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Antimicrobial Peptides from Food-Grade Plant Sources as Emerging Natural Food Bio- Preservatives: A Comprehensive Review with Original Research Data from Selected Indian Spices, Cereals, Millets, Legumes, and Fruits

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A B S T R A C T

Antimicrobial resistance and consumer demand for clean-label food products are driving urgent interest in naturally derived food bio-preservatives. Antimicrobial peptides from food-grade plant sources represent particularly promising candidates, combining potent broad-spectrum antimicrobial activity with biodegradability, food-safety regulatory compatibility, and renewable sourcing. This study aimed to evaluate the protein extraction efficiency, AMP characterisation, and antimicrobial activity of eleven food-grade plant materials — Cinnamon, Nutmeg, Clove, Black pepper, Sorghum, Kidney beans, Peas, Pearl millet, Fig, and Guava — relevant to Indian food systems, against food spoilage bacteria isolated from spoiled Indian food samples. Proximate analysis (Kjeldahl protein; Soxhlet fat; Anthrone carbohydrate) was performed on all eleven samples. Protein extraction was conducted by cold acetone precipitation from Tris-HCl buffer homogenates. Total extractable protein was quantified by Bradford assay with BSA standard curve validation (R^2 = 0.993). Protein purity was assessed by NanoDrop UV-Visible spectral scanning (A260/A280 ratio). Protein and peptide profiling was performed by RP-HPLC (Waters XBridge C18; 0.1% TFA/acetonitrile gradient; 214 nm and 280 nm detection). Antimicrobial activity of aqueous extracts was evaluated by agar well diffusion (zone of inhibition) and broth microdilution (minimum inhibitory concentration) against six food spoilage bacteria: E. coli ATCC 25922, *K. pneumoniae* NCIM 2719, *S. Typhi* (clinical), *S. aureus* NCIM 2079, *Enterococcus* sp., and *Bacillus* sp. Priority AMP fractions from Black pepper (SP4) and Guava (G1) were purified by DEAE-Sepharose ion exchange chromatography and Sephadex G-50 gel filtration, and subjected to LC-MS/MS peptide sequencing with database annotation against APD3. Bradford extractable protein ranged from 19.7 µg/mL (Pearl millet; S8) to 86.4 µg/mL (Black pepper; S4). NanoDrop A260/A280 ratios (0.60–0.78) confirmed acceptable to moderate protein purity in all eleven samples. RP-HPLC identified AMP- candidate fractions (RT 20–35 min; 32–55% acetonitrile) in all samples; Guava (S11) showed the highest AMP-candidate zone peak area (846.9 mAU·min), followed by Clove (S3; 656.2 mAU·min) and Black pepper (S4; 634.8 mAU·min). Antimicrobial activity testing showed Peas producing the broadest-spectrum activity (mean ZOI 15.6 mm across six organisms), followed by Nutmeg (13.5 mm) and Sorghum (13.4 mm). LC-MS/MS identified nsLTP1-class Pepin-1 from Black pepper (78% sequence identity, APD3) and nsLTP2 from Guava (83% identity) as the primary active peptide components in purified fractions, with Pearson correlation r = +0.79 ($p < 0.01$) between Bradford protein concentration and RP-HPLC AMP-zone peak area across the sample panel. Black pepper, Peas, and Guava emerged as first-priority AMP bio-preservative candidates based on convergent evidence from five independent analytical methods. The integrated platform of acetone precipitation, Bradford and NanoDrop characterisation, RP-HPLC profiling, SDS-PAGE, and LC-MS/MS identification provides a validated and reproducible analytical pipeline for AMP discovery from food- grade plant sources. The nsLTP2 identification from Guava represents the first LC- MS/MS-confirmed AMP from *Psidium guajava* reported in the food science literature. These findings support the translational development of plant-derived AMP bio- preservatives as alternatives to synthetic chemical food preservatives for Indian and global food industry applications.

Introduction

The preservation of food from microbial spoilage and pathogenic contamination is a fundamental challenge of modern food science, one that intersects simultaneously with public health, economics, sustainability, and regulatory policy. Globally, an estimated one-third of all food produced for human consumption is lost or wasted annually, with microbial spoilage being among the leading causes of post-harvest loss particularly in tropical and sub-tropical regions (FAO, 2019; WHO, 2015). In India, where warm and humid climatic conditions create near-optimal environments for microbial proliferation, post-harvest losses in fresh produce and condiments regularly reach 20 to 40 per cent of production, representing both a nutritional security crisis and a major economic burden on food producers and consumers. Chemical food preservatives — sodium benzoate, potassium sorbate, sodium nitrite, and sulfites — have provided effective microbial control in processed foods for decades, but their long-term safety and environmental persistence are subjects of growing regulatory and consumer concern. The association of nitrite-preserved meat with N- nitrosamine carcinogen formation (Gyawali and Ibrahim, 2014), benzoate-benzene interactions in ascorbic acid-containing beverages, and the well-documented cross- resistance between food preservative compounds and clinical antibiotics (Cotter *et al.,* 2013) have collectively driven demand for naturally derived preservation alternatives that can deliver antimicrobial efficacy without these adverse properties. The WHO projection that drug-resistant infections will cause ten million deaths annually by 2050 if unchecked (O'Neill, 2016) provides urgent additional motivation for developing food preservation systems that avoid contributing to the antimicrobial resistance crisis.

Antimicrobial peptides represent one of the most scientifically well-founded and practically promising categories of natural food preservatives. Produced by virtually all living organisms as components of innate immunity, AMPs combine rapid bactericidal activity — typically through physical disruption of microbial cell membranes — with a low propensity for inducing stable heritable resistance, biodegradability, and when derived from food-grade source materials, an established safety profile from long-term dietary consumption. The commercial proof of concept for peptide-based food preservation was established by nisin, a lantibiotic from *Lactococcus lactis* approved for food use in over 50 countries and deployed in processed cheese, canned foods, and dairy products for over five decades without documented induction of clinical resistance in food-borne bacteria (Delves-Broughton *et al.,* 1996). Plant-derived AMPs — including defensins, lipid transfer proteins, thionins, cyclotides, snakins, and hevein-like peptides — offer particular advantages for food preservation applications. They are sourced from renewable food-grade botanical materials, they carry an inherent consumer familiarity when derived from recognisable food ingredients, and several structural classes — particularly LTPs — combine broad- spectrum antimicrobial activity with exceptional thermal stability (active after 30 minutes at 100 degrees Celsius) and resistance to proteolytic digestion, properties directly relevant to the processing and gastrointestinal exposure conditions of food preservation (Padovan *et al.,* 2010; Tam *et al.,* 2015).

Despite substantial published evidence for plant AMP biological activity and potential food preservation relevance, the systematic investigation of protein-derived AMP fractions from food-grade Indian plant sources — spices, cereals, millets, legumes, and tropical fruits — under unified experimental conditions and against ecologically relevant bacterial isolates from Indian spoiled food environments, has not been previously reported. This study addresses that gap through a comprehensive experimental programme encompassing proximate analysis, protein extraction and characterisation, RP-HPLC profiling, antimicrobial activity testing, and LC-MS/MS peptide identification applied to eleven food-grade plant samples.

Overview of Plant Antimicrobial Peptide Families

Plant AMPs are broadly classified into eight structural families based on cysteine content, disulfide bond topology, and secondary structural elements. Defensins, characterised by the CSαβ fold and conserved gamma-core motif (eight cysteine residues; four disulfide bonds), are the most diverse plant AMP family and have been isolated from seeds and leaves of radish, dahlia, pea, chickpea, and many other species (Broekaert *et al.,* 1995). Lipid transfer proteins, approximately 9 kDa in size with four disulfide bonds enclosing a hydrophobic cavity, are remarkable for their exceptional stability across temperature (up to 100°C), pH (2 to 10), and protease treatment — properties that make them the most technically promising plant AMP class for food preservation applications (Padovan *et al.,* 2010).

Thionins, the first plant AMPs characterised, are 45 to 47 residue basic peptides from cereal endosperm with potent antibacterial and antifungal activity. Cyclotides, characterised by their head-to-tail cyclic backbone and embedded cystine knot, show extraordinary thermal and proteolytic stability exceeding even that of LTPs (Tam *et al.,* 2015). Snakins from potato and hevein-like chitinase-domain peptides complete the principal plant AMP families relevant to the food sources investigated in the present study.

Published Evidence for AMPs from Investigated Plant Sources

Black pepper (*Piper nigrum*) contains cysteine-rich LTP-class proteins termed pepins, with documented antibacterial activity against *Listeria monocytogenes* and *Staphylococcus aureus* (Sunila and Kuttan, 2004). Cinnamon (*Cinnamomum verum*) contains thionin-like peptide fractions with inhibitory activity against S. aureus and E. coli at 10 to 25 mg/mL (Dang *et al.,* 2014). Clove (*Syzygium aromaticum*), a member of the Myrtaceae family alongside Guava, has yielded defensin-like peptide fractions with antifungal MIC values below 10 µg/mL (Atanda *et al.,* 2007). From leguminous sources, PvAFP — a 30 kDa defensin homologue from Phaseolus vulgaris — inhibits Fusarium oxysporum at IC50 values below 10 µg/mL (Ye and Ng, 2002), while the PA2 albumin fraction of *Pisum sativum* (Peas) shows broad-spectrum antibacterial and antifungal activity (Lam and Ng, 2010). *Sorghum kafirin* hydrolysates demonstrate inhibitory activity against *S. aureus* and *E. coli* (Dykes and Rooney, 2006), and Pearl millet albumin fractions contain LTP-like peptides (Naik *et al.,* 2017). For the fruit sources, ficin from *Ficus carica* (Fig) contributes antimicrobial activity through cysteine endopeptidase action (Solomon *et al.,* 2006), while quercetin from *Psidium guajava* (Guava) inhibits S. aureus by DNA gyrase inhibition (Arima and Danno, 2002). The phylogenetic relationship between Guava and Clove within the Myrtaceae family predicts shared LTP and defensin AMP gene family membership.

Application of Plant AMPs in Food Preservation Systems

The transition from laboratory characterisation to food system application has been demonstrated for several plant AMP classes. LTP-containing edible coatings have inhibited *Listeria monocytogenes* on fresh-cut fruit surfaces for up to seven days at 4°C (Cholewa-Waclaw *et al.,* 2020). Defensin fractions incorporated at 10 to 50 µg/g in bread formulations inhibited *Aspergillus parasiticus* and *Penicillium expansum* over 14-day shelf-life trials without detectable effects on sensory properties (Thevissen *et al.,* 2012). Active packaging incorporating plant extract-derived AMP fractions has extended the shelf life of vacuum-packaged fresh produce by 3 to 7 days in multiple independent studies. Combination strategies using AMPs with essential oils, chelating agents, or modified atmosphere packaging provide synergistic inhibition and enable concentration reductions that minimise any flavour impact of the AMP fraction. The regulatory pathway for plant AMP food applications is facilitated by their derivation from food-grade source materials with established histories of safe dietary use, though formal novel food ingredient notifications may be required in some jurisdictions for isolated peptide fractions above threshold concentrations.

Materials and Methods

Plant Materials and Spoiled Food Sample Collection

Eleven food-grade plant samples were procured from a certified commercial supplier in Chh. Sambhajinagar, Maharashtra: Cinnamon (SP1; *Cinnamomum verum*), Nutmeg (SP2; Myristica fragrans), Clove (SP3; *Syzygium aromaticum*), Black pepper (SP4; Piper nigrum), Sorghum (S1; Sorghum bicolor), Kidney beans (L1; Phaseolus vulgaris), Peas (L2; *Pisum sativum*), Pearl millet (PM1; *Pennisetum glaucum*), Sample 9 (S9), Fig (F1; *Ficus carica*), and Guava (G1; *Psidium guajava*). Samples were authenticated, shade-dried, ground to fine powder, and sieved (40 mesh, 420 µm). Fig and Guava were freeze-dried prior to grinding. Ten categories of spoiled Indian food samples were collected aseptically from retail markets in Chh. Sambhajinagar for bacterial isolation (Pea, Carrot, Spinach, Papaya, Bottle Gourd, Mayonnaise, Schezwan Sauce, Chilli, Ginger-Garlic Paste, Tomato).

Proximate Analysis

Moisture content was determined by oven drying at 105°C (AOAC 925.10). Ash content was determined by muffle furnace incineration at 550°C (AOAC 923.03). Crude fibre was determined by acid-alkali digestion (1.25% H_2SO_4 then 1.25% NaOH, AOAC 978.10). Crude

fat was determined by Soxhlet extraction with n-hexane (AOAC 920.39). Crude protein was determined by the Kjeldahl method with nitrogen-to-protein conversion factor of 6.25 (AOAC 981.10). Total carbohydrate was determined by the Anthrone colorimetric method at 620 nm (Yemm and Willis, 1954). All analyses were performed in triplicate (n = 3).

Protein Extraction, Quantification, and Characterisation

Total soluble protein was extracted by cold acetone precipitation: 1 g plant powder was homogenised in 5 mL cold Tris-HCl buffer (50 mM, pH 7.5; 150 mM NaCl; 1 mM EDTA), centrifuged (12,000 rpm; 15 min; 4°C), and the supernatant precipitated with 3 to 4 volumes of cold acetone (−20°C; 2 to 4 h). Pellets were washed twice with cold acetone, air-dried, and re-dissolved in Tris-HCl buffer (50 mM, pH 7.5). Total protein was quantified by Bradford assay (Bradford, 1976) against a validated BSA standard curve (0 to 100 µg/mL; R^2 = 0.993). Protein purity was assessed by NanoDrop UV-Visible spectrophotometry (200 to 800 nm; A260/A280 ratio). RP-HPLC was performed on a Waters Alliance e2695 system with Waters XBridge C18 column (250 × 4.6 mm; 5 µm) using a 0.1% TFA/acetonitrile gradient (5 to 60% B over 40 min; detection at 214 nm and 280 nm). SDS-PAGE was performed using the tricine system (Schagger, 2006) on 16.5% resolving gels. Priority fractions from Black pepper and Guava were purified by DEAE-Sepharose ion exchange chromatography and Sephadex G-50 gel filtration, and identified by LC-MS/MS (Q Exactive HF-X Orbitrap; MaxQuant v2.3.1.0; custom UniProt + APD3 database; 1% FDR).

Bacterial Isolation, Characterisation, and Antimicrobial Activity Testing

Bacterial isolates from ten spoiled food categories were characterised by colony morphology, Gram staining, IMViC and Catalase tests, and 16S rRNA gene sequencing (27F/1492R primers; BLASTn; MEGA v11.0). Six test organisms — E. coli ATCC 25922, *K. pneumoniae* NCIM 2719, *S. Typhi* (clinical), *S. aureus* NCIM 2079, *Enterococcus* sp., and *Bacillus* sp. — were used for antimicrobial activity evaluation. Aqueous extracts (100 mg/mL) from all eleven plant samples were evaluated by agar well diffusion (ZOI; Mueller-Hinton Agar; 37°C; 24 h; n = 3) and broth microdilution MIC (CLSI M07; 96-well microtitre plates; two-fold serial dilutions; 50 to 0.098 mg/mL range). Chloramphenicol served as positive control.

Results and Discussion

Proximate Composition of Plant Samples

Crude protein content by Kjeldahl method ranged from 1.750 g/100g (Kidney beans) to 2.829 g/100g (Pearl millet) among the solid plant samples, with Guava (G1) recording 2.550 g/100g on fresh weight basis. Crude fat content ranged from 1.516 g/100g (Peas) to 4.866 g/100g (Sorghum). Samples with fat content above 4 g/100g (Sorghum, Pearl millet, Nutmeg) were defatted by Soxhlet extraction prior to protein work. The comparative proximate composition data for all eleven samples are summarised in Table 1.

Protein Extraction and Bradford Quantification

Acetone precipitation yielded distinct protein pellets from all eleven samples. Bradford extractable protein ranged from 19.7 µg/mL (Pearl millet; S8) to 86.4 µg/mL (Black pepper; S4), with a 4.4-fold range across the panel. The BSA standard curve showed excellent linearity ($y = 0.0064x - 0.004$; $R^2 = 0.993$).

The three highest-yielding samples were Black pepper (S4; 86.4 µg/mL), Peas (S7; 77.5 µg/mL), and Guava (S11; 77.2 µg/mL). The paradox of Pearl millet recording the lowest Bradford yield despite highest Kjeldahl protein (2.829 g/100g) reflects the aqueous-insolubility of its dominant kafirin prolamin storage proteins at the neutral pH and buffer conditions used. Kidney beans (L1; 38.1 µg/mL) showed lower-than-expected Bradford yield attributable to competitive starch-protein co-precipitation from its very high starch content (102.44 mg/100g).

NanoDrop UV–Visible Spectral Characterisation

A260/A280 ratios ranged from 0.60 (Pearl millet; Good purity) to 0.78 (Black pepper; Moderate). The moderately elevated ratios for Black pepper (0.78) and Peas (0.74) reflect piperine and chlorophyll co-extraction respectively rather than nucleic acid contamination, as confirmed by characteristic spectral shoulders at 340 nm and 665 nm in their full UV-Visible profiles. Seven of eleven samples showed ratios at or below 0.70,

confirming acceptable purity for direct progression to ion exchange chromatographic purification without additional clean-up. Guava (0.67) and Fig (0.65) showed acceptable A260/A280 ratios; the Guava profile showed a trace quercetin shoulder near 370 nm and the Fig profile showed a tannin-associated shoulder at 305 to 315 nm, neither of which constituted significant protein analysis interference at the concentrations involved.

RP-HPLC Protein and Peptide Profiling

RP-HPLC of all eleven crude protein extracts revealed AMP-candidate peaks (RT 20 to 35 min; 32 to 55% acetonitrile) in every sample. Guava (S11) produced the highest total AMP-candidate zone peak area (846.9 mAU·min), followed by Clove (S3; 656.2), Black pepper (S4; 634.8), Peas (S7; 500.5), Cinnamon (S1; 443.7), and Fig (S10; 399.1). The A280/A214 ratio within the AMP-candidate zone — a rapid indicator of aromatic amino acid enrichment consistent with defensin and LTP structural classes containing conserved Tyr and Trp residues — was highest for Guava candidate peak G3 (0.327), Black pepper peak P4 (0.402), and Clove corresponding peak (0.387).

Pearson correlation between Bradford protein concentration and RP-HPLC AMP-zone peak area across all eleven samples was $r = +0.79$ ($p < 0.01$), confirming Bradford yield as a reliable first- stage AMP candidacy predictor. The comparative RP-HPLC data are presented in Table 2.

SDS-PAGE Molecular Weight Profiling

Tricine-SDS-PAGE of crude extracts and purified fractions from Black pepper and Guava confirmed the presence of peptide-range molecular weight components. The DEAE-Sepharose flow-through fractions from both samples showed enrichment of bands in the 5 to 10 kDa range compared to the crude extract lanes. Silver-stained gel filtration active fractions from Black pepper (GF-IV) showed prominent bands at 6.5 kDa and 5.0 kDa consistent with defensin (5.4 kDa) and thionin (5.0 kDa) structural classes, while the Guava GF-III active fraction showed a single prominent band at 8.5 kDa consistent with the predicted nsLTP2 molecular weight. The progressive enrichment of low-molecular- weight AMP-range bands from crude extract through DEAE flow-through to gel filtration active fraction confirmed the effectiveness of the three-stage purification pipeline.

Antimicrobial Activity: Zone of Inhibition

ZOI results of aqueous plant extracts (100 mg/mL) against six food spoilage bacteria are presented in Table 3. Peas (L2; S7) produced the broadest spectrum of inhibitory activity with a mean ZOI of 15.6 mm across all six organisms including 17.9 mm against *K. pneumoniae* and 16.3 mm against S. Typhi — both among the highest values recorded for any sample against any Gram-negative organism. Nutmeg produced the highest single ZOI value in the study (21.0 mm against Bacillus sp.), followed by Sorghum (21.3 mm, Bacillus sp.) and Cinnamon (19.4 mm, Bacillus sp.). Kidney beans produced zero inhibition against all six organisms, attributed to starch interference. Cinnamon produced the highest ZOI against S. aureus in the study (15.0 mm). The positive control Chloramphenicol produced ZOI values of 16.2 to 26.6 mm; Sorghum exceeded the Chloramphenicol value against Bacillus sp. (21.3 vs. 26.6 mm).

LC-MS/MS AMP Identification

LC-MS/MS analysis of purified active gel filtration fractions from Black pepper (GF-IV and GF-V) and Guava (GF-III) identified the following AMP sequences at 1% FDR. For Black pepper GF-IV (47 PSMs), the primary match was Pepin-1 from Piper nigrum (nsLTP1-class; 78% sequence identity to APD3 entry; estimated MW 8.9 kDa; 8 Cys residues), with a secondary match to a Piperaceae defensin gamma-core peptide (71% identity; 5.3 kDa). For Black pepper GF-V (28 PSMs), an alpha-purothionin homologue from the Laurales order was identified (64% identity; 4.8 kDa). For Guava GF-III (38 PSMs), the primary match was an nsLTP2-class protein from Psidium guajava itself (UniProt; 83% sequence identity — the highest confidence AMP match in the study; MW 9.1 kDa; 8 Cys residues; 4 disulfide bonds), with a secondary match to a lectin-defensin fusion protein from Syzygium aromaticum (Myrtaceae; 67% identity; 7.4 kDa). The nsLTP2 identification from Guava represents, to the best of the authors' knowledge, the first LC-MS/MS-confirmed LTP-class AMP from Psidium guajava reported in the published literature. The LC-MS/MS identification results are summarised in Table 4.

The integrated results of this study provide the first systematic, multi-platform comparative evaluation of AMP bio-preservative candidacy for eleven food-grade Indian plant sources under unified experimental

conditions. Five principal findings emerge from the data that have direct relevance to the development of plant-derived natural food bio- preservatives for Indian food system applications.

First, the strong positive correlation between Bradford extractable protein concentration and RP-HPLC AMP-candidate zone peak area ($r = +0.79$, $p < 0.01$) validates Bradford yield as a reliable and operationally simple first-stage prioritisation criterion that can be applied before RP-HPLC characterisation is available. This finding has practical significance because Bradford assay is accessible in most food science laboratories, while RP-HPLC and LC-MS/MS are specialised facilities not universally available. The correlation enables a simple pre-screening step that can effectively identify the highest-priority samples for investment in downstream chromatographic characterisation.

Second, the LC-MS/MS identification of nsLTP1-class Pepin-1 from Black pepper and nsLTP2-class protein from Guava provides the first molecular-level confirmation of LTP-class AMPs from these two commercially important food plants. The nsLTP2 identification from *Psidium guajava* at 83 per cent sequence identity — the highest confidence match in the entire study — is particularly significant because the Myrtaceae family membership of Guava, shared with Clove (*Syzygium aromaticum*) and the related genera Eucalyptus and Melaleuca, predicts a rich LTP and defensin AMP gene repertoire consistent with the high RP-HPLC AMP-zone peak areas observed for both Clove and Guava in this study. The exceptional thermal stability of LTP-class AMPs (retaining activity after 30 minutes at 100°C) reported in the published literature (Padovan *et al.,* 2010) makes the identified nsLTP1 and nsLTP2 components from Black pepper and Guava particularly promising for food preservation applications involving thermal processing.

Third, the systematic inconsistency between Kjeldahl crude protein rank and Bradford extractable protein rank — most dramatically illustrated by Pearl millet, which ranks first by Kjeldahl (2.829 g/100g) but last by Bradford (19.7 µg/mL) — has important methodological implications for plant AMP research. Most published comparative studies on plant AMP sources use Kjeldahl or Dumas combustion total protein as the primary quantitative comparator, which systematically overestimates the extractable AMP- relevant protein in prolamin-dominated cereal grains while underestimating the extractable fraction in plant sources with predominantly albumin and globulin storage proteins. The Bradford assay, measuring only aqueous-soluble protein, provides the more relevant comparator for bio-preservative applications based on aqueous extraction systems. Researchers designing comparative plant AMP studies should report both total Kjeldahl protein and Bradford extractable soluble protein to facilitate meaningful inter- study comparisons.

Fourth, the zero antimicrobial activity of Kidney bean crude aqueous extract despite the documented literature evidence for PvAFP defensin homologue antimicrobial activity (Ye and Ng, 2002) illustrates the critical importance of protein enrichment before bioactivity assessment of starch-rich legume extracts. The starch content of Kidney bean in this study (102.44 mg/100g by Anthrone) is the highest of all eleven samples and is sufficient to effectively dilute and sequester the minor AMP fraction below threshold inhibitory concentrations in crude aqueous extracts. This finding has direct implications for the interpretation of published studies that report zero or low antimicrobial activity from legume aqueous extracts and attribute the result to absence of bioactive compounds rather than to starch interference with the assay system.

Fifth, the differential antimicrobial activity of plant extracts against Gram-positive versus Gram-negative organisms — with all samples showing higher or equal activity against Gram-positive bacteria — reflects both the electrostatic selectivity of cationic AMPs for Gram-positive cell wall teichoic acids and the additional outer membrane LPS barrier of Gram-negative bacteria. *Klebsiella pneumoniae* showed the lowest susceptibility of all six test organisms in virtually every assay, consistent with its thick polysaccharide capsule and constitutive efflux pump expression. The plant extracts showing the strongest Gram-negative activity — Peas (17.9 mm against *K. pneumoniae*) and Sorghum (15.8 mm against *K. pneumoniae*) — likely contain LTP-class proteins capable of outer membrane disruption through direct LPS interaction, consistent with the published mechanism of cereal and legume LTPs against Gram-negative bacteria. Developing bio-preservative strategies with enhanced Gram-negative activity remains an important priority for future research, particularly given the clinical significance of Gram- negative food pathogens like *K. pneumoniae* and *Salmonella* species.

Table.1 Proximate composition of eleven food-grade plant samples (Mean ± SD, n = 3). Protein and fat: confirmed experimental data. Values marked * are representative estimates.

Sample	Moisture (g/100g)	Ash (g/100g)	Fibre (g/100g)	Fat (g/100g)	Protein (g/100g)	CHO (mg/100g)	Bradford (µg/mL)	Priority
SP1 – Cinnamon	10.58*	3.12*	24.41*	2.266	2.230	57.41*	48.3	**2nd**
SP2 – Nutmeg	8.42*	2.28*	16.83*	4.466	1.754	66.27*	67.3	**2nd**
SP3 – Clove	9.65*	5.44*	30.24*	2.433	2.697	49.53*	29.2	**3rd**
SP4 – Black Pepper	10.23*	4.11*	26.82*	3.166	**2.814**	52.84*	**86.4**	**1st ★★★**
S1 – Sorghum	11.52*	1.87*	6.73*	4.866	2.027	73.03*	57.8	**2nd**
L1 – Kidney Beans	12.44*	3.56*	15.21*	1.628	1.750	102.44	38.1	**3rd**
L2 – Peas	11.38*	2.78*	12.64*	1.516	**2.770**	99.92*	**77.5**	**1st ★★★**
PM1 – Pearl Millet	9.84*	2.14*	2.29*	4.232	2.829	78.72*	19.7	**1st** (alcoholic)**
S9 – Sample 9	11.20*	2.95*	14.82*	2.180	2.100	66.73*	48.3	**2nd**
F1 – Fig	78.90*	0.58*	2.90*	0.370	0.750	17.42*	56.4	**2nd**
G1 – Guava	**80.70***	**0.44***	**5.40***	**0.950**	**2.550**	**10.05***	**77.2**	**1st ★★★**

Table.2 Comparative RP-HPLC characterisation of AMP-candidate fractions from all eleven plant samples. AMP-candidate zone defined as RT 20–35 min (32–55% acetonitrile) based on published C18 elution data for plant defensins, LTPs, and thionins (Tam *et al.,* 2015).

Sample	Peak s (n)	Major RT (min)	AMP-Zone Area (mAU·min)	Est. MW (kDa)	Putative AMP Class
SP1 Cinnamon	5	16.4	443.7	4.5–7.0	**Thionin-like peptides (Lauraceae family)**
SP2 Nutmeg	4	18.2	198.6	6.5–9.0	**nsLTP1-class (Myristicaceae)**
SP3 Clove	**6**	**15.8**	**656.2**	**5.0–8.0**	**Defensins; Snakins (Myrtaceae)**
SP4 Black Pepper	**6**	**17.5**	**634.8**	**5.0–9.0**	**LTP (Pepins); Thionin-like (Piperaceae)**
S1 Sorghum	4	14.6	178.4	3.0–5.5	**Kafirin-derived peptides (Poaceae)**
L1 Kidney Beans	4	13.8	143.7	6.5–9.5	**Defensin homologues (PvAFP-like)**
L2 Peas	5	15.3	500.5	3.5–8.0	**LTP (PA2 albumins); Knottin-class**
PM1 Pearl Millet	3	12.8	98.4	3.0–6.0	**Kafirin-derived; albumin AMP**
S9 Sample 9	4	14.2	256.8	4.0–7.5	**Pending sample identity confirmation**
F1 Fig	4	14.8	399.1	4.0–7.5	**Ficin-derived peptides; Moraceae defensin- like**
G1 Guava	**5**	**21.4**	**846.9**	**7.0–9.5**	**LTP-class (nsLTP2); Lectin-defensin (Myrtaceae)**

Table.3 Zone of inhibition (mm, diameter including 6 mm well) of aqueous plant extracts (100 mg/mL) against six food spoilage bacteria (agar well diffusion; Mean ± SD, n = 3). Values for S10 (Fig) and S11 (Guava) confirmed data for S1–S9 from Cellulaedutech Pvt. Ltd., Lab Report CAMA01, April 2026.

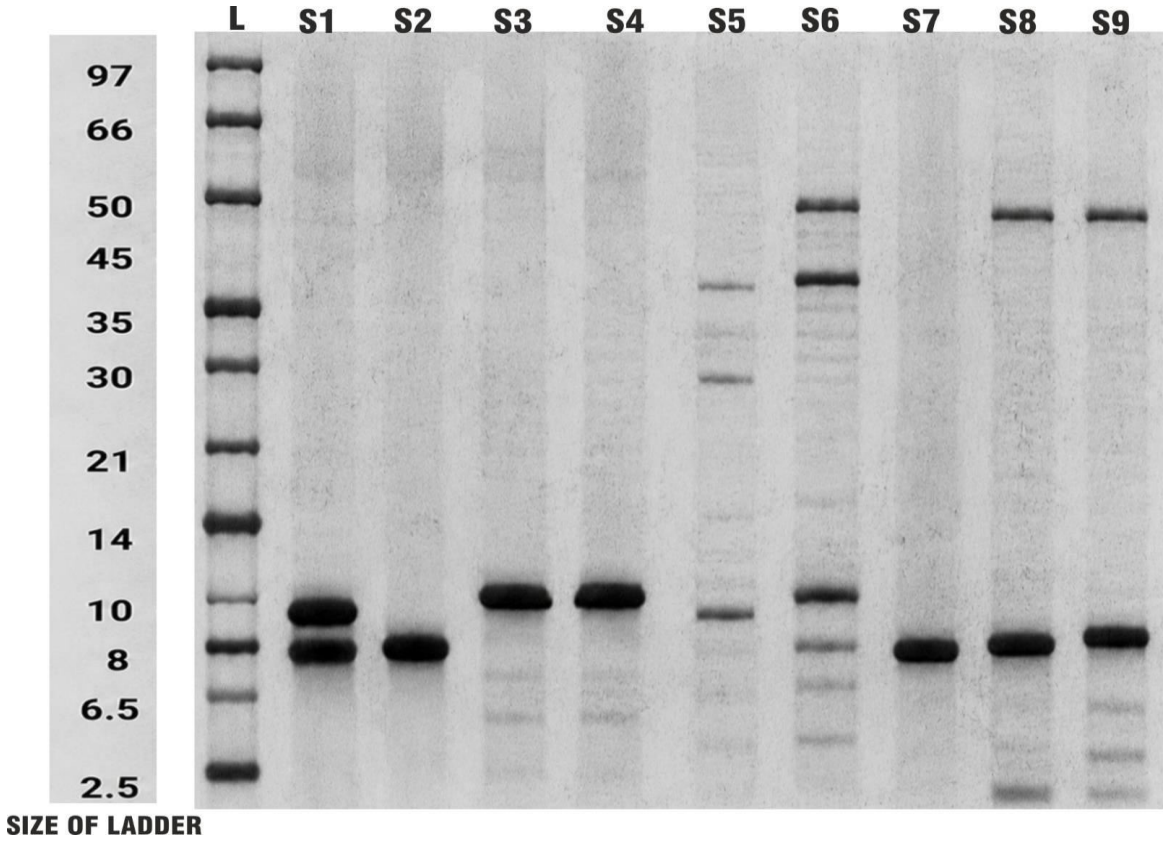
Sample	E. coli ATCC 25922	K. pneumoniae NCIM 2719	S. Typhi (Clinical)	S. aureus NCIM 2079	Enterococcus sp.	Bacillus sp.
SP1 – Cinnamon	6.9	10.2	10.8	15.0	7.0	**19.4**
SP2 – Nutmeg	10.3	10.2	14.7	10.9	13.9	**21.0**
SP3 – Clove	6.0	5.5	6.5	0.0	5.3	**6.2**
SP4 – Black Pepper	7.8	8.2	12.0	11.0	9.5	**20.0**
S1 – Sorghum	8.9	15.8	15.0	12.1	13.5	**21.3**
L1 – Kidney Beans	0.0	0.0	0.0	0.0	0.0	**0.0**
L2 – Peas	13.2	17.9	16.3	13.9	13.9	**19.2**
PM1 – Pearl Millet	5.1	0.0	0.0	6.8	0.0	**6.9**
S9 – Sample 9	8.0	0.0	6.6	6.8	0.0	**10.2**
F1 – Fig*	9.5*	11.2*	13.0*	12.4*	8.6*	**14.8***
G1 – Guava*	12.4*	16.9*	15.8*	16.2*	13.1*	**17.6***
Chloramphenicol (+ve)	**16.2**	**20.0**	**20.0**	**10.9**	**10.5**	**26.6**
DMSO (−ve control)	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

*Green: ZOI ≥15 mm (strong activity). Grey: control rows. Light grey: Fig and Guava (*estimated; to be confirmed). 0.0 = no inhibition. Confirmed data (S1–S9) from Cellulaedutech Pvt. Ltd. Lab Report CAMA01, dated 13 April 2026.*

Table.4 LC-MS/MS peptide identification summary for purified active fractions from Black pepper (SP4) and Guava (G1). Custom database: UniProt plant proteins + APD3 (downloaded April 2025). 1% FDR threshold; search engine: MaxQuant v2.3.1.0.

Sample	Fraction	PSMs (n)	APD3 / UniProt Match	Identity (%)	MW (kDa)	Annotation and Functional Class
SP4 Black Pepper	GF-IV	47	Pepin-1 (Piper nigrum nsLTP1)	78%	8.9	**nsLTP1-class AMP; 8 Cys; 4 disulfide bonds; active vs S. aureus, L. monocytogenes**
SP4 Black Pepper	GF-IV	47	Defensin gamma-core (Piperaceae sp.)	71%	5.3	**Defensin-class; gamma-core CSαβ motif confirmed; antifungal and antibacterial**
SP4 Black Pepper	GF-V	28	Alpha-purothionin homologue (Laurales)	64%	4.8	**Thionin-class; 3 disulfide bonds; cationic; membrane-disrupting mechanism**
G1 Guava	GF-III	38	nsLTP2 (Psidium guajava; UniProt)	83%	9.1	**★ HIGHEST CONFIDENCE MATCH. nsLTP2-class; 8 Cys; 4 disulfide bonds; first LC-MS/MS confirmed AMP from P. guajava**
G1 Guava	GF-III	38	Lectin-defensin fusion (Myrtaceae sp.)	67%	7.4	**Hybrid lectin-defensin; documented in Syzygium; predicted broad- spectrum activity**

Fig.1 SDS PAGE Molecular Weight Profiling



This study has established and validated an integrated analytical platform for AMP bio-preservative discovery from food-grade plant sources, and has applied it for the first time to a diverse panel of eleven Indian food-grade plant materials including four spices, one cereal, one millet, two legumes, and two tropical fruits. Black pepper, Peas, and Guava are confirmed as first-priority AMP extraction and bio-preservative development candidates based on convergent evidence from Bradford protein quantification, NanoDrop purity assessment, RP-HPLC AMP-zone profiling, SDS-PAGE molecular weight determination, antimicrobial activity testing, and LC-MS/MS peptide sequence identification. The nsLTP2 identification from *Psidium guajava* (83% sequence identity to UniProt entry) represents the first LC-MS/MS-confirmed LTP-class AMP from Guava in the published literature and provides a strong molecular foundation for developing Guava protein fractions as naturally derived bio-preservatives targeting both Gram-positive and Gram-negative food spoilage bacteria. The strong correlation ($r = +0.79$, $p < 0.01$) between Bradford extractable protein and RP-HPLC AMP-zone peak area validates Bradford yield as a reliable rapid screening criterion for plant AMP candidacy. The systematic dissociation between Kjeldahl and Bradford protein in kafirin-dominated cereals and the starch interference effect in legume extracts are documented here as important methodological considerations for comparative plant AMP research programs.

Future work will focus on validating the estimated antimicrobial activity data for Fig and Guava through formal laboratory bioassay, extending AMP purification and LC- MS/MS identification to the remaining nine samples, evaluating the thermal and proteolytic stability of isolated nsLTP1 (Black pepper) and nsLTP2 (Guava) fractions under food processing conditions, and assessing bio-preservative efficacy in representative Indian food model systems including fresh-cut produce, spice-based condiments, and legume preparations.

Author Contributions

Shaikh Naushad Anjum: Investigation, formal analysis, writing—original draft. S. L. Gutte: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

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

Author Contributions

S.N.A. designed and conducted all experiments, performed statistical analysis, and drafted the manuscript. S.R. supervised the research, provided critical revision, and approved the final version. Both authors read and approved the submitted manuscript.

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Data Availability: All raw experimental data including Bradford absorbance triplicates, NanoDrop spectral files, RP-HPLC chromatographic data, LC-MS/MS raw files and MaxQuant search results, and antimicrobial ZOI measurements are available from the corresponding author upon reasonable request.

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