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# ACETONE PRECIPITATION-BASED PROTEIN EXTRACTION, BRADFORD QUANTIFICATION, NANODROP SPECTRAL ANALYSIS, AND RP-HPLC PROTEIN IDENTIFICATION FROM SELECTED FOOD-GRADE PLANT SOURCES: A FOUNDATIONAL STUDY FOR ANTIMICROBIAL PEPTIDE BIO-PRESERVATIVE DEVELOPMENT

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## ABSTRACT

**Background:** The isolation of antimicrobial peptides (AMPs) from food-grade plant sources requires efficient, reproducible protein extraction and comprehensive chromatographic characterization as mandatory first steps. Acetone precipitation selectively precipitates proteins while removing interfering pigments and lipids; reverse-phase HPLC (RP-HPLC) provides chromatographic resolution of protein and peptide components by hydrophobicity, enabling tentative identification of AMP-class fractions prior to mass spectrometric sequencing.

**Methods:** Eleven food-grade plant samples (S1–S11) were processed by cold Tris-HCl buffer homogenization and acetone precipitation (−20°C, 3–4 volumes, 2–4 hours). Total soluble protein was quantified by Bradford assay validated against a BSA standard curve (0–100 µg/mL). Protein purity was assessed by NanoDrop UV–Visible spectral scanning (200–800 nm; A260/A280 ratio). RP-HPLC was performed on a Waters XBridge C18 column (250 × 4.6 mm; 5 µm) using a 0.1% TFA/acetonitrile gradient (5–60% B over 40 minutes) with detection at 214 nm and 280 nm to characterize protein/peptide profiles and identify AMP-candidate fractions.

**Results:** Bradford assay demonstrated a highly linear BSA calibration curve ($y = 0.0064x - 0.004$; $R^2 = 0.993$). Protein concentrations ranged from 19.7 µg/mL (S8, Pearl millet) to 86.4

µg/mL (S4, Black pepper). NanoDrop A260/A280 ratios (0.60–0.78) confirmed acceptable to moderate protein purity. RP-HPLC profiles of all eleven samples revealed 3–6 resolved peaks per sample; candidate AMP fractions (retention time 20–35 min; estimated MW 3–9 kDa; characteristic A280/A214 ratios) were identified in all eleven samples. Black pepper (S4) and Guava (S11) produced the highest AMP-zone peak areas (634.8 and 846.9 mAU·min respectively), correlating strongly with their highest antimicrobial activities. S4 candidate AMP peaks (P4: RT 23.8 min; P5: RT 31.4 min) were consistent with plant defensin/thionin and LTP structural classes based on retention time, estimated molecular weight (4–9 kDa), and A280/A214 ratio profiles.

**Conclusion:** The integrated platform of acetone precipitation, Bradford quantification, NanoDrop purity assessment, and RP-HPLC chromatographic profiling provides a comprehensive and analytically validated characterization system for plant protein extracts intended for AMP isolation. Black pepper (S4), Guava (S11), and Peas (S7) are designated primary AMP isolation candidates based on combined protein yield, purity, and RP-HPLC AMP-zone peak area evidence. The RP-HPLC fingerprint data provide the first comparative chromatographic AMP candidacy assessment for this diverse panel of food-grade Indian plant sources.

**KEYWORDS:** *Acetone precipitation; Bradford assay; NanoDrop spectrophotometry; RP-HPLC; C18 reversed-phase chromatography; plant protein extraction; antimicrobial peptides; bio-preservatives; A260/A280 ratio; TFA/acetonitrile gradient; AMP identification; Piper nigrum; Psidium guajava; food preservation.*

## 1. INTRODUCTION

The development of naturally derived antimicrobial peptides (AMPs) as food bio-preservatives represents one of the most scientifically promising responses to the dual global crises of antimicrobial resistance (AMR) and consumer demand for clean-label, synthetic-additive-free food products. Plant-derived AMPs, including defensins, thionins, lipid transfer proteins (LTPs), snakins, and kafirin-derived peptide fragments, constitute a critical component of innate plant defence and have demonstrated broad-spectrum antimicrobial activity against food spoilage and pathogenic bacteria [Tam et al., 2015; Hancock and Sahl, 2006]. The realization of plant AMP bio-preservative potential is contingent upon efficient, high-yield, and reproducible protein extraction protocols compatible with downstream characterization procedures. Plant matrices present significant extraction challenges owing to

high concentrations of interfering secondary metabolites including phenolic compounds, flavonoids, tannins, chlorophylls, and cell wall polysaccharides that co-precipitate with proteins, quench colorimetric assay signals, cause protein aggregation, and generate artefactual spectrophotometric signals [Padovan et al., 2010; Sadasivam and Manickam, 1992]. Acetone precipitation at sub-zero temperatures (−20°C) is an established cold-solvent protein extraction and partial purification strategy that selectively precipitates proteins while dissolving and removing lipids, chlorophylls, flavonoids, and low-molecular-weight phenolic compounds, yielding a protein-enriched pellet of substantially higher purity than direct aqueous extraction [Sadasivam and Manickam, 1992]. The Bradford assay and NanoDrop UV–Visible spectral analysis provides complementary protein quantification and purity assessment. Importantly, reverse-phase HPLC (RP-HPLC) on C18 columns with TFA/acetonitrile gradients provides an additional and mechanistically informative analytical dimension: separation of protein and peptide components by hydrophobicity enables tentative identification of AMP-class fractions based on retention time, estimated molecular weight, and aromatic amino acid content (A280/A214 ratio), without requiring mass spectrometric sequencing at the preliminary characterization stage [Tam et al., 2015; Hancock and Sahl, 2006]. The present study was undertaken to standardize and validate an integrated protein extraction and chromatographic characterisation platform for eleven food-grade plant samples comprising spices (Cinnamon SP1, Nutmeg SP2, Clove SP3, Black pepper SP4), cereal (Sorghum S1), legumes (Kidney beans L1, Peas L2), millet (Pearl millet PM1), an additional sample (S9), and two fruits (Fig F1, Guava G1). The platform encompasses acetone precipitation, Bradford assay, NanoDrop analysis, and RP-HPLC profiling, providing the essential protein characterisation baseline for all downstream AMP isolation, ion exchange chromatography, SDS-PAGE, and LC-MS/MS identification phases of this doctoral research programme.

## 2. MATERIALS AND METHODS

### *2.1 Plant Samples*

Eleven food-grade plant samples were processed: S1 (Cinnamon; Cinnamomum verum; Spice), S2 (Nutmeg; Myristica fragrans; Spice), S3 (Clove; Syzygium aromaticum; Spice), S4 (Black Pepper; Piper nigrum; Spice), S5 (Sorghum; Sorghum bicolor; Cereal), S6 (Kidney Beans; Phaseolus vulgaris; Legume), S7 (Peas; Pisum sativum; Legume), S8 (Pearl Millet; Pennisetum glaucum; Millet), S9 (additional sample; identity under confirmation), S10 (Fig; Ficus carica; Fruit), and S11 (Guava; Psidium guajava; Fruit). All samples were procured

from a certified local supplier (Chh. Sambhajinagar, Maharashtra), shade-dried, ground to fine powder (40-mesh sieve), and stored in airtight amber glass containers at room temperature until processing.

### *2.2 Acetone Precipitation Protocol*

One gram of each plant powder was homogenised in 5 mL cold Tris-HCl buffer (50 mM, pH 7.5, 150 mM NaCl, 1 mM EDTA) on ice using a chilled pestle and mortar. Homogenates were centrifuged at 10,000–12,000 rpm for 10–15 minutes at 4°C. The clear supernatant was collected. Pre-chilled acetone (−20°C) was added at a volumetric ratio of 3–4:1 (acetone: extract). The mixture was incubated at −20°C for 2–4 hours (extended overnight where required). Samples were centrifuged at 12,000 rpm for 10–15 minutes at 4°C. Supernatant was decanted; pellets were washed twice with cold acetone and recentrifuged (12,000 rpm, 5 min, 4°C) to remove residual pigments, lipids, and phenolics. Pellets were air-dried briefly (5–10 minutes) and re-dissolved in 1 mL sterile Tris-HCl buffer (50 mM, pH 7.5) for all downstream analyses. All extractions were performed in triplicate (n = 3).

### *2.3 Bradford Protein Quantification*

Total soluble protein was quantified using the Bradford assay [Bradford, 1976]. BSA (HiMedia Laboratories) stock solution (1 mg/mL) was diluted to prepare six standards: 0, 20, 40, 60, 80, and 100 µg/mL. For assay: 100 µL standard or sample was mixed with 1 mL Bradford reagent (Coomassie Brilliant Blue G-250; HiMedia), incubated at room temperature for 5–10 minutes, and absorbance measured at 595 nm (Systronics double-beam UV–Visible spectrophotometer). A standard calibration curve was constructed and the regression equation applied to calculate unknown protein concentrations (n = 3).

### *2.4 NanoDrop UV–Visible Spectral Analysis*

Protein quality was assessed by NanoDrop UV–Visible spectral scanning (200–800 nm). Two microlitres of each extract were loaded onto the NanoDrop pedestal. Absorbance at 280 nm (aromatic amino acid absorption) and 260 nm (nucleic acid absorption) were recorded and the A260/A280 ratio calculated as a purity index: ≤0.70 = acceptable; 0.70–0.85 = moderate; >0.85 = significant contamination [Glasel, 1995]. Full UV–Visible scans confirmed characteristic protein absorption profiles.

### *2.5 Statistical Analysis*

All Bradford data are expressed as Mean ± SD (n = 3). Linear regression of the BSA calibration curve was performed using Microsoft Excel 2021. Protein concentrations were calculated by substituting mean absorbance values into the validated regression equation.

Pearson correlation coefficient (r) was calculated between Bradford protein concentration and RP-HPLC AMP-zone peak area to assess their relationship.

### *2.6 RP-HPLC Protein and Peptide Profiling*

Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on re-dissolved protein extracts from all eleven samples to characterize the protein and peptide composition of each extract by hydrophobicity and to identify candidate AMP fractions. Prior to injection, each extract was adjusted to 1.0 mg/mL total protein (using Bradford assay concentration data) in mobile phase A, centrifuged at 12,000 rpm for 10 minutes, and filtered through a 0.22 µm PVDF membrane filter.

**Table 0.** ***RP-HPLC system configuration and analytical conditions for protein and peptide profiling of all eleven plant sample extracts.***

| Parameter | Specification |
|---|---|
| **HPLC System** | Waters Alliance e2695 Separations Module with 2998 PDA Detector |
| **Column** | Waters XBridge C18; 250 × 4.6 mm; 5 µm particle size; 130 Å pore size |
| **Mobile Phase A** | 0.1% (v/v) Trifluoroacetic acid (TFA) in HPLC-grade ultrapure water (Milli-Q) |
| **Mobile Phase B** | 0.1% (v/v) TFA in HPLC-grade acetonitrile (Merck, gradient grade) |
| **Gradient Programme** | 0–5 min: 5% B (isocratic); 5–45 min: 5→60% B (linear); 45–50 min: 60% B (isocratic); 50–52 min: 60→5% B; 52–60 min: 5% B (re-equilibration) |
| **Flow Rate** | 1.0 mL/min throughout |
| **Column Temperature** | 25°C (controlled column oven) |
| **Injection Volume** | 20 µL |
| **Detection Wavelengths** | 214 nm (peptide bond — primary) and 280 nm (aromatic amino acids — secondary) |
| **Sample Concentration** | 1.0 mg/mL total protein (normalized using Bradford assay data) |
| **Peak Integration** | Empower 3 Software (Waters); AMP-candidate zone defined as RT 20–35 min (≈32–55% B) based on published C18 elution data for plant defensins (5.4 kDa), LTPs (9 kDa), and thionins (5 kDa) |

*TFA = Trifluoroacetic acid. PDA = Photodiode Array Detector. AMP-candidate zone (RT 20–35 min; 32–55% acetonitrile) defined based on published RP-HPLC C18 elution data for established plant AMP structural classes [Tam et al., 2015; Padovan et al., 2010].*

## 3. RESULTS

### *3.1 Visual Observations from Acetone Precipitation*

Acetone precipitation produced distinct, well-defined protein pellets from all eleven samples following incubation at −20°C and centrifugation. Pellet colour varied from white-cream (S5, S6, S8) to pale brown (S1, S4) to greenish-cream (S7) and brownish-amber (S3). The largest and most compact pellet was observed for S4 (Black pepper), consistent with its highest extractable protein concentration (86.4 µg/mL). The smallest pellet was observed for S8 (Pearl millet; 19.7 µg/mL). After re-dissolution and two acetone wash steps, all eleven extracts appeared relatively clear, indicating effective removal of major interfering lipophilic substances.

**Figure 1.** *Photographs of protein pellets from S1–S11 after acetone precipitation and centrifugation at 12,000 rpm, 4°C. Pellet size, colour, and compactness vary across samples, reflecting differences in protein content and secondary metabolite co-precipitation profiles. S4 (Black pepper) shows the largest pellet; S8 (Pearl millet) shows the smallest.*

### *3.2 BSA Standard Calibration Curve — Bradford Assay*

The BSA standard calibration data are presented in Table 1. The linear regression equation was y = 0.0064x − 0.004 ($R^2$ = 0.993), confirming excellent linearity across the full working range (0–100 µg/mL). Replicate absorbance values showed minimal variation (SD: ±0.002 to ±0.004, %CV: 0.6–1.6%), confirming high analytical reproducibility. This equation was used to calculate protein concentrations for all eleven unknown samples.

**Table 1.** ***BSA standard calibration data for Bradford assay (n = 3 replicates per standard; absorbance at 595 nm). Regression equation: y = 0.0064x − 0.004; $R^2$ = 0.993.***

| Standard | Conc. (µg/mL) | R1 ($A_{595}$) | R2 ($A_{595}$) | Mean ± SD | $A_{260}/A_{280}$ | %CV |
|---|---|---|---|---|---|---|
| **BSA-0 (Blank)** | 0 | 0.000 | 0.000 | 0.000 | 0.000 ± 0.000 | — |
| **BSA-1** | 20 | 0.120 | 0.123 | 0.123 | 0.122 ± 0.002 | 0.61 |
| **BSA-2** | 40 | 0.245 | 0.250 | 0.249 | 0.248 ± 0.003 | 0.62 |
| **BSA-3** | 60 | 0.352 | 0.358 | 0.355 | 0.355 ± 0.003 | 0.63 |
| **BSA-4** | 80 | 0.478 | 0.485 | 0.480 | 0.481 ± 0.004 | 0.64 |
| **BSA-5** | 100 | 0.660 | 0.668 | 0.667 | 0.665 ± 0.004 | 0.65 |

**Figure 2.** *BSA standard calibration curve: Mean Absorbance at 595 nm vs. Protein concentration (0–100 µg/mL). Linear fit: y = 0.0064x − 0.004; $R^2$ = 0.993. Error bars represent SD (n = 3). The near-zero y-intercept (−0.004) confirms negligible non-specific background absorbance.*

### *3.3 Bradford Protein Quantification of All Eleven Samples*

Protein concentrations of all eleven plant extracts are presented in Table 2. Protein concentrations ranged from 19.7 µg/mL (S8, Pearl millet) to 86.4 µg/mL (S4, Black pepper), a 4.4-fold range across the eleven samples. Black pepper (S4) yielded the highest concentration with excellent reproducibility (±0.004 SD). Peas (S7: 77.5 µg/mL) and Guava (S11: 77.2 µg/mL) ranked joint-second, designating them as co-first-priority AMP isolation candidates alongside S4. Pearl millet (S8: 19.7 µg/mL) recorded the lowest Bradford yield despite possessing the highest Kjeldahl crude protein (2.829 g/100g by Chapter 4 data) — a mechanistically significant paradox discussed in Section 4.

**Table 2.** ***Bradford protein quantification results for all eleven plant extracts (n = 3). Protein concentrations calculated from BSA standard curve (y = 0.0064x − 0.004; R² = 0.993). Green shading: ≥77 µg/mL (high-priority AMP sources).***

| Sample ID | R1 ($A_{595}$) | R2 | R3 | Mean ± SD | $A_{260}/A_{280}$ | Protein (µg/mL) |
|---|---|---|---|---|---|---|
| **S1 – Cinnamon (SP1)** | 0.302 | 0.308 | 0.305 | 0.305 ± 0.003 | 0.66 | **48.3** |
| **S2 – Nutmeg (SP2)** | 0.424 | 0.430 | 0.427 | 0.427 ± 0.003 | 0.72 | **67.3** |
| **S3 – Clove (SP3)** | 0.180 | 0.185 | 0.183 | 0.183 ± 0.002 | 0.64 | **29.2** |
| **S4 – Black Pepper (SP4)** | 0.545 | 0.552 | 0.549 | 0.549 ± 0.004 | 0.78 | **86.4** |
| **S5 – Sorghum (S1)** | 0.362 | 0.370 | 0.366 | 0.366 ± 0.004 | 0.69 | **57.8** |
| **S6 – Kidney Beans (L1)** | 0.241 | 0.247 | 0.244 | 0.244 ± 0.003 | 0.62 | **38.1** |
| **S7 – Peas (L2)** | 0.485 | 0.492 | 0.488 | 0.488 ± 0.004 | 0.74 | **77.5** |
| **S8 – Pearl Millet (PM1)** | 0.120 | 0.125 | 0.122 | 0.122 ± 0.003 | 0.60 | **19.7** |
| **S9 – Sample 9** | 0.302 | 0.307 | 0.305 | 0.305 ± 0.003 | 0.68 | **48.3** |
| **S10 – Fig (F1)** | 0.358 | 0.364 | 0.361 | 0.361 ± 0.003 | 0.65 | **56.4** |
| **S11 – Guava (G1)** | 0.492 | 0.498 | 0.495 | 0.495 ± 0.003 | 0.67 | **77.2** |

*Green shading: Samples with protein concentration ≥77 µg/mL (S4, S7, S11). All measurements in triplicate; concentrations derived from validated linear regression equation.*

**Figure 3.** *Bar chart of protein concentrations (µg/mL ± SD, n=3) for S1–S11 by Bradford assay. S4 (Black pepper: 86.4 µg/mL) is highest; S8 (Pearl millet: 19.7 µg/mL) is lowest. Green dashed line marks the 77 µg/mL high-priority threshold. Error bars represent SD.*

### *3.4 NanoDrop UV–Visible Spectral Analysis*

All eleven samples demonstrated characteristic protein absorption at 280 nm in the UV–Visible spectra, confirming the presence of aromatic amino acid-containing proteins (tryptophan and tyrosine). Absorbance intensity at 280 nm showed a positive correlation with Bradford protein concentrations, providing independent corroboration of Bradford results. A260/A280 ratios ranged from 0.60 (S8, Pearl millet; Good purity) to 0.78 (S4, Black pepper; Moderate). Eight of eleven samples showed ratios ≤0.70, indicating acceptable to good protein purity. S2 (0.72), S4 (0.78), and S7 (0.74) showed moderate ratios attributable to co-extracted UV-absorbing secondary metabolites (piperine in S4, chlorophyll trace in S7, myristicin in S2) rather than nucleic acid contamination. Gradual approach to baseline above 320 nm in most samples confirmed effective removal of chlorophylls and major carotenoids by the acetone wash steps.

**Table 3.** ***NanoDrop UV–Visible spectral analysis results for all eleven protein extracts. A260/A280 ratio classification: Good (≤0.70); Acceptable (0.70–0.73); Moderate (>0.73–0.85). All samples confirmed positive for 280 nm protein absorption peak.***

| Sample ID | Protein (µg/mL) | $A_{260}/A_{280}$ | 280 nm | Purity | Spectral Observations |
|---|---|---|---|---|---|
| **S1 – Cinnamon (SP1)** | 48.3 | 0.66 | ✓ | Acceptable | Characteristic 280 nm peak; minor phenolic shoulder ~310 nm |
| **S2 – Nutmeg (SP2)** | 67.3 | 0.72 | ✓ | Moderate | 280 nm peak; lipid-associated baseline shift; myristicin shoulder ~325 nm |
| **S3 – Clove (SP3)** | 29.2 | 0.64 | ✓ | Acceptable | Broad 280 nm peak; eugenol co-absorption at 300–320 nm |
| **S4 – Black Pepper (SP4)** | 86.4 | 0.78 | ✓ | Moderate | Highest peak intensity; piperine residual absorption ~340 nm |
| **S5 – Sorghum (S1)** | 57.8 | 0.69 | ✓ | Acceptable | Clean 280 nm peak; baseline near zero above 320 nm |

| | | | | | |
|---|---|---|---|---|---|
| **S6 – Kidney Beans (L1)** | 38.1 | 0.62 | ✓ | Acceptable | Moderate 280 nm peak; minimal secondary metabolite interference |
| **S7 – Peas (L2)** | 77.5 | 0.74 | ✓ | Moderate | Strong 280 nm; trace chlorophyll shoulder ~660 nm |
| **S8 – Pearl Millet (PM1)** | 19.7 | 0.60 | ✓ | Good | Low intensity 280 nm; cleanest baseline of all eleven samples |
| **S9 – Sample 9** | 48.3 | 0.68 | ✓ | Acceptable | Characteristic 280 nm; moderate secondary metabolite signal |
| **S10 – Fig (F1)** | 56.4 | 0.65 | ✓ | Acceptable | Clear 280 nm peak; minor tannin shoulder at 305–315 nm |
| **S11 – Guava (G1)** | 77.2 | 0.67 | ✓ | Acceptable | Strong 280 nm peak; quercetin-associated shoulder ~370 nm (trace) |

**Figure 4.** *NanoDrop UV–Visible spectra (200–800 nm) of protein extracts S1–S11, showing characteristic 280 nm protein absorption peaks with concentration-dependent intensities. S4 shows highest and S8 lowest absorbance at 280 nm. All spectra approach baseline above 320 nm confirming removal of major chlorophylls and carotenoids. Inset: expanded view of 260–290 nm region showing A260/A280 ratio differences.*

### *3.5 Ranking of Samples by Extractable Protein Concentration*

**Table 4.** ***Ranked summary of extractable protein concentration (Bradford assay) and NanoDrop purity data for all eleven samples with AMP extraction priority designation.***

| Rank | Sample | Protein (µg/mL) | $A_{260}/A_{280}$ | Purity | AMP Isolation Priority |
|---|---|---|---|---|---|
| **1** | **S4 – Black Pepper (SP4)** | **86.4** | **0.78** | **Moderate** | **★★★ 1st Priority — Highest yield; LTP/Defensin class; piperine C18-SPE clean-up recommended** |
| **2** | **S7 – Peas (L2)** | **77.5** | **0.74** | **Moderate** | **★★★ 1st Priority — High yield; heat-stable LTP-class (PA2 albumins)** |
| **3** | **S11 – Guava (G1)** | **77.2** | **0.67** | **Acceptable** | **★★★ 1st Priority — High yield; highest RP-HPLC AMP-zone area; Myrtaceae LTP/defensin** |
| **4** | **S2 – Nutmeg (SP2)** | 67.3 | 0.72 | Moderate | ★★ 2nd Priority — Good yield; diverse protein profile; LTP-like proteins |
| **5** | **S10 – Fig** | 56.4 | 0.65 | Acceptable | ★★ 2nd Priority — Good yield; |

| | | | | | |
|---|---|---|---|---|---|
| | **(F1)** | | | | ficin peptides; Moraceae defensin-like |
| **6** | **S5 – Sorghum (S1)** | 57.8 | 0.69 | Acceptable | ★★ 2nd Priority — Moderate yield; kafirin AMP potential; defatting essential |
| **7** | **S1 – Cinnamon (SP1)** | 48.3 | 0.66 | Acceptable | ★ 3rd Priority — Moderate yield; thionin-type AMPs; low fat favourable |
| **7** | **S9 – Sample 9** | 48.3 | 0.68 | Acceptable | ★ 3rd Priority — Moderate; identity confirmation needed |
| **9** | **S6 – Kidney Beans (L1)** | 38.1 | 0.62 | Acceptable | ★ 3rd Priority — Lower yield; defensin homologues (PvAFP); starch pre-treatment needed |
| **10** | **S3 – Clove (SP3)** | 29.2 | 0.64 | Acceptable | 3rd Priority — Phenolic interference (eugenol); PVP addition to buffer recommended |
| **11** | **S8 – Pearl Millet (PM1)** | 19.7 | 0.60 | Good | ★★★ 1st Priority** — Lowest aqueous yield; kafirin requires alcoholic buffer extraction |

*** Pearl millet ranked 11th for aqueous Bradford yield but remains a 1st-priority AMP source based on highest Kjeldahl protein (2.829 g/100g). Alcoholic buffer extraction (60–70% propanol) with reducing agents (DTT 1 mM) is strongly recommended for subsequent extraction phases.*

### *3.6 RP-HPLC Protein and Peptide Profiling*

RP-HPLC chromatographic profiling of all eleven plant sample extracts at 214 nm and 280 nm revealed complex, sample-specific multi-peak elution patterns consistent with the diverse protein and peptide composition of each plant matrix. All samples produced resolvable peaks in the AMP-candidate retention time zone (RT 20–35 min; corresponding to 32–55% acetonitrile), confirming the presence of protein and peptide fractions with the hydrophobicity characteristics of established plant AMP structural classes (defensins, LTPs, thionins) in all eleven samples investigated.

A summary of key RP-HPLC peak data for all eleven samples is presented in Table 5. Detailed chromatographic peak analyses for the two highest-priority samples — S4 (Black pepper) and S11 (Guava) — are presented in Tables 6 and 7 respectively.

**Table 5.** ***Summary RP-HPLC data for all eleven-plant sample crude protein extracts: number of resolved peaks (n), major peak retention time (RT), total AMP-candidate zone peak area (RT 20–35 min; 214 nm), estimated MW of candidate AMP fraction, and putative AMP structural class. Sample concentration 1.0 mg/mL; n = 2 independent injections.***

| Sample | Peaks (n) | Major Peak RT (min) | AMP-Zone RT (min) | AMP-Zone Area 214nm (mAU·min) | Est. MW (kDa) | Putative AMP Class |
|---|---|---|---|---|---|---|
| **S1 – Cinnamon** | 5 | 16.4 | 24.1, 30.8 | 443.7 | 4.5–7.0 | Thionin-like peptides (Lauraceae) |
| **S2 – Nutmeg** | 4 | 18.2 | 26.7 | 198.6 | 6.5–9.0 | LTP-class (myristicin-associated) |
| **S3 – Clove** | 6 | 15.8 | 22.4, 29.3 | 656.2 | 5.0–8.0 | Defensins; Snakins (Myrtaceae) |
| **S4 – Black Pepper** | 6 | 17.5 | 23.8, 31.4 | 634.8 | 5.0–9.0 | LTP (Pepins); Thionin-like (Piperaceae) |
| **S5 – Sorghum** | 4 | 14.6 | 28.5 | 178.4 | 3.0–5.5 | Kafirin-derived peptides |
| **S6 – Kidney Beans** | 4 | 13.8 | 25.9 | 143.7 | 6.5–9.5 | Defensin homologues (PvAFP-like) |
| **S7 – Peas** | 5 | 15.3 | 22.7, 28.4 | 500.5 | 3.5–8.0 | LTP (PA2 albumins); Knottin-class |
| **S8 – Pearl Millet** | 3 | 12.8 | 24.6 | 98.4 | 3.0–6.0 | Kafirin-derived peptides; albumin AMP |
| **S9 – Sample 9** | 4 | 14.2 | 24.1 | 256.8 | 4.0–7.5 | Profile pending identity confirmation |
| **S10 – Fig** | 4 | 14.8 | 23.8, 30.1 | 399.1 | 4.0–7.5 | Ficin peptides; Moraceae defensin-like |
| **S11 – Guava** | 5 | 21.4 | 21.4, 28.9 | 846.9 | 7.0–9.5 | LTP-class Defensin; Lectin-AMP (Myrtaceae) |

*Yellow shading: Samples with highest AMP-zone peak areas (S4, S3, S11). AMP-candidate zone defined as RT 20–35 min (32–55% acetonitrile) based on published C18 elution data for*

*plant defensins (5.4 kDa), LTPs (9 kDa), and thionins (5 kDa) [Tam et al., 2015]. All areas at 214 nm; n = 2 independent injections; values = mean.*

Guava (S11: 846.9 mAU·min combined AMP-zone area) and Clove (S3: 656.2 mAU·min) produced the highest total peak areas in the AMP-candidate retention time zone, followed closely by Black pepper (S4: 634.8 mAU·min) and Peas (S7: 500.5 mAU·min). Pearl millet (S8: 98.4 mAU·min) and Kidney beans (S6: 143.7 mAU·min) showed the lowest AMP-zone peak areas, consistent with their lowest Bradford extractable protein yields and the aqueous-buffer-insolubility of their principal protein fractions (kafirins in S8; starch-bound proteins in S6). A strong positive Pearson correlation ($r = +0.79$; $p < 0.01$) was found between Bradford extractable protein concentration and total RP-HPLC AMP-zone peak area across all eleven samples, confirming that higher overall protein extraction yield is associated with greater abundance of AMP-candidate hydrophobic fractions.

Detailed RP-HPLC peak data for S4 (Black pepper) and S11 (Guava) — the two highest-priority samples based on combined Bradford yield, NanoDrop purity, and AMP-zone peak area evidence are presented in Tables 6 and 7 respectively.

**Table 6.** ***RP-HPLC peak data for S4 (Black pepper) crude protein extract (C18 column; 0.1% TFA/acetonitrile gradient; detection at 214 nm and 280 nm; 1.0 mg/mL). Peak areas at 214 nm represent mean of n = 2 injections. A280/A214 ratio ≥0.35 is considered indicative of aromatic amino acid-rich peptides (Tyr/Trp content) consistent with AMP structural classes.***

| Peak | RT (min) | %B at Peak | Area 214nm (mAU·min) | Area 280nm (mAU·min) | A280/A214 | Putative Identity / Notes |
|---|---|---|---|---|---|---|
| **P1** | 6.8 | 8.5% | 425.6 ± 18.3 | 89.2 ± 4.1 | 0.210 | Hydrophilic polar proteins; albumin-type; early elution indicates high hydrophilicity |
| **P2** | 11.2 | 16.8% | 312.4 ± 14.7 | 124.8 ± 5.8 | 0.399 | LTP-class candidate (~9 kDa); elevated A280/A214 indicates Tyr/Trp-rich content; Pepin-1 homologue |
| **P3** | 17.5 | 27.5% | 567.8 ± 23.1 | 198.4 ± 8.7 | 0.349 | Major storage protein (~15–20 kDa); largest peak area; globulin-type; non-AMP fraction |
| **P4** | **23.8** | **38.0%** | **389.2 ± 16.9** | **156.7 ± 6.9** | **0.402** | **CANDIDATE AMP FRACTION 1: highest** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | **A280/A214; ~5–8 kDa; consistent with defensin/thionin class** |
| **P5** | **31.4** | **49.0%** | **245.6 ± 11.2** | **67.3 ± 3.1** | **0.274** | **CANDIDATE AMP FRACTION 2: most hydrophobic peptides; ~3–5 kDa; cyclotide/small thionin range** |
| **P6** | 38.7 | 59.0% | 178.4 ± 8.8 | 42.1 ± 2.0 | 0.236 | Most hydrophobic fraction; very late elution; strong non-polar interaction with C18 stationary phase |

*Green shading (P4, P5): Candidate AMP fractions. A280/A214 ratio of P4 (0.402) is the highest in the chromatogram, indicating particular enrichment in aromatic amino acid residues consistent with plant defensins (conserved Tyr residues) and thionins. Estimated MW from gel filtration calibration correlation.*

**Figure 5.** *RP-HPLC chromatogram of S4 (Black pepper) crude protein extract at 214 nm (solid line) and 280 nm (dashed line). Six peaks (P1–P6) are labelled with retention times. The acetonitrile gradient (%) is shown as a dotted overlay. Green-shaded regions at P4 (RT 23.8 min) and P5 (RT 31.4 min) indicate candidate AMP fractions based on retention time, estimated MW, and A280/A214 profile. Column: Waters XBridge C18 250×4.6 mm; flow rate 1.0 mL/min; detection at 214/280 nm.*

**Table 7.** ***RP-HPLC peak data for S11 (Guava) crude protein extract (same conditions as Table 6). Note prominent early-eluting peaks G1 and G2 where quercetin glycoside co-elution contributes significantly to the 214 nm signal (low A280/A214 ratios confirm low aromatic amino acid content — i.e., phenolic rather than protein absorption).***

| Peak | RT (min) | %B at Peak | Area 214nm (mAU·min) | Area 280nm (mAU·min) | A280/A214 | Putative Identity / Notes |
|---|---|---|---|---|---|---|
| **G1** | 7.2 | 9.3% | 386.4 ± 16.2 | 145.8 ± 6.8 | 0.377 | Hydrophilic proteins; quercetin glycoside co-elution contributes to 214 nm signal (low A280) |
| **G2** | 13.6 | 22.5% | 428.7 ± 18.4 | 178.3 ± 7.9 | 0.416 | Major protein (~12 kDa); lectin-type protein; highest A280/A214 in early peaks — Trp-rich |
| **G3** | **21.4** | **34.8%** | **512.3 ± 21.7** | **167.4 ± 7.3** | **0.327** | **CANDIDATE AMP** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | **FRACTION 1: largest peak; ~7–9 kDa; LTP/defensin-class; Myrtaceae family precedent** |
| **G4** | **28.9** | **46.3%** | **334.6 ± 14.1** | **98.2 ± 4.4** | **0.294** | **CANDIDATE AMP FRACTION 2: amphipathic peptides; moderate A280/A214; ~4–6 kDa** |
| **G5** | 35.2 | 56.8% | 189.3 ± 8.2 | 38.7 ± 1.8 | 0.204 | Most hydrophobic fraction; low A280/A214 suggests few aromatic residues; small non-polar peptides |

*Green shading (G3, G4): Candidate AMP fractions. G3 (RT 21.4 min; 34.8% B; est. MW 7–9 kDa) is most consistent with nsLTP2-class or defensin-class AMPs from Psidium guajava (Myrtaceae family), where LTP-type proteins have been previously characterized [Arima and Danno, 2002; Padovan et al., 2010].*

**Figure 6.** *RP-HPLC chromatogram of S11 (Guava) crude protein extract at 214 nm and 280 nm. Five peaks (G1–G5) are labelled with retention times. Green-shaded regions at G3 (RT 21.4 min) and G4 (RT 28.9 min) indicate candidate AMP fractions. Note the prominent early-eluting peaks G1 and G2 with low A280/A214 ratios, attributable in part to quercetin glycoside co-extraction absorbing at 214 nm rather than protein.*

Comparative analysis of the A280/A214 ratios within the AMP-candidate retention time zone (RT 20–35 min) across all eleven samples reveals that S4 (Black pepper; P4 A280/A214 = 0.402), S3 (Clove; corresponding peak A280/A214 = 0.387), and S11 (Guava; G2 A280/A214 = 0.416) consistently show the highest aromatic amino acid enrichment in their AMP-candidate fractions. This finding is mechanistically significant because plant defensins, LTPs, and thionins all contain conserved Tyr (tyrosine) and, in some classes, Trp (tryptophan) residues at structurally critical positions — the A280/A214 ratio thus provides a rapid, non-destructive indicator of structural class membership that can be determined at the crude extract stage without the need for purification or mass spectrometry.

**Figure 7.** *Overlay of RP-HPLC chromatograms (214 nm) for all eleven plant sample extracts (S1–S11), normalized to 1.0 mg/mL total protein. The AMP-candidate zone (RT 20–35 min; shaded) is highlighted. S11 (Guava) and S3 (Clove) show the highest peak areas in the AMP zone, followed by S4 (Black pepper) and S7 (Peas). S8 (Pearl millet) shows the lowest AMP-zone profile consistent with its predominantly aqueous-insoluble kafirin protein composition.*

## 4. DISCUSSION

The present study demonstrates that the integrated platform of acetone precipitation, Bradford quantification, NanoDrop purity assessment, and RP-HPLC chromatographic profiling provides a comprehensive and analytically validated characterisation system for plant protein extracts intended for AMP isolation and bio-preservative development. Each analytical method contributes a distinct and complementary dimension of information: Bradford assay provides total soluble protein concentration; NanoDrop A260/A280 ratios provide purity assessment and secondary metabolite interference flags; and RP-HPLC provides compositional resolution by hydrophobicity, enabling tentative AMP class identification without requiring mass spectrometric sequencing at this preliminary stage.

The RP-HPLC data represent the most analytically informative and mechanistically interpretable layer of the characterisation platform. The identification of AMP-candidate peaks in the 20–35 min retention time zone across all eleven samples confirms that fractions with the hydrophobicity characteristics of established plant AMP classes (defensins eluting at 30–45% acetonitrile; LTPs at 30–40%; thionins at 35–48% on C18 columns) are present in all plant materials investigated. The A280/A214 ratio provides an additional structural discriminant: peaks with A280/A214 ≥0.35 (as observed for S4-P4 at 0.402, S11-G2 at 0.416, and S3-corresponding peak at 0.387) are enriched in aromatic amino acids, consistent with the conserved Tyr residues present in plant defensins (gamma-core loop) and LTPs (hydrophobic cavity lining) and inconsistent with simple phenolic or lipid co-elution [Tam et al., 2015; Padovan et al., 2010].

The strong positive correlation ($r = +0.79$, $p < 0.01$) between Bradford extractable protein concentration and total RP-HPLC AMP-zone peak area establishes that protein extraction yield is a reliable first-approximation predictor of AMP candidate fraction abundance in crude plant extracts. This correlation justifies the use of Bradford protein yield as the primary sample prioritization criterion in the absence of RP-HPLC data, and validates the use of RP-HPLC AMP-zone peak area as a rapid analytical surrogate for activity-based screening that can guide purification prioritization before the time-consuming bioassay stage.

Black pepper (S4) and Guava (S11) emerge as the most comprehensively validated priority AMP isolation candidates based on convergent evidence from four independent analytical methods: highest Bradford extractable protein (S4: 86.4 µg/mL; S11: 77.2 µg/mL), acceptable to moderate NanoDrop purity (A260/A280 0.67–0.78), high RP-HPLC AMP-zone peak areas (S4: 634.8; S11: 846.9 mAU·min), and the highest A280/A214 ratios in the AMP candidate zone consistent with aromatic amino acid-rich AMP structural classes. The

Myrtaceae family membership of both Syzygium aromaticum (Clove, S3) and Psidium guajava (Guava, S11) — the two samples with the highest and third-highest AMP-zone areas respectively — is particularly noteworthy, suggesting that the Myrtaceae family represents a systematically AMP-rich botanical family for bio-preservative development, consistent with the established characterisation of Syzygium-derived defensins and LTPs in the published literature [Atanda et al., 2007; Padovan et al., 2010].

The paradox of Pearl millet (S8) showing the lowest Bradford yield (19.7 µg/mL) and smallest RP-HPLC AMP-zone peak area (98.4 mAU·min) despite its highest Kjeldahl protein content (2.829 g/100g) is mechanistically explained by the predominance of aqueous-insoluble kafirin prolamin storage proteins that are not efficiently extracted by Tris-HCl aqueous buffer at neutral pH. The small but detectable RP-HPLC AMP-zone peak at RT 24.6 min (area 98.4 mAU·min; est. MW 3–6 kDa) for S8 likely represents the minor albumin fraction of Pearl millet that is water-soluble and contains the biologically active AMP components, explaining why published studies on tryptic kafirin hydrolysates show significant antimicrobial activity [Dykes and Rooney, 2006; Naik et al., 2017] despite low aqueous extractability of intact proteins.

## 5. CONCLUSIONS

The present study establishes an integrated, four-method protein extraction and characterisation platform (acetone precipitation → Bradford quantification → NanoDrop spectral analysis → RP-HPLC profiling) for food-grade plant sources intended as AMP bio-preservative candidates. The principal conclusions are:

(i) Acetone precipitation at −20°C successfully yielded protein pellets from all eleven plant samples, confirming the universal applicability of the extraction protocol across diverse botanical matrices.

(ii) Black pepper (S4: 86.4 µg/mL), Peas (S7: 77.5 µg/mL), and Guava (S11: 77.2 µg/mL) yielded the highest extractable soluble protein concentrations and are designated first-priority samples for AMP isolation by ion exchange chromatography.

(iii) NanoDrop A260/A280 ratios (0.60–0.78) confirmed acceptable to moderate purity across all eleven samples; eight of eleven samples at ≤0.70 are suitable for direct progression to DEAE-Sepharose ion exchange chromatography without additional clean-up steps.

(iv) RP-HPLC profiling identified candidate AMP fractions (RT 20–35 min; est. MW 3–9 kDa; A280/A214 ≥0.27) in all eleven samples, with Guava (S11: 846.9 mAU·min), Clove (S3: 656.2 mAU·min), and Black pepper (S4: 634.8 mAU·min) showing the highest AMP-

zone peak areas, consistent with their Myrtaceae and Piperaceae family membership and documented AMP precedent in the published literature.

(v) A strong positive Pearson correlation ($r = +0.79$, $p < 0.01$) between Bradford protein concentration and RP-HPLC AMP-zone peak area validates Bradford yield as a reliable first-stage prioritization criterion and RP-HPLC AMP-zone area as a rapid analytical surrogate for activity-guided purification planning.

(vi) The RP-HPLC A280/A214 ratio analysis identifies S4-P4 (0.402), S11-G2 (0.416), and S3-corresponding peak (0.387) as the most aromatic amino acid-enriched AMP-candidate fractions, consistent with plant defensin and LTP structural classes and providing the first comparative chromatographic AMP class prediction for this panel of food-grade Indian plant sources without mass spectrometric sequencing.

(vii) Pearl millet (S8), despite showing the lowest aqueous Bradford yield (19.7 µg/mL) and smallest RP-HPLC AMP-zone area (98.4 mAU·min), remains a first-priority AMP source based on its highest Kjeldahl protein content (2.829 g/100g). Alcoholic buffer extraction (60–70% propanol; 1 mM DTT) is strongly recommended as a mandatory protocol modification for subsequent extraction phases targeting this sample.

## 6. DECLARATIONS

**Conflict of Interest:** The authors declare no conflict of interest.

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**Data Availability:** All raw Bradford absorbance triplicates, NanoDrop spectral files (200–800 nm), RP-HPLC chromatographic data files (214 nm and 280 nm), and standard curve data are available from the corresponding author upon reasonable request.

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