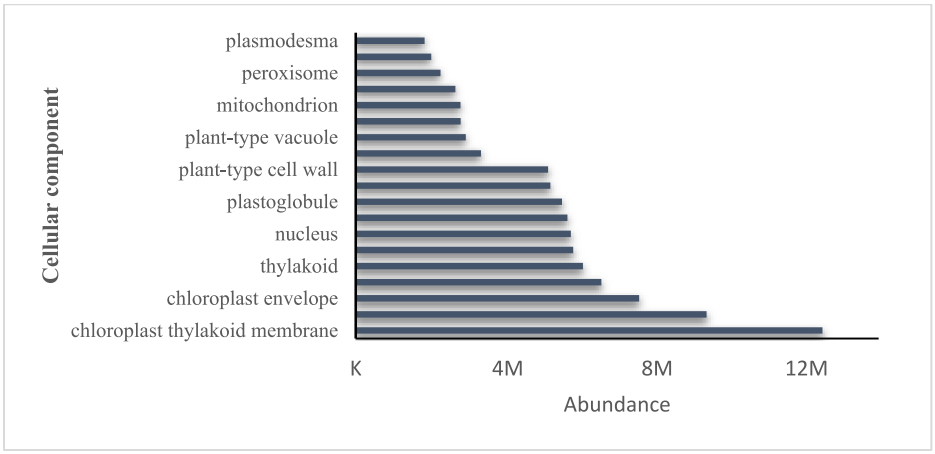
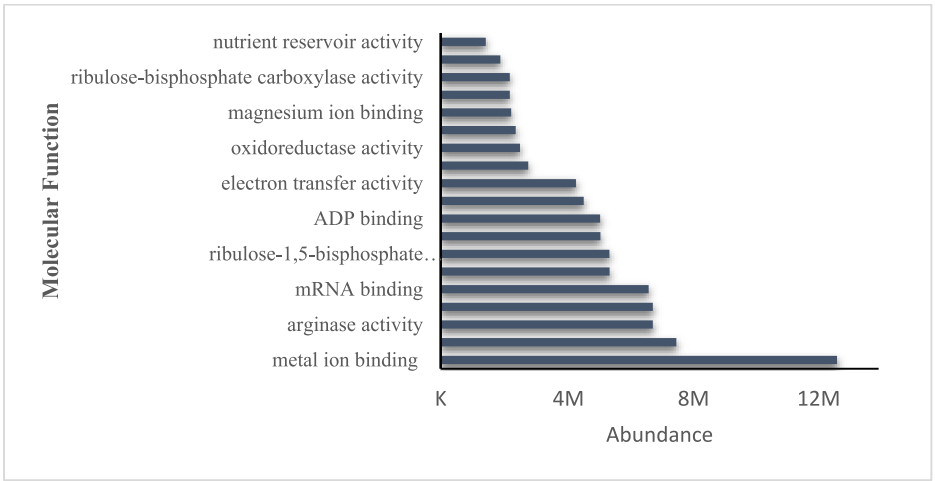


Fig. 3 Pellet formed during extraction. **a** Mixture of powdered *T. gigantea* with extraction buffer for all extraction methods. **b** Protein pellet formed after first washing step in all extraction types **c** Brownish/yellowish protein pellet that will interfere downstream process **d** Final protein pellet after washing (**e**) *T. gigantea* plant

response. The most abundant molecular functions identified were metal ion binding (approx. 12.67 million) and ATP binding (approx. 7.53 million). Proteins driving these functions likely include abundant Photosystem subunits (requiring metal cofactors for electron transport) and high concentrations of ATP synthases, kinases, and ATP-dependent Heat Shock Proteins (HSPs) necessary for energy production and protein quality control (Ravet & Pilon 2013).

The successful identification of these core proteins, which are often difficult to extract in metabolite-rich tropical species, confirms that the optimized protocol effectively removes interfering polyphenols and polysaccharides. While this identification set represents the high-abundance "housekeeping" proteome, its successful recovery demonstrates that the extraction pipeline is robust and provides a suitable foundation for future, more in-depth





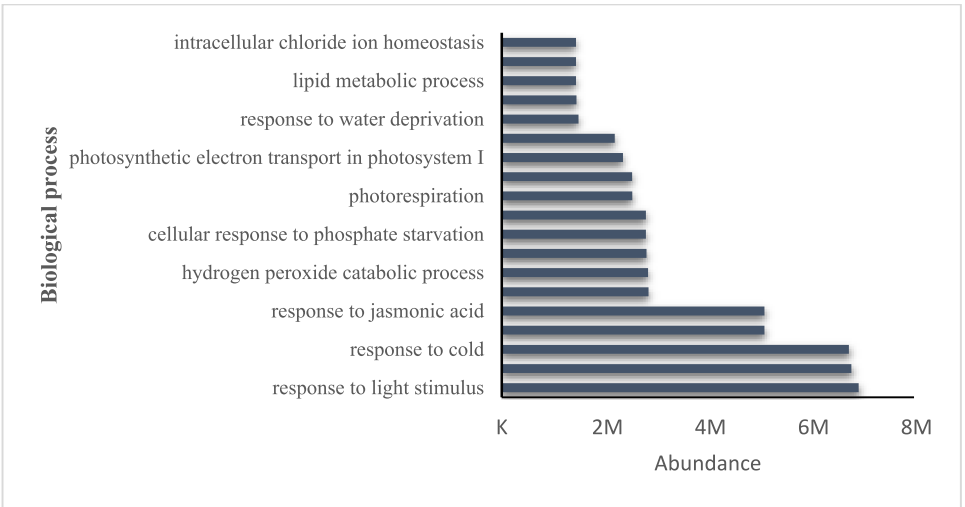


Fig. 4 Total cumulative MS1 intensity per GO term in *T. gigantea* proteins identified using LC–MS/MS. Bar chart describes the distribution of proteins in *T. gigantea* to three GO categories: Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)

proteomic studies as specific genomic resources for *T. gigantea* become available. Based on Table 6, the top most abundant 20 proteins with the most abundance identified in *T. gigantea* were listed. Detail protein list is supplied in the Supplementary. Proteins were only reported if they were identified by at least two peptides.

From a poultry nutrition standpoint, the quality of protein is primarily assessed based on the composition of essential amino acids, especially lysine and methionine, which are frequently limiting in plant-based diets. Research indicates that *T. gigantea* has significant amounts of leucine (8.7 g/16 g N), valine (6.1 g/16 g N), and isoleucine (4.9 g/16 g N), along

Table 6 List of the most abundant proteins detected in LC–MS/MS from *A. thaliana* database

	Protein accession	Protein description	Average abundance (a.u.)	Molecular weight (kDa)	Protein score	Sequence coverage (%)	Identified peptides
1	P10896	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	5,096,681.962	51.9	50.043	10	MGINPIMMSAGELESGNAGEPAK; MCCLFIND-LDAGAGR; VPLILGIWGGK
2	A0A8F5V8L5	Photosystem I iron-sulfur center	2,351,212.6	9	24.531	64	RCESACPTDFLSVR; IYDTCIGCTQCVR; VYLWHETTR; ACPTDVLEMIPWDGCK; CESACPTDFLSVR
3	A0A8F5GFN2	Ribulose bisphosphate carboxylase large chain	2,209,938.799	52.9	21.287	22	WSPELAAACEVWK; DNGLLLHIHR; FLFCAE-AIYK
4	A0A178UUD0	Catalase	1,769,416.992	56.9	20.935	17	SIWISYWSQADK; WIDALSDPR; LGP-NYLQLPVNAPK; EGNFDLVGNNFPVFFIR; GPILLEDYHLVEK; FDFDPLDVTK; APGVQT-PVIVR
5	C0Z2I6	Catalase	961,589.4427	56.9	13.649	10	LGPNYLQLPVNAPK; GPVLLEDYHLIEK; FDFDPLDVTK; APGVQTPVIVR
6	A0A178UC65	Hypothetical protein	652,586.4831	18.4	10.808	17	KVPFISEDIALECEGK; FPENFTGCQDLAK; VPFISEDIALECEGK
7	A0A7G2FJJ0	Cytochrome *f*	438,592.3698	35.3	5.717	9	NILVIGPVPGQK; SNNTVYNATAGGIISK
8	Q9LR30	Glutamate–glyoxylate aminotransferase 1	322,514.832	53.3	20.729	20	LLEATGISTVPGSGFGQK; VPDVFYCLK; DGYPSDPELIFLTDGASK; AMVIINPGNPTGQ-CLSEANIR; EVQLVSFHTVSK; HYLSLTSGGL-GAYSDSR
9	A0A654ENJ7	Ribulose bisphosphate carboxylase/oxygenase activase	297,466.987	48.2	9.407	7	IPLILGIWGGK; VPLIVTGNDFSTLYAPLIR
10	A0A8F5V9E3	Cytochrome *f*	283,893.2975	35.3	9.045	12	YSEITFPILSPDPATK; QVVDIIPPGPELLVS-EGESIK
11	A0A5S9Y7G7	Serine hydroxymethyltransferase	251,361.1797	57.4	8.808	8	YYGGNEYIDMAETLCQK; NTVPGDVSAMVPG-GIR; QFPTIGFEK
12	A0A384L7B8	Hypothetical protein	228,673.668	15	7.158	31	LPSACVGDMVMATVK; MSLGLPVAATVN-CADNTGAK
13	A0A5S9YBP6	Malate dehydrogenase	159,192.7409	35.6	6.827	11	VLVVANPANTNALILK; NVIIWGNHSSTQYPD-VNHATVK
14	A0A8F5V9F8	ATP synthase subunit alpha, chloroplastic	157,278.1374	55.5	11.825	11	IAQIPVSEAYLGR; LIESPAPGIISR; IVNTGTV-LQVGDGIAR
15	A0A654F988	Small ribosomal subunit protein uS5c	154,698.125	32.6	7.174	11	IVLEMAGVENALGK; VMLRPASPGTGVIAG-GAVR
16	A0A8F5NZX9	ATP synthase subunit beta, chloroplastic	150,243.8451	53.7	16.139	24	MPSAVGYQPTLSTEMGSLQER; LSIFETGIK; VVDLLAPYR; VALVYGQMNEPPGAR; GIYPAVDPLDSTSTMLQPR; GMEVIDTGAPLS-VPVGGATLGR; FVQAGSEVSALLGR

Table 6 (continued)

	Protein accession	Protein description	Average abundance (a.u.)	Molecular weight (kDa)	Protein score	Sequence coverage (%)	Identified peptides
17	A0A178W4Q1	Phosphoglycerate kinase	142,974.1164	49.9	15.387	19	IGVIESLLEK; ELDYLVGAVSNPK; ADLNV-PLDDNQNITDDTR; AQGLSVGSSLVEEDK; GVTTIIGGGDSVAAVEK; LASLADLYVNDAF-GTAHR
18	A0A8F5P3R8	Photosystem II CP47 reaction center protein	125,085.6442	55.9	8.459	7	YQWDQGYFQQEIYR; AQLGEIFELDR; LAFY-DYIGNNPAK
19	O80988	Glycine dehydrogenase (decarboxylating) 2, mitochondrial	117,771.056	113.7	7.462	7	DLSLCHSMIPLGSCTMK; NVCIIPVSAHGT-NPASAAMCGMK
20	A0A5S9WSF4	Lactoylglutathione lyase	110,447.3392	39.4	5.652	10	ITACLDPDGWK; TVIAFIEDPDGYK; GPTPEPL-CQVMLR

with moderate levels of lysine (4.0 g/16 g N). However, sulfur-containing amino acids like methionine (2.0 g/16 g N) are present in lower quantities (Rosales 1997). The current proteomic analysis highlights a prevalence of soluble, photosynthesis-related proteins such as RuBisCO, which are generally regarded as digestible plant proteins. Although this study did not directly evaluate digestibility or amino acid bioavailability, the available evidence suggests that *T. gigantea* protein could play an important role in poultry feed formulations when properly combined with complementary protein sources.

4 Conclusion

In this study, three extraction types were compared, all were preliminary chosen based on previous reports on their potential in woody plants. Despite being the gold standard in protein plant extraction, phenol extract gave the lowest yield on *T. gigantea* while TCA/acetone and modified SDS-TCA/Acetone respectively gave promising yield of protein. Surprisingly, modified extraction able to give outstanding protein yield in root which TCA/acetone unable to extract. Optimization was carried out on TCA/acetone extract using sonication at different interval. The sonication treatment able to enhance protein extraction at short durations (10–20 min), but extended sonication (≥ 30 min) reduces yield across all organs. Hence, it can be concluded that optimized method with TCA/acetone + 10–20 min sonication was necessary to overcome the low solubility/incomplete pellet dissolution, thus making it the final workflow choice for obtaining high protein yield in leaf. Meanwhile, technical validation via LC–MS/MS confirmed that the optimized protocol effectively removes interfering secondary metabolites, yielding MS-grade protein suitable for high-throughput proteomics. Although the reliance on cross-species homology limited the identification count to 134 high-abundance proteins, the successful recovery of core metabolic and stress-response proteins which are dominated by metal ion and ATP binding functions demonstrates the robustness of the pipeline. This study provides the first proteomic overview of *T. gigantea* and hence, highlight the species' adaptive strategy for thriving in tropical conditions and identify key metabolic nodes for future improvement of stress tolerance and protein productivity for livestock nutrition.

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Author contributions CNMCZ, PQMY, & NAZ conducted technical validation experimental work and collected data. CNMCZ performed statistical analysis and drafting the manuscript. NMANAR supervised the project, and involved in experimental design. CNMCZ conceptualized the study, drafting and revising the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated and analyzed during the current study are available from the corresponding author (NMANAR) upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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