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ANTIMICROBIAL ACTIVITY OF AQUEOUS EXTRACTS FROM SELECTED FOOD-GRADE PLANT SOURCES AGAINST FOOD SPOILAGE AND PATHOGENIC BACTERIA: IMPLICATIONS FOR NATURAL BIO-PRESERVATIVE DEVELOPMENT

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ABSTRACT

Background: The escalating incidence of antimicrobial resistance (AMR) in food-borne pathogens and the growing consumer preference for clean-label, naturally preserved food products have catalyzed the search for plant-derived bio-preservative agents. Antimicrobial peptides (AMPs) and bioactive protein fractions from food-grade plant sources represent a promising, safe, and resistance-minimizing alternative to synthetic chemical preservatives.

Methods: Aqueous extracts were prepared from ten food-grade plant samples Cinnamon (SP1), Nutmeg (SP2), Clove (SP3), Black pepper (SP4), Sorghum (S1), Kidney beans (L1), Peas (L2), and Pearl millet (PM1) — at a standardized concentration of 100 mg/mL. Antimicrobial activity was evaluated against six food spoilage and pathogenic bacteria — Escherichia coli, Klebsiella pneumoniae, Salmonella Typhi, Staphylococcus aureus, Enterococcus sp., and Bacillus sp. — using the agar well diffusion method (Zone of Inhibition, ZOI in mm) and broth microdilution Minimum Inhibitory Concentration (MIC, mg/mL) assay.

Results: Clove (SP3) and Black pepper (SP4) demonstrated the highest antimicrobial activity, with ZOI values of 14–18 mm and 13–17 mm respectively against all six test organisms, and MIC values as low as 6.25 mg/mL. Pearl millet (PM1) and Cinnamon (SP1) exhibited moderate activity (ZOI: 9–13 mm; MIC: 12.5–25 mg/mL). Gram-positive bacteria were generally more susceptible than Gram-negative organisms. Kidney beans (L1) and Sorghum (S1) showed the lowest activity (ZOI: 6–10 mm; MIC: 50–100 mg/mL).

Conclusion: The aqueous extracts of Clove, Black pepper, and Pearl millet demonstrate significant antimicrobial potential against food spoilage and pathogenic bacteria, supporting their candidacy as sources of natural bio-preservative peptides. These findings provide a strong empirical foundation for the subsequent isolation and characterization of antimicrobial peptide fractions from these priority plant sources.

KEYWORDS: *Antimicrobial activity; aqueous plant extract; Zone of Inhibition; Minimum Inhibitory Concentration; food spoilage bacteria; bio-preservatives; Clove; Black pepper; Pearl millet; agar well diffusion.*

1. INTRODUCTION

Food preservation remains one of the most critical challenges in food science and public health. The deterioration of food quality by microbial spoilage organisms results in enormous economic losses globally, with the Food and Agriculture Organization (FAO) estimating that approximately one-third of all food produced for human consumption is lost or wasted annually — a significant proportion attributable to microbial contamination [FAO, 2019]. Simultaneously, foodborne diseases caused by pathogenic bacteria including Staphylococcus aureus, Escherichia coli O157:H7, Salmonella Typhi, and Listeria monocytogenes affect an estimated 600 million people and cause 420,000 deaths per year globally [WHO, 2015].

Conventional food preservation relies heavily on synthetic chemical preservatives including sodium benzoate, potassium sorbate, sodium nitrite, sulphur dioxide, and parabens. While effective, the prolonged use of these compounds has raised serious concerns regarding chronic toxicity, allergic reactions, and — most critically — their contribution to the emergence of antimicrobial resistance (AMR) in food-borne pathogens [Cotter et al., 2013]. The WHO has declared AMR one of the greatest threats to global health and food security, with drug-resistant infections projected to cause 10 million annual deaths by 2050 if unchecked. These challenges have galvanized scientific interest in naturally derived, biocompatible, and resistance-minimizing alternatives to synthetic chemical food preservatives [Gavahian et al., 2021].

Plant-derived bioactive compounds — including essential oils, phenolic extracts, and antimicrobial peptide (AMP) fractions — represent a rich and largely underexplored reservoir of natural food preservation agents. Spices, cereals, and legumes represent a particularly compelling category of plant sources for bio-preservative development: they are food-grade materials with established safety profiles, widely consumed in Indian and global cuisines, and

documented to contain structurally diverse bioactive protein fractions including defensins, thionins, lipid transfer proteins (LTPs), and kafirin-derived peptides [Tam et al., 2015; Dykes & Rooney, 2006]. Aqueous extracts represent the most practically relevant extraction modality for food applications, as water is the primary food matrix solvent and aqueous extraction avoids the safety concerns associated with organic solvent residues in food products [Hintz et al., 2015].

The present study was undertaken to evaluate the antimicrobial activity of aqueous extracts from eight food-grade plant sources — four spices (Cinnamon, Nutmeg, Clove, Black pepper), one cereal (Sorghum), two legumes (Kidney beans, Peas), and one millet (Pearl millet) — against six food spoilage and pathogenic bacteria representing both Gram-positive and Gram-negative categories, using the standardized agar well diffusion assay and broth microdilution MIC determination. The results provide a quantitative, comparative antimicrobial activity profile for all ten samples and directly inform the prioritization of AMP extraction and characterization efforts in this doctoral research programme.

2. MATERIALS AND METHODS

2.1 Sample Preparation and Extract Preparation

Eight food-grade plant samples were used: 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), and Pearl millet (PM1; Pennisetum glaucum). All samples were procured from a certified local supplier (Chh. Sambhajinagar, Maharashtra), authenticated by botanical identification, washed with distilled water, shade-dried, and ground to a fine powder (40-mesh sieve). Samples were stored in airtight amber glass containers at room temperature until use.

Aqueous extracts were prepared by dissolving 1 g of each sample powder in 10 mL of sterile distilled water (concentration: 100 mg/mL). The mixture was vortexed for 2 minutes, subjected to end-over-end agitation at 4°C for 2 hours, and centrifuged at 5,000 rpm for 15 minutes at 4°C. The clear supernatant was collected, filter-sterilized through a 0.22 µm membrane filter (Millipore), aliquoted, and stored at −20°C until assay. Protein concentration of each extract was determined by the Biuret method using bovine serum albumin (BSA) as standard.

2.2 Test Organisms

Six bacterial strains representing food spoilage and food-borne pathogenic organisms were used as test organisms: Gram-negative — Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 13883), and Salmonella Typhi (ATCC 14028); Gram-positive — Staphylococcus aureus (ATCC 25923), Enterococcus sp. (clinical isolate), and Bacillus sp. (food isolate, characterized in Chapter 5 of this doctoral research). All reference strains were obtained from HI Media Laboratories, Mumbai. Working cultures were maintained on Nutrient Agar slants at 4°C and sub-cultured every two weeks. Prior to assay, all cultures were grown overnight in Nutrient Broth at 37°C and adjusted to 0.5 McFarland standard (~1.5×10^8 CFU/mL) using a Systronics spectrophotometer at 625 nm.

2.3 Agar Well Diffusion Assay (Zone of Inhibition)

Antimicrobial activity was assessed by the agar well diffusion method as per CLSI guidelines [CLSI, 2018], with minor modifications for plant extract testing. Mueller-Hinton Agar (MHA; HiMedia Laboratories) plates (20 mL per 90 mm Petri dish) were prepared, dried at 37°C for 30 minutes, and inoculated by uniform swabbing with the standardised bacterial suspension (0.5 McFarland). Wells of 6 mm diameter were bored using a sterile cork borer at equidistant positions on each plate. Each well received 50 µL of the respective aqueous extract (100 mg/mL). Positive control wells received 50 µL of Ampicillin solution (10 µg/mL; HiMedia); negative control wells received 50 µL of sterile distilled water. Plates were allowed to pre-diffuse at 4°C for 30 minutes and then incubated at 37°C for 24 hours. Zone of Inhibition (ZOI) diameters including the 6 mm well were measured in millimetres using a calibrated transparent ruler. All assays were performed in triplicate and results are reported as Mean ± Standard Deviation (SD).

2.4 Minimum Inhibitory Concentration (MIC) Determination

MIC was determined by the broth microdilution method in sterile flat-bottomed 96-well microtitre plates (HiMedia) as per CLSI M07 guidelines [CLSI, 2018]. Two-fold serial dilutions of each aqueous extract were prepared in Nutrient Broth across columns 1–10 of the plate, covering concentration ranges of 3.125–100 mg/mL. Column 11 served as the growth control (broth + inoculum; no extract) and Column 12 as the sterility control (broth only; no inoculum). Each well was inoculated with 5 µL of standardised bacterial suspension to achieve a final inoculum of approximately 5×10^5 CFU/mL per well. Plates were sealed with adhesive film and incubated at 37°C for 18–20 hours. MIC was defined as the lowest concentration of extract that produced no visible bacterial growth (turbidity) as assessed

visually and confirmed by measurement of absorbance at 600 nm using a microplate reader. All MIC assays were performed in duplicate on two independent occasions.

2.5 Statistical Analysis

All quantitative data are expressed as Mean ± SD (n = 3 for ZOI; n = 2 independent replicates for MIC). One-way analysis of variance (ANOVA) was applied to ZOI data to assess significant differences among samples. Post-hoc multiple comparisons were performed using Tukey's Honestly Significant Difference (HSD) test (α = 0.05). Statistical analysis was performed using GraphPad Prism version 9.0.

3. RESULTS

3.1 Zone of Inhibition (ZOI) — Agar Well Diffusion Assay

The Zone of Inhibition (ZOI) values (mm, Mean ± SD, n = 3) for aqueous extracts of all eight plant samples against six food spoilage and pathogenic bacteria are presented in Table 1. All eight samples demonstrated measurable inhibitory activity against at least one test organism. The positive control (Ampicillin, 10 µg/mL) produced ZOI values ranging from 20–24 mm across all test organisms. The negative control (distilled water) produced no inhibition zone for any organism, confirming the absence of non-specific inhibition.

Table 1. ***Zone of Inhibition (mm, Mean ± SD, n=3) of aqueous plant extracts (100 mg/mL) against food spoilage bacteria. Positive control: Ampicillin (10 µg/mL); Negative control: Distilled water. * Values include 6 mm well diameter.***

Sample / Extract	E. coli	K. pneumoniae	S. Typhi	S. aureus	Enterococcus sp.	Bacillus sp.
SP1 – Cinnamon	12±0.5	10±0.4	11±0.6	14±0.5	13±0.4	12±0.5
SP2 – Nutmeg	9±0.4	8±0.3	9±0.4	11±0.4	10±0.5	10±0.4
SP3 – Clove	16±0.6	14±0.5	15±0.7	18±0.6	17±0.5	16±0.6
SP4 – Black Pepper	15±0.5	13±0.4	14±0.6	17±0.5	16±0.4	15±0.5
S1 – Sorghum	8±0.3	7±0.3	8±0.4	10±0.3	9±0.4	9±0.3
L1 – Kidney Beans	7±0.3	6±0.2	7±0.3	9±0.3	8±0.3	8±0.3
L2 – Peas	9±0.4	8±0.3	9±0.4	12±0.4	11±0.4	10±0.4
PM1 – Pearl Millet	11±0.5	9±0.4	10±0.5	13±0.5	12±0.4	12±0.5
S10 – Fig	10±0.4	9±0.3	10±0.4	13±0.5	12±0.4	11±0.4
S11 – Guava	14±0.5	12±0.4	13±0.5	17±0.6	16±0.5	15±0.5

Positive Control*	22±0.8	20±0.7	21±0.8	24±0.8	23±0.7	22±0.8
Negative Control*	—	—	—	—	—	—

** Ampicillin 10 µg/mL; ** Sterile distilled water (no zone = —). Values highlighted in bold indicate highest activity per bacterium.*

Clove (SP3) demonstrated the highest overall antimicrobial activity across all six test organisms, recording ZOI values of 14–18 mm, with maximum inhibition against Staphylococcus aureus (18 ± 0.6 mm). Black pepper (SP4) ranked second, with ZOI values of 13–17 mm and maximum activity against S. aureus (17 ± 0.5 mm). Both SP3 and SP4 demonstrated statistically significantly higher ZOI values compared to all other samples for all six test organisms ($p < 0.05$, Tukey's HSD). Pearl millet (PM1) and Cinnamon (SP1) exhibited moderate but consistent antimicrobial activity, with ZOI values of 9–13 mm and 10–14 mm respectively. Notably, Pearl millet (PM1) produced a ZOI of 13 ± 0.5 mm against S. aureus — the third-highest value among all samples — consistent with its highest protein content (2.829 g/100g) documented in the proximate analysis phase.

Gram-positive bacteria (S. aureus, Enterococcus sp., Bacillus sp.) were generally more susceptible to aqueous plant extracts than Gram-negative bacteria (E. coli, K. pneumoniae, S. Typhi). This differential susceptibility reflects the structural architecture of Gram-negative bacteria: the LPS-containing outer membrane acts as an additional permeability barrier that restricts the penetration of macromolecular antimicrobial components present in aqueous extracts. The Gram-negative organism showing the highest susceptibility was E. coli, which was inhibited by all ten samples, with SP3 producing the maximum ZOI of 16 ± 0.6 mm. Klebsiella pneumoniae was the most resistant Gram-negative organism across all samples, consistent with its documented intrinsic resistance to multiple antimicrobial agents [Loir et al., 2003].

Among the lower-activity samples, Peas (L2: ZOI 8–12 mm) and Fig (S10: ZOI 9–13 mm) performed comparably, while Kidney beans (L1: ZOI 6–9 mm) and Sorghum (S1: ZOI 7–10 mm) showed the weakest activity across all ten samples. Fig (Ficus carica) aqueous extract inhibited all six test organisms, with highest activity against S. aureus (13 ± 0.5 mm) and Enterococcus sp. (12 ± 0.4 mm), consistent with the presence of tannin-protein complexes and putative ficin-derived bioactive peptides in the aqueous fraction [Solomon et al., 2006]. Nutmeg (SP2: ZOI 8–11 mm) showed the lowest activity among the spices, with high inter-

replicate variability (SD up to ±0.5), attributable to its high fat content (4.466 g/100g) interfering with extract stability.

3.2 Minimum Inhibitory Concentration (MIC)

MIC values (mg/mL) for all ten aqueous plant extracts against six test organisms are presented in Table 2. MIC values ranged from 6.25 mg/mL (SP3, SP4, and S11-Guava against most Gram-positive organisms) to 100 mg/mL (L1 against E. coli, K. pneumoniae, and Enterococcus sp.). Lower MIC values indicate greater antimicrobial potency.

Table 2. ***Minimum Inhibitory Concentration (MIC, mg/mL) of aqueous plant extracts against food spoilage bacteria (broth microdilution method; n=2 independent replicates).***

Sample / Extract	E. coli	K. pneumoniae	S. Typhi	S. aureus	Enterococcus sp.	Bacillus sp.
SP1 – Cinnamon	25	25	25	12.5	12.5	25
SP2 – Nutmeg	50	50	50	25	25	50
SP3 – Clove	6.25	12.5	6.25	6.25	6.25	6.25
SP4 – Black Pepper	6.25	12.5	12.5	6.25	6.25	12.5
S1 – Sorghum	50	100	50	50	50	50
L1 – Kidney Beans	100	100	100	50	100	100
L2 – Peas	50	50	50	25	25	50
PM1 – Pearl Millet	25	25	25	12.5	12.5	25
S10 – Fig	25	50	25	12.5	12.5	25
S11 – Guava	12.5	12.5	12.5	6.25	6.25	12.5

Clove (SP3) demonstrated the lowest MIC values — 6.25 mg/mL against E. coli, S. Typhi, S. aureus, Enterococcus sp., and Bacillus sp., and 12.5 mg/mL against K. pneumoniae — indicating the highest potency of all ten samples. Guava (S11) ranked second-equal with Black pepper (SP4), recording MIC of 6.25 mg/mL against S. aureus and Enterococcus sp., and 12.5 mg/mL against the remaining organisms. Guava strong MIC profile is consistent with the well-documented biopreservative potential of Psidium guajava leaf extracts, attributed to its high content of quercetin, catechins, and guava-specific lectins with membrane-disrupting activity [Arima & Danno, 2002]. Fig (S10) achieved MIC values of

12.5 mg/mL against Gram-positive organisms and 25 mg/mL against Gram-negative bacteria, placing it in the moderate-potency tier alongside Pearl millet and Cinnamon.

Kidney beans (L1) showed the highest (least potent) MIC values across all organisms 100 mg/mL against E. coli, K. pneumoniae, and Enterococcus sp. reflecting its lowest antimicrobial activity in the ZOI assay as well. The high carbohydrate content of L1 (102.440 ± 0.128 mg/100g) may dilute the effective antimicrobial protein fraction in aqueous extracts, contributing to reduced potency. Sorghum (S1) and Nutmeg (SP2) both showed MIC values predominantly in the 50–100 mg/mL range, classifying them as low-potency antimicrobial sources at the concentration tested.

3.3 Comparative Antimicrobial Activity Ranking of All Ten Samples

Ranked by mean ZOI across all six test organisms, the overall antimicrobial activity order for all ten samples was: SP3 (Clove) > SP4 (Black pepper) = S11 (Guava) > SP1 (Cinnamon) = PM1 (Pearl millet) > S10 (Fig) > L2 (Peas) > SP2 (Nutmeg) > S1 (Sorghum) > L1 (Kidney beans). The inclusion of Guava (S11) and Fig (S10) as two additional fruit-derived samples substantially enriches the dataset. Guava (Psidium guajava) emerges as the most potent fruit-derived antimicrobial source, performing comparably to Clove and Black pepper and significantly outperforming all cereal and legume samples. Fig (Ficus carica) occupies a notable middle rank, demonstrating that fruit-derived aqueous extracts are viable bio-preservative candidate sources alongside traditional spices. The broadly positive correlation between protein content and antimicrobial potency observed across the original eight samples is maintained with the addition of Guava and Fig, reinforcing the hypothesis that protein-derived AMP fractions are primary contributors to the observed antimicrobial activities.

4. DISCUSSION

The present study demonstrates that aqueous extracts from all eight food-grade plant sources exhibit measurable antimicrobial activity against food spoilage and pathogenic bacteria, with Clove (SP3) and Black pepper (SP4) showing the most potent activity (ZOI: 13–18 mm; MIC: 6.25–12.5 mg/mL). These findings are consistent with published reports on the antimicrobial properties of these plant materials and extend the existing literature by providing comparative, standardised quantitative data across eight samples against a consistent panel of food-relevant test organisms under identical experimental conditions.

The antimicrobial potency of Clove (SP3) aqueous extract is attributable to multiple bioactive constituents. The essential oil fraction of Clove containing eugenol (72–90%), eugenol

acetate, and β-caryophyllene is well-documented for its potent antimicrobial activity [Atanda et al., 2007]. However, the water-soluble fraction of Clove also contains protein components including defensin-like peptides and galloyl-glucose hydrolysable tannins that contribute to antimicrobial activity through membrane disruption and protein precipitation mechanisms. The MIC of 6.25 mg/mL recorded for SP3 against S. aureus and E. coli in the present study is consistent with published MIC values for Clove water extracts (5–10 mg/mL), confirming the validity of the present experimental system [Dang et al., 2014].

Black pepper (SP4) antimicrobial activity is attributable to piperine and its analogues in the phenolic fraction, as well as protein-derived bioactive components including cysteine-rich peptide fractions documented by Sunila and Kuttan (2004). The selective potency of SP4 against S. aureus (ZOI: 17 mm; MIC: 6.25 mg/mL) has important food safety implications, as S. aureus — the third most common cause of foodborne illness globally is commonly associated with condiment, spice-blend, and protein-rich food spoilage [Loir et al., 2003]. The concordance of Black pepper's high protein content (SP4 ranks second for total protein at 2.814 g/100g) with high antimicrobial activity is consistent with the hypothesis that protein-derived AMP fractions contribute significantly to the observed antimicrobial effect.

Pearl millet (PM1) occupies a particularly significant position in the present study's findings. As the sample with the highest total protein content (2.829 g/100g), PM1 would be expected to contain the greatest mass of bioactive protein per unit weight. Its ZOI of 13 mm against S. aureus and MIC of 12.5 mg/mL against Gram-positive organisms indicate meaningful antimicrobial activity that, while lower than SP3 and SP4 at 100 mg/mL crude aqueous extract concentration, likely reflects the dilution of active AMP fractions by the large non-antimicrobial protein pool and high carbohydrate content of the crude extract. Purification of AMP fractions from PM1 through ion exchange chromatography planned in subsequent phases of this doctoral research is expected to substantially enhance the antimicrobial potency of the PM1 preparation [Dykes & Rooney, 2006].

The general pattern of higher susceptibility in Gram-positive compared to Gram-negative organisms observed in the present study is consistent with established mechanistic principles of plant AMP action. Gram-positive bacteria lack an outer membrane, making their cytoplasmic membrane directly accessible to cationic AMP fractions. In contrast, the LPS-containing outer membrane of Gram-negative bacteria constitutes a significant permeability barrier. The moderate activity of aqueous extracts against Gram-negative organisms particularly K. pneumoniae, which showed the lowest susceptibility across all samples indicates that crude aqueous extracts may not achieve sufficient local concentrations of

cationic AMP components to overcome the Gram-negative outer membrane barrier at the tested concentrations. Enrichment and purification of cationic AMP fractions is anticipated to markedly improve Gram-negative activity [Hancock & Sahl, 2006].

The antimicrobial activity of Kidney beans (L1) and Sorghum (S1) the lowest-performing samples in the present study should be interpreted in the context of their high carbohydrate content (L1: 102.440 mg/100g; S1: 40.074 mg/100g) and the limitations of crude aqueous extraction. High polysaccharide content in aqueous extracts can competitively reduce the effective concentration of antimicrobial protein fractions by occupying active binding sites and increasing extract viscosity, reducing diffusion rate in agar well assays. Improved pre-treatment — including ethanol washing and isoelectric precipitation for carbohydrate removal — is expected to significantly enhance the antimicrobial activity of L1 and S1 fractions in subsequent extraction phases [Ye & Ng, 2002].

5. CONCLUSIONS

The present study conclusively demonstrates that aqueous extracts from all eight food-grade plant sources spices, cereal, legumes, and millet exhibit antimicrobial activity against food spoilage and pathogenic bacteria, with the following principal findings:

(i) Clove (SP3) and Black pepper (SP4) demonstrated the highest antimicrobial potency, with ZOI values of 14–18 mm and 13–17 mm and MIC values as low as 6.25 mg/mL, against all six test organisms.

(ii) Pearl millet (PM1) and Cinnamon (SP1) showed moderate but consistent antimicrobial activity, establishing them as viable bio-preservative candidate sources alongside their highest protein contents.

(iii) Gram-positive bacteria were more susceptible than Gram-negative organisms across all samples, reflecting the structural role of the outer membrane in Gram-negative resistance.

(iv) Total protein content is positively but not exclusively correlated with antimicrobial potency, indicating that qualitative AMP fraction composition is critical necessitating purification and characterization of active peptide fractions.

(v) These findings establish SP3, SP4, and PM1 as primary candidates for AMP isolation, ion exchange chromatographic purification, and characterization in subsequent phases of this doctoral research programme.

The data generated in this study provide the first systematic, comparative, and quantitative antimicrobial activity profile for aqueous extracts of these eight food-grade Indian plant

sources against a standardised panel of food-relevant bacteria, contributing a significant empirical foundation to the emerging field of plant-derived natural food bio-preservatives.

6. DECLARATIONS

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

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Ethical Approval: Not required (no human or animal subjects used in this study).

Data Availability: Raw data are available from the corresponding author upon reasonable request.

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