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Antibiofilm and Phytochemical Profile of *Piper umbellatum* Flavonoid Fractions against Multidrug Resistant *Proteus* and *Aeromonas* Biotypes

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ABSTRACT

The global rise of multidrug-resistant (MDR) pathogens and biofilm-associated infections presents a critical public health challenge, driving the search for new antimicrobial agents from natural sources. *Piper umbellatum* L., a plant known for its rich secondary metabolites and traditional medicinal uses, shows promising pharmacological potential. This study screened 500 clinical samples collected from two Nigerian hospitals for MDR *Proteus* and *Aeromonas* species. *Proteus* species predominated in urine (59.1%), while *Aeromonas* was primarily isolated from hand swabs (55.6%). Biochemical profiling revealed significant biotype variability, with 43.8% and 37.5% for *Proteus* and *Aeromonas*, respectively. Biofilm analysis indicated 28% of *Proteus* and 37.5% of *Aeromonas* isolates as strong biofilm producers, particularly from urine and hand swabs. Phytochemical screening of *Piper umbellatum* fractions identified flavonoids, including quercetin, with root fractions showing consistent antimicrobial and antibiofilm activity, inhibiting ≥50% biofilm formation in MDR isolates. These findings demonstrate the potentials of *P. umbellatum* as a source of bioactive compounds to combat MDR infections and biofilm-associated challenges. This study underscores the urgent need for novel antimicrobial strategies and advocating for integrated approaches in combining ethnopharmacological and conventional therapies.

Keywords: *Piper umbellatum*, Flavonoids, *Proteus*, *Aeromonas*, multidrug resistance, biofilm, traditional medicine.

Introduction

Plants are a rich source of secondary metabolites, such as polyphenols, flavonoids, terpenoids, alkaloids, and tannins, which are known for their antimicrobial properties (Cowan, 1999; Abachi *et al.*, 2016; Sharma *et al.*, 2010; Zuo *et al.*, 2018). These bioactive compounds have shown promising therapeutic potential, particularly in combating multidrug-resistant (MDR) pathogens and biofilm-associated infections (Wijesundara *et al.*, 2018). *Piper umbellatum* L. (cow foot leaf), a lesser-known species within the Piperaceae family, is predominantly found in tropical climates and has gained attention for its nutritional and medicinal uses. Its roots and aerial parts contain several bioactive compounds, including nerolidylcatechol, which demonstrates antioxidant and chemopreventive properties (Ejele, *et al.*, 2014; Oliveira, *et al.*, 2013; Karuppusamy, *et al.*, 2020; Silva, *et al.*, 2022).

Pathogenic bacteria, particularly those acquired in clinical settings or from the community, often form biofilms which are complex bacterial structures embedded in an extracellular matrix that adhere to surfaces and provide protection against antimicrobial agents (Maheshwasi *et al.*, 2014; Zhang *et al.*, 2024; Van Meervenn *et al.*, 2014; Aun *et al.*, 2019). These biofilms contribute to persistent infections, enhance resistance to chemical, biological, and mechanical agents, and facilitate horizontal gene transfer (HGT) between bacterial species. The prevalence of MDR bacterial pathogens in clinical samples has been reported globally; these infections are commonly community- or hospital-acquired (Ghosh *et al.*, 2024).

In this study, the antimicrobial resistance profiles and biofilm formation abilities of 50 MDR *Proteus* and 8 MDR *Aeromonas* biotypes from clinical samples were investigated. The antibacterial and antibiofilm activities. The leaf, stem bark, and root fractions of *P. umbellatum* were evaluated.

Citation: Pipi *et al*. (2025). Antibiofilm and phytochemical profile of *Piper umbellatum* flavonoid fractions against multidrug resistant *Proteus* and *Aeromonas* biotypes. *International Journal of Microbiology and Applied Sciences*. *4(3)*: 27 – 42.

Phytochemical profiling was conducted using qualitative and quantitative assays, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method.

This study highlights the potential of *P. umbellatum* as a source of bioactive compounds capable of combating MDR pathogens and disrupting biofilm formation, contributing to the search for novel antimicrobial agents.

Materials and Methods

Collection and Processing of Clinical Samples

Clinical samples were collected over 12 months from the Federal Medical Centre (n = 400) and Abia State Specialist Hospital and Diagnostic Centre, Umuahia (n = 100), Nigeria. A total of 500 samples were obtained: midstream clean-catch urine (n = 150), rectal stool (n = 150), blood (n = 100), and hand swabs (n = 100). Samples were immediately transported to the postgraduate laboratory of the Department of Zoology and Environmental Biology, Michael Okpara University of Agriculture, Umudike, for microbiological analysis.

For bacterial isolation, samples were inoculated onto Cysteine Lactose Electrolyte Deficient (CLED) agar and Aeromonas Differential Agar (ADA; TM Media®, Titan Biotech Ltd, India), then incubated at 37 °C for 24 h (Nayeeb *et al.*, 2015).

Pure cultures were examined based on colonial morphology and subjected to Gram staining and a battery of biochemical tests, modified from Gul *et al*. (2004), for presumptive identification of *Proteus* and *Aeromonas* species. Biochemical tests included arginine dihydrolase, lysine and ornithine decarboxylase, citrate utilization, urease activity, tryptophan deaminase, indole production, gelatin hydrolysis, various sugar fermentations, oxidase, and Voges–Proskauer reactions.

Collection and Extraction of Plant Materials

Healthy, mature *Piper umbellatum* leaves, stem bark, and roots were harvested from Okporoenyi, Isieke, and Osisioma (Abia State, Nigeria), and authenticated at the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike. A voucher specimen (#56477) was deposited in the departmental herbarium.

Plant parts (leaves, stem bark and roots) were cleaned, air-dried at room temperature for two weeks, pulverized (750 g each), and sequentially macerated in methanol, ethyl acetate, butanol, hexane and distilled water fractions (5 × 1.5 L) at room temperature. The extracts were filtered, concentrated under reduced pressure using a rotary evaporator, and stored at −20 °C until use. The different plant part extracts of methanol, ethyl acetate, butanol, hexane, and distilled water were designated F1, F2, F3, F4, and F5 respectively. Control used in this study was the crude extract of the plant parts.

Phytochemical Screening

Qualitative and quantitative phytochemical analyses, including total flavonoid were conducted as described by Wijesundara *et al.* (2017).

Total Flavonoids

The flavonoid content of *P. umbellatum* extracts was quantified as it acts as a major antioxidant in plants, reducing oxidative stress, as described by Zhishen *et al.* (1999). Exactly 100 µg/mL of various *P. umbellatum* extracts were taken in different test tubes. To each extract, 2 mL of distilled water was added. Then, 150 µL of 5% $NaNO_2$ was added to all test tubes, followed by incubation at room temperature for 6 minutes. After incubation, 150 µL of 10% $AlCl_3$ was added to all test tubes, including the blank. All test tubes were then incubated for 6 minutes at room temperature. Afterwards, 2 mL of 4% NaOH was added, and the volume was made up to 5 mL using distilled water. The reaction mixtures were vortexed well and allowed to stand for 15 minutes at room temperature. The appearance of pink color was recorded and measured spectrophotometrically at 510 nm. The quantity of flavonoids was calculated in mg rutin equivalents (RE)/g DW.

The absorbance values of the extracts were being used to calculate the flavonoid concentration (C) in µg/mL from the calibration curve equation. [C = {A - c}{m}] were:

- (C) = flavonoid concentration in the extract (µg/mL),
- (A) = absorbance of the extract,
- (m) = slope of the calibration curve,
- (c) = y-intercept of the calibration curve.

The concentration was being converted to Quercetin Equivalents per gram of dry weight (QE/g DW) using the following equation:

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[\text{Flavonoid content (QE/g DW)} = \frac{C \times V \times D}{W \times 1000}] where:

- (C) = flavonoid concentration from the calibration curve (µg/mL),
- (V) = total volume of the extract (mL),
- (D) = dilution factor (if applicable),
- (W) = dry weight of the sample (g),
- 1000 = conversion factor from µg to mg.

The flavonoid content for each extract (leaf, stem bark, roots) in QE/g DW was being calculated. Results were being recorded in a tabular format, including sample type, dry weight, absorbance, calculated concentration, and final flavonoid content (QE/g DW).

Calculations were being verified for accuracy and consistency.

All measurements were being performed in triplicate to account for variability.

Consistent experimental conditions (e.g., temperature, incubation time) were being maintained for reliable results. Extracts and reagents were stored properly to prevent degradation.

Determination of Biofilm Biomass

A modified crystal violet microtiter plate assay was employed to quantify biofilm formation. Following incubation, planktonic cells were removed, wells were washed with sterile distilled water, air-dried, and stained with 0.1% crystal violet for 20 minutes. The dye was then solubilized with 33% acetic acid, and absorbance was measured at 550 nm using a Jenway 6400 UV-Vis spectrophotometer. Sterile media controls (TSB,) were included as blanks.

Proteus and *Aeromonas* clinical isolates were grown overnight in TSB, diluted 1:100 (~1×10^7 CFU/mL), and incubated in 96-well polystyrene plates at 37 °C for 24 h. Biofilm formation was quantified using crystal violet staining (1% w/v, 5 min), followed by solubilization with 33% glacial acetic acid. OD was measured at 570 nm using a Jenway 6400 UV-Vis spectrophotometer. Negative controls included sterile TSB. Each isolate was tested in nonuplets and repeated on three separate days.

For qualitative assessment, isolates were also streaked onto Congo Red Agar after overnight growth in TSB, with black/brown colonies indicating slime production.

Anti-Biofilm Activity

The biofilm-forming ability of *Proteus* and *Aeromonas* strains and the anti-biofilm activity of *Piper umbellatum* fractions were evaluated using a modified microtiter plate assay. Overnight cultures grown on blood agar were suspended in sterile saline, adjusted to 5×10^5 CFU/mL, and mixed 1:1 with TSB supplemented with 1% glucose (TSBG) or with TSBG containing sub-MIC concentrations of *P. umbellatum* fractions.

Aliquots (200 µL) were dispensed into 96-well flat-bottom polystyrene microtiter plates and incubated at 37 °C for 20–24 h without agitation. Following incubation, non-adherent cells were removed by gentle washing with sterile water (twice), and adherent biofilm cells were fixed with 200 µL methanol for 15 min. Wells were stained with 0.4% crystal violet for 5 min, rinsed under running water, and air-dried. The bound stain was solubilized with 160 µL of 33% glacial acetic acid, and OD was measured at 630 nm. The Biofilm inhibition (%) was calculated using the formula:

$$\text{Inhibition (\%)} = [(OD_{control} - OD_{treated}) / OD_{control}] \times 100$$

All treatments were performed in triplicate. An additional assay using OD600-adjusted cultures confirmed the anti-biofilm potential of *P. umbellatum* fractions at sub-MIC levels, with absorbance measured at 570 nm using a Jenway 6400 UV-Vis spectrophotometer.

Statistical Analysis

All experiments in this study were carried out in triplicate. The figures and graphs shown in this report were elaborate using Microsoft Excel tools, and all results obtained and indicated were presented as means ± standard deviation (SD). Statistical analysis of the data was performed using a one-way ANOVA and other descriptive tools.

Results

The results of the Morphological, physiological characteristics of *Proteus* and *Aeromonas* species isolated from clinical samples are presented in Table 1.

The results of the prevalence or distribution of *Proteus* and *Aeromonas* isolates from the clinical samples during the study are presented in Table 2.

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Table 1: Morphological and physiological characteristics of *Proteus* and *Aeromonas* species isolated from clinical samples

Isolate code	Gram reaction	Shape	L-arginine	L-Lysine	L-ornithine	Citrate	Urease	L-tryptophan	Indole	Gelatin	D-glucose	D-mannitol	D-sorbitol	L-rhamnose	D-saccharose	L-arabinose	Oxidase	Voges Proskaur	Biotype	Presumptive Organism
CLSS 21.15P	-	Rod	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	5	*Proteus mirabilis*
CLSU 23.28P	-	Rod	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	4	*Proteus vulgaris*
CLSU 24.18P	-	Rod	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	2	*Proteus mirabilis*
CLSS 23.16P	-	Rod	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	1	*Proteus mirabilis*
CLSU 22.13P	-	Rod	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	3	*Proteus mirabilis*
NCHS 22.13A	-	Rod	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	3	*Aeromonas hydrophila*
NCHS 22.14A	-	Rod	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	1	*Aeromonas hydrophila*
CLSU 23.15A	-	Rod	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	4	*Aeromonas hydrophila*
NCHS 23.9A	-	Rod	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	5	*Aeromonas hydrophila*
CLSU 24.15A	-	Rod	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	2	*Aeromonas hydrophila*

Note: CLSS = Clinical stool sample; CLSU = Clinical urine sample; NCHS = non-Clinical hand swab, A = *Aeromonas* isolates; P = *Proteus* isolates

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Table 2: Prevalence of *Proteus* and *Aeromonas* isolates from Clinical Samples

Sample	Number of samples	*Proteus*		*Aeromonas*	
		Number of isolates	Percentage Prevalence	Number of isolates	Percentage Prevalence
Urine	150	68	59.1	5	4.3
Stool	150	31	27	3	2.6
Blood	100	5	4.3	0	0
Hand swab	100	11	9.6	0	8.4
Total	500	**115**	**23**	**18**	**3.6**

Colonies on Aeromonas Differential Agar (ADA) presented red centres with purple peripheries and were confirmed as motile, Gram-negative bacilli—presumptively *Aeromonas* spp., (Table 1). Phenotypic characterization identified 23% of the isolates as *Proteus* species, comprising *P. mirabilis* (biotypes 1, 2, and 4) and *P. vulgaris* (biotypes 1 and 4), while *Aeromonas hydrophila* was identified with five biotypes (1–5), representing 3.6% of all positive samples. Among the 16 biochemical tests conducted, seven (43.8%) showed biotype-specific variations for *Proteus* spp., including ornithine decarboxylase, citrate utilization, urease production, indole production, gelatin hydrolysis, glucose fermentation, and saccharose fermentation (Appendix 1). *Aeromonas* biotypes differed in six tests (37.5%) including indole utilization, fermentation of mannitol, rhamnose, saccharose, arabinose, and the Voges–Proskauer reaction (Table 1).

During the study period, a total of 115 presumptive *Proteus* isolates and 18 *Aeromonas* strains were recovered from 500 clinical samples. *Proteus* species were predominantly isolated from urine (59.1%), followed by stool (27.0%), hand swabs (9.6%), and least from blood samples (4.3%).

Conversely, *Aeromonas* isolates were primarily found in hand swabs (n = 10), with fewer from urine (n = 5) and stool (n = 3); no *Aeromonas* isolates were detected in blood samples Table 2.

Results of the qualitative screening of flavonoids contents in *Piper umbellatum* leaf, stem bark and root solvent fractions are presented in Table 3. While results of the quantitative screening of *P. umbellatum* leaf, stem bark and root solvent fractions are presented in Tables 4, 5 and 6.

Table 3 revealed the presence of several bioactive compounds including and quercetin, kaempferol and rutin across the leaf, stem bark, and root extracts. Flavonoids were detected in all root fractions, with moderate presence (++) in F1 and F2, and low presence (+) in F3, F4, and F5. In stem bark, flavonoids were absent (-) in fractions F1, F2, and F3, with low presence (+) in F4 and F5. Leaf fractions exhibited the greatest variability, with high flavonoid presence (+++) in F3, moderate presence (++) in F1, low presence (+) in F2 and F4, and absence (-) in F5. There was differential flavonoid distribution across plant parts, with roots showing consistent flavonoid presence, stem bark displaying limited distribution, and leaves exhibiting the highest flavonoid concentration in F3 (Table 3).

Table 3: Qualitative Analysis of total Flavonoid in *P. umbellatum* root, stem bark and leaf fractions

Extraction Solvent	Presence of Flavonoid		
	Leaf Extract	Stem bark Extract	Root Extract
Methanol (F1)	++	-	++
Ethyl Acetate (F2)	+	-	++
Butanol (F3)	+++	-	+
Hexane (F4)	+	+	+
Aqueous (F5)	-	+	+

Note: +++ = High flavonoid presence, ++ = Moderate presence, + = Low presence, - = Absent

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Table 4: Quantitative Analysis of Quercetin (QE/g DW) in *P. umbellatum* Leaf, stem bark, and Root

Extraction Solvent	Quercetin (QE/g DW)					
	Leaf Fraction		Stem bark Fraction		Root Fraction	
	Leaf	Control	Stem bark	Control	Root	Control
F1	20.93 ± 9.60	12.93 ± 10.83	9.78 ± 6.71	33.95 ± 16.10	23.69 ± 2.72	33.95 ± 16.10
F2	10.94 ± 4.63	16.36 ± 16.97	8.09 ± 12.50	33.81 ± 20.29	13.34 ± 4.28	33.81 ± 20.29
F3	31.82 ± 4.79	33.75 ± 23.28	15.02 ± 21.00	14.89 ± 0.42	7.96 ± 11.63	14.89 ± 0.42
F4	27.22 ± 1.36	332.71 ± 5.76	36.78 ± 22.55	13.26 ± 8.79	5.91 ± 8.96	13.26 ± 8.79
F5	41.0 ± 20.66	19.36 ± 5.91	34.61 ± 47.74	21.39 ± 11.70	7.68 ± 6.13	21.39 ± 11.70

Note: F1 = Methanol, F2 = Ethyl Acetate, F3 = Butanol, F4 = Hexane, F5 = Aqueous

Table 5: Quantitative Analysis of Kaempferol (QE/g DW) in *P. umbellatum* Leaf, steam bark, and Root

Extraction Solvent	Kaempferol (QE/g DW)					
	Leaf Fraction		Stem bark Fraction		Root Fraction	
	Leaf	Control	Stem bark	Control	Root	Control
F1	31.32 ± 8.11	12.93 ± 10.83	19.44 ± 6.42	33.95 ± 16.10	26.97 ± 4.19	21.40 ± 10.92
F2	35.92 ± 6.26	16.36 ± 16.97	24.81 ± 17.26	33.81 ± 20.29	40.44 ± 8.44	10.87 ± 1.50
F3	34.93 ± 6.28	33.75 ± 23.28	36.33 ± 6.92	14.89 ± 0.42	28.66 ± 4.50	11.44 ± 3.03
F4	24.80 ± 15.09	332.71 ± 5.76	26.15 ± 21.27	13.26 ± 8.79	36.54 ± 9.86	14.47 ± 2.90
F5	17.67 ± 17.21	19.36 ± 5.91	31.62 ± 10.11	21.39 ± 11.70	14.05 ± 8.81	18.73 ± 7.34

Note: F1 = Methanol, F2 = Ethyl Acetate, F3 = Butanol, F4 = Hexane, F5 = Aqueous

Table 6: Quantitative Analysis of Rutin (QE/g DW) in *P. umbellatum* Leaf, steam bark, and Root

Extraction Solvent	Rutin (QE/g DW)					
	Leaf Fraction		Stem bark Fraction		Root Fraction	
	Leaf	Control	Stem bark	Control	Root	Control
F1	8.19 ± 8.67	33.95 ± 16.10	8.19 ± 8.67	33.95 ± 16.10	22.16 ± 9.41	21.40 ± 10.92
F2	7.72 ± 16.20	33.81 ± 20.29	7.72 ± 16.20	33.81 ± 20.29	25.38 ± 0.27	10.87 ± 1.50
F3	13.11 ± 19.62	14.89 ± 0.42	13.11 ± 19.62	14.89 ± 0.42	26.51 ± 5.76	11.44 ± 3.03
F4	6.56 ± 9.95	13.26 ± 8.79	6.56 ± 9.95	13.26 ± 8.79	35.57± 12.15	14.47 ± 2.90
F5	7.92 ± 10.50	21.39 ± 11.70	7.92 ± 10.50	21.39 ± 11.70	42.91 ± 1.66	18.73 ± 7.34

Note: F1 = Methanol, F2 = Ethyl Acetate, F3 = Butanol, F4 = Hexane, F5 = Aqueous

Biofilm formation by MDR *Proteus* and *Aeromonas* isolates

The results of the ability of MDR *Proteus* isolates to form biofilms are presented in Figure 1.

The results of the ability of MDR *Aeromonas* isolates to form biofilms are presented in Figure 2.

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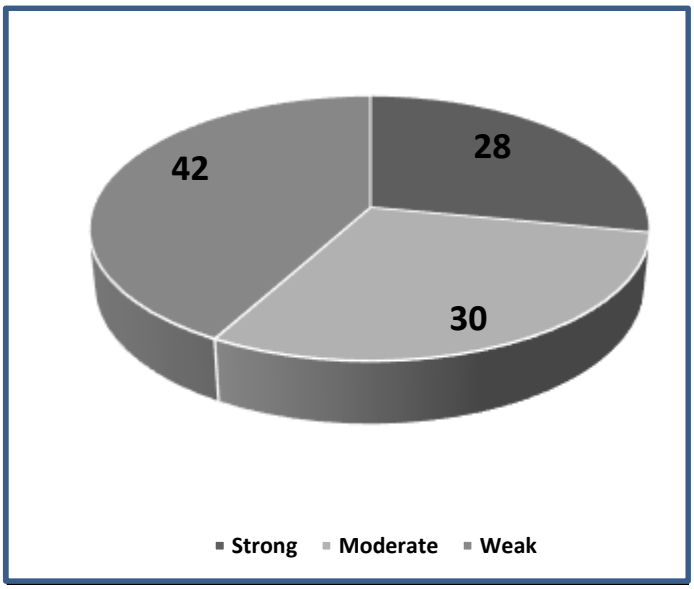

Figure 1: Biofilm formation (%) by MDR *Proteus* isolates

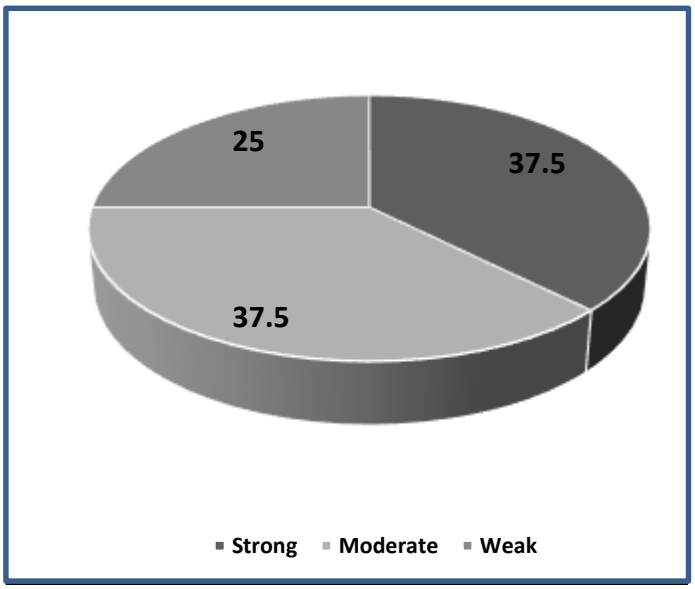

Figure 2: Biofilm formation (%) by MDR *Aeromonas* isolates

Approximately 26% of the *Proteus* isolates demonstrated the ability to form biofilms on glass surfaces. Congo red agar analysis revealed slime production in 28% of the isolates, indicating strong biofilm-forming potential. Safranin staining stratified *Proteus* biofilm production into strong (28%), moderate (30%), and weak (42%) categories. Analysis of the distribution of strong biofilm-producing *Proteus* isolates showed that 71.4% were recovered from urine samples, with stool and hand swab samples accounting for 14.3% each. No strong biofilm producers were observed in blood-derived isolates. Among moderate biofilm producers, urine samples exhibited the highest prevalence (86.7%) (Figure 1).

For MDR *Aeromonas* isolates, 37.5% showed positive slime production on CRA. The safranin assay similarly classified 37.5% of isolates as strong biofilm producers, 37.5% as moderate, and 25% as weak biofilm producers. All *Aeromonas* isolates identified as strong biofilm producers were recovered from hand swab samples (100%).

Moderate biofilm-producing *Aeromonas* were also predominantly isolated from hand swabs (66.7%) (Figure 2).

Antibiofilm formation activities of *P. umbellatum* leaf, stem bark and root fractions

Spectrophotometric analysis of antibiofilm activity showed that all root fractions inhibited ≥50% biofilm formation by multidrug-resistant (MDR) *Proteus* and *Aeromonas* strains. Stem bark fractions F1, F2, F4, and F5 (Figure 3), as well as leaf fractions F2, F3, and F4 (Figure 4), exhibited ≥50% inhibition against MDR *Proteus* isolates. Root fractions, especially the less-polar F1 and F2, exhibit consistent and potent antibiofilm activity against multidrug-resistant *Proteus* isolates (Figure 5).

Notably, all stem bark (Figure 6), leaf (Figure 7) and roots (Figure 8) fraction F4 achieved ≥50% inhibition against MDR *Aeromonas* isolates.

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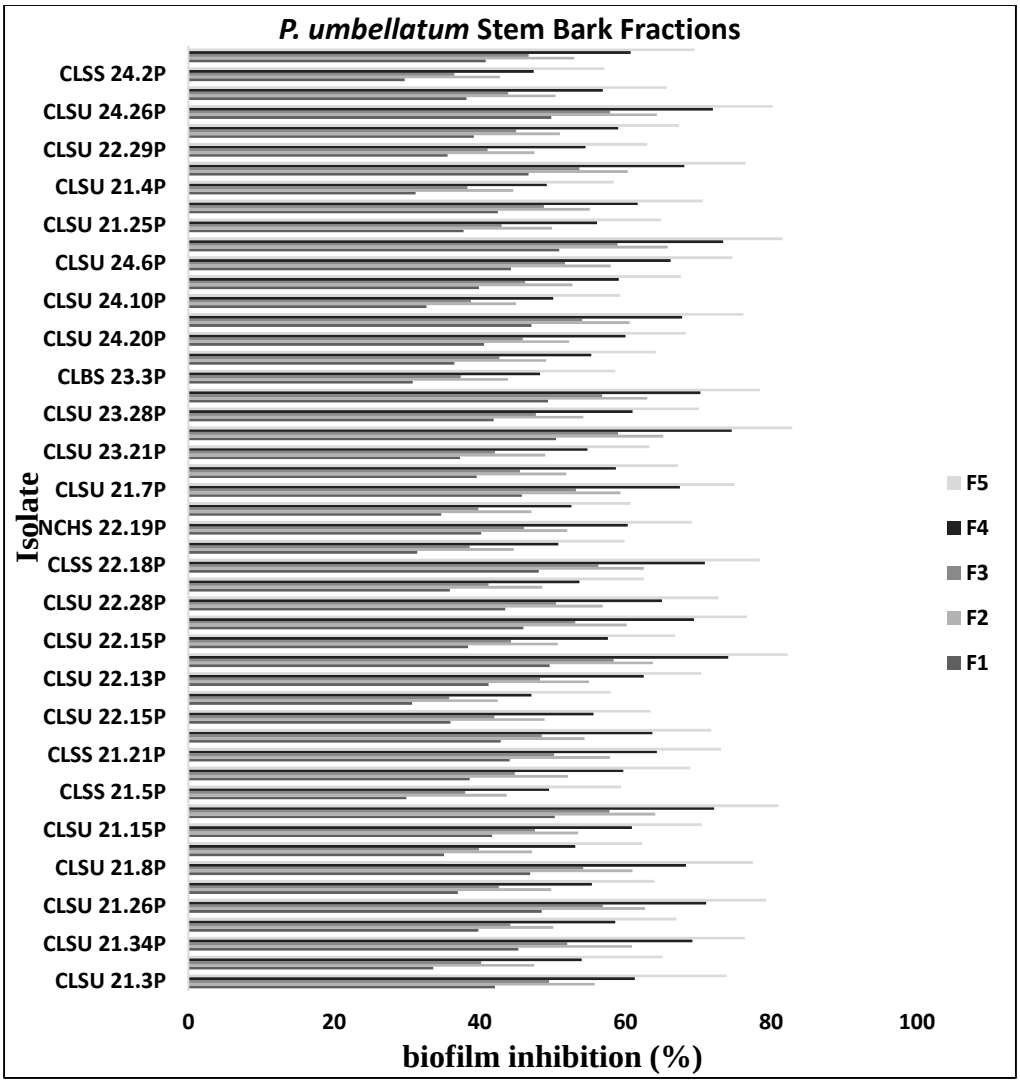

Figure 3: Antibiofilm activities of *P. umbellatum* stem bark fractions on MDR *Proteus* isolates

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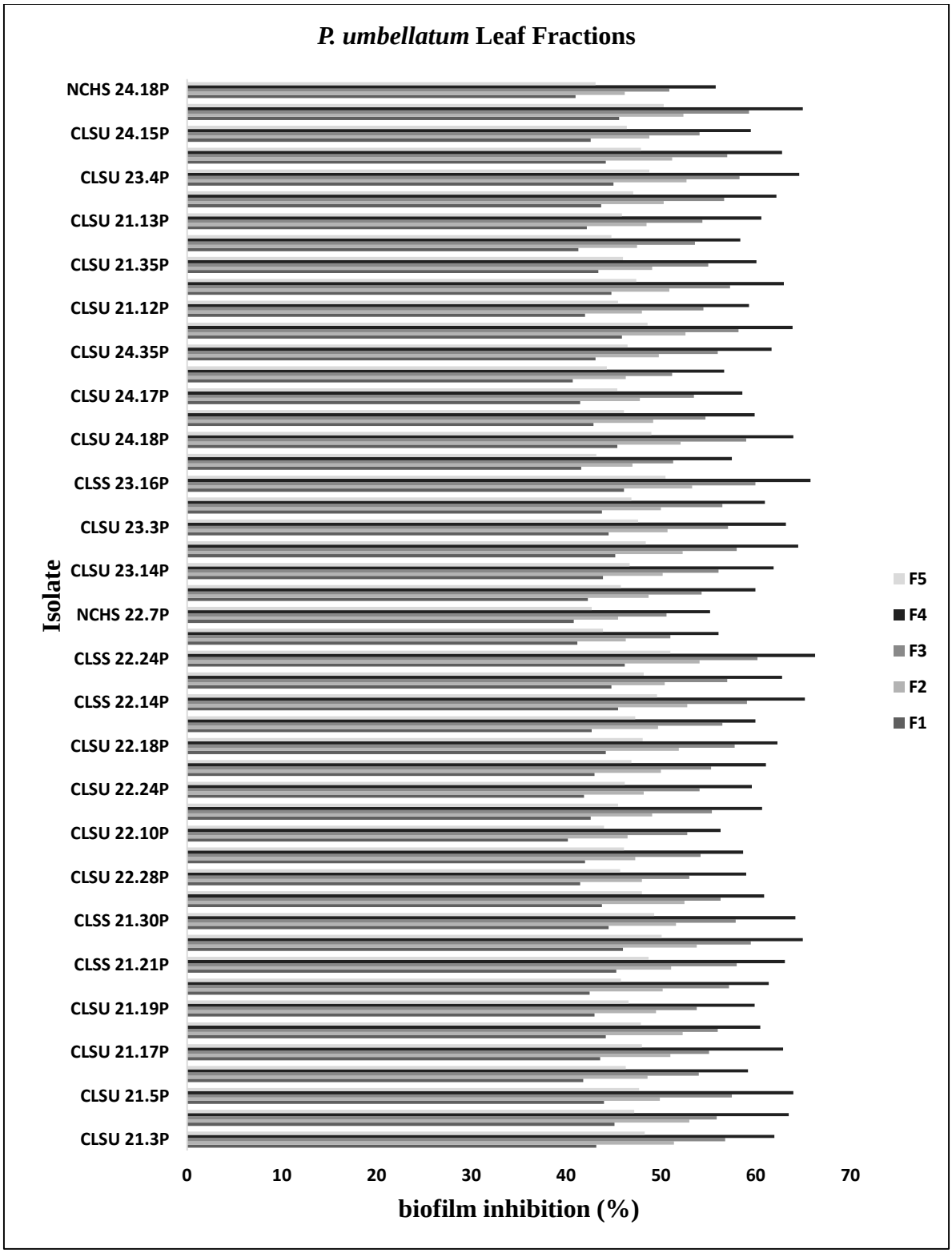

Figure 4: Antibiofilm activities of *P. umbellatum* Leaf fractions on MDR *Proteus* isolates

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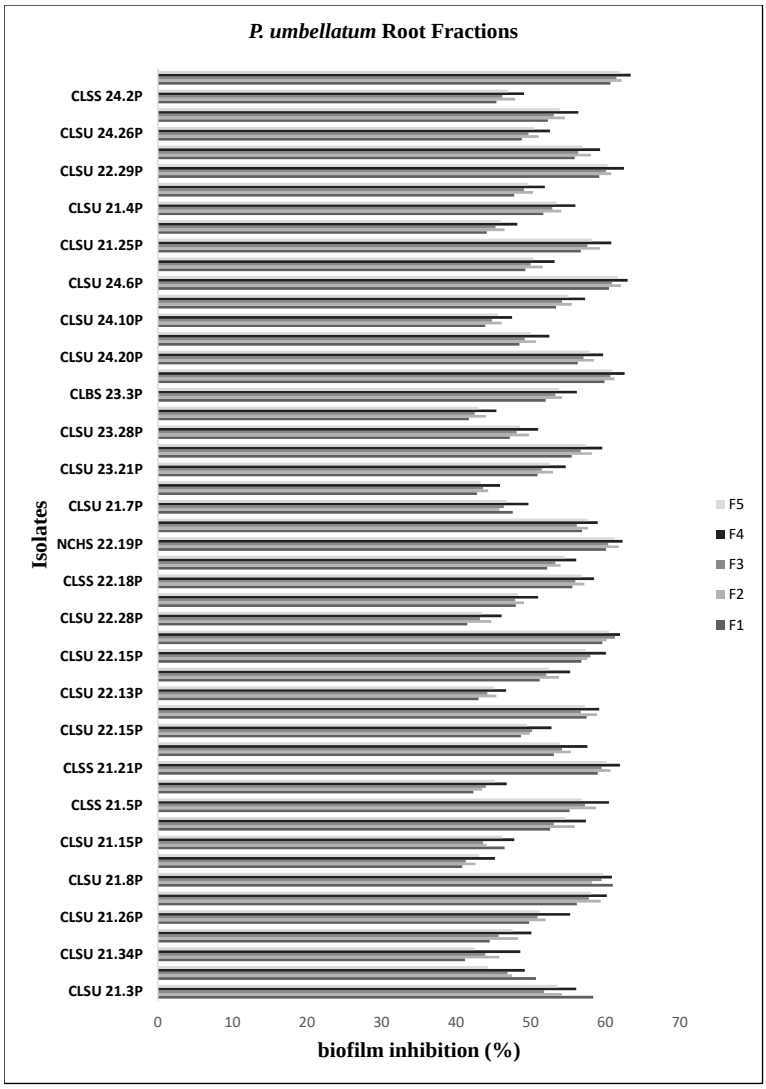

Figure 5: Antibiofilm activities of *P. umbellatum* Root fractions on MDR *Proteus* isolates

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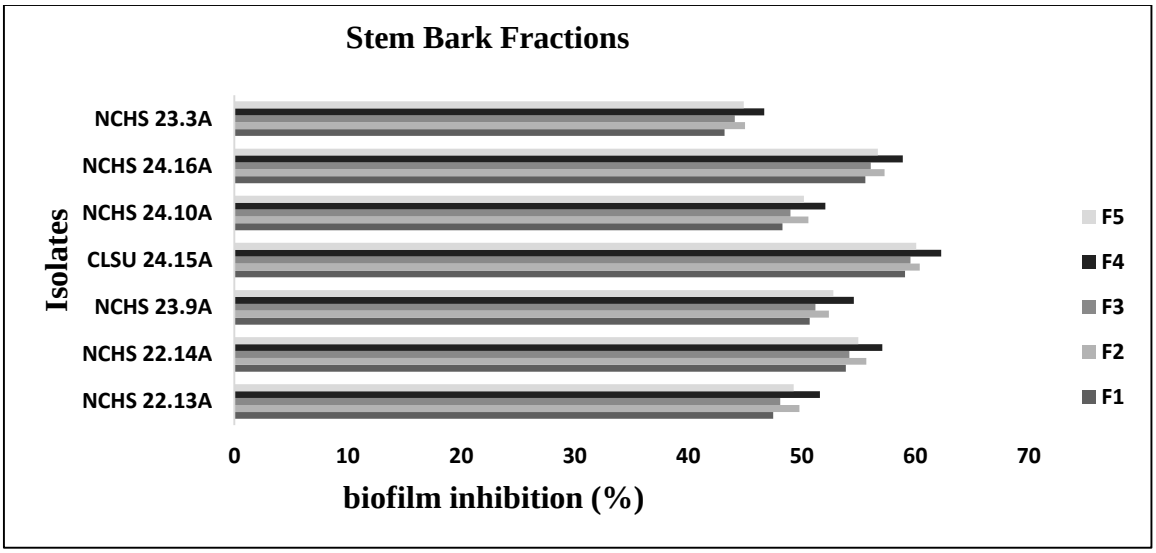

Figure 6: Antibiofilm activities of *P. umbellatum* stem bark fractions on MDR *Aeromonas* isolates

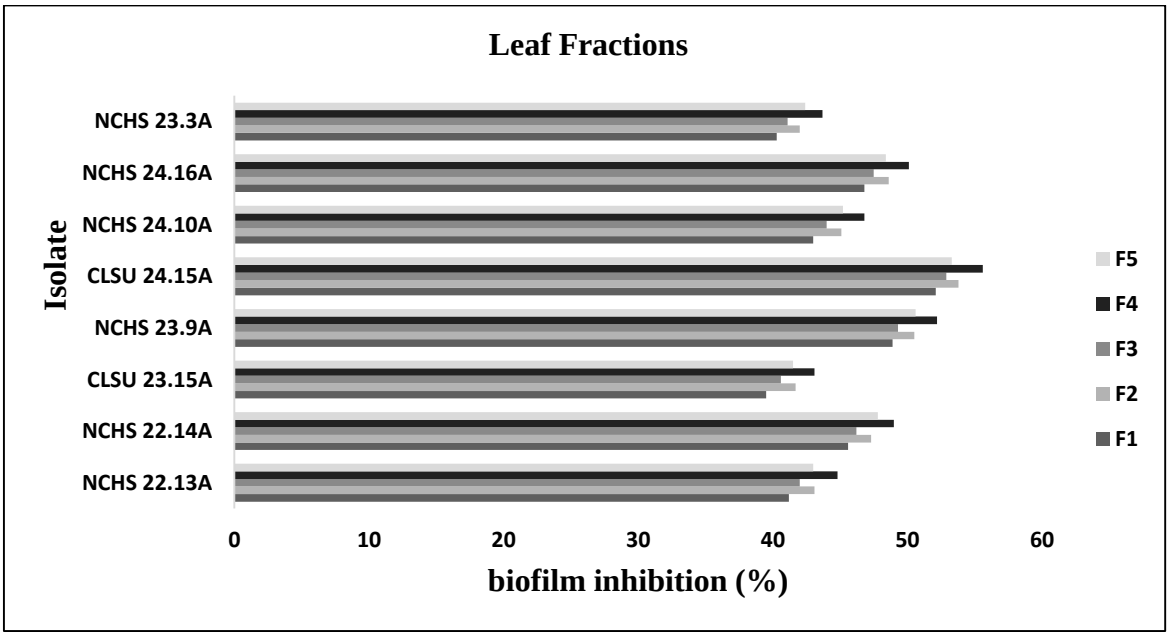

Figure 7: Antibiofilm activities of *P. umbellatum* leaf fractions on MDR *Aeromonas* isolates

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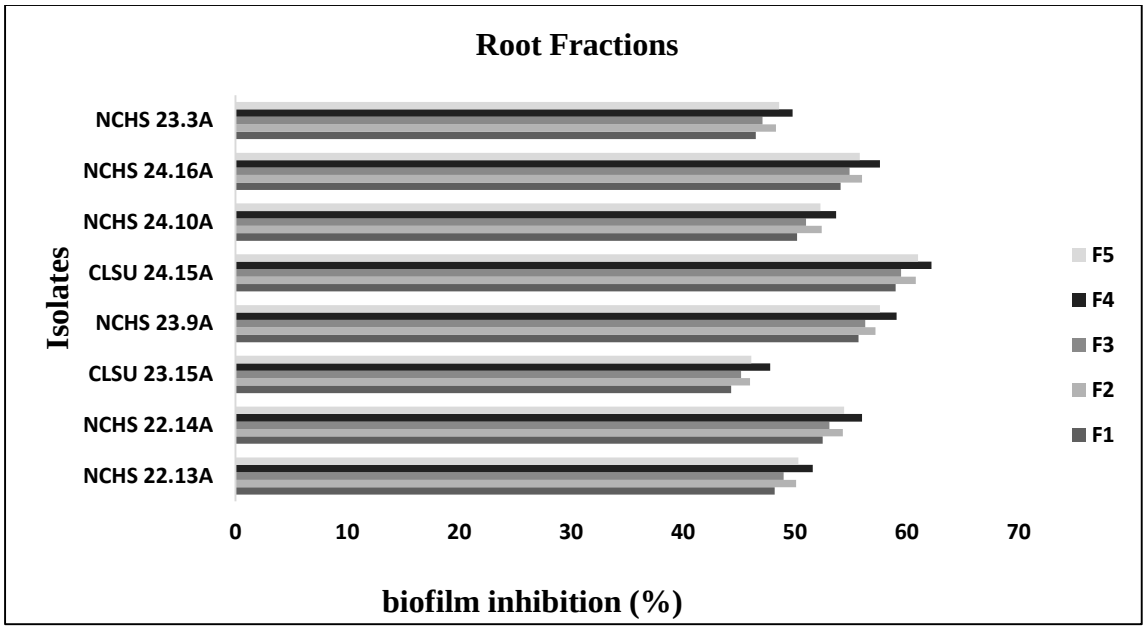

Figure 8: Antibiofilm activities of *P. umbellatum* Root fractions on MDR *Aeromonas* isolates

Discussion

This comprehensive study provides critical insights into the growing threat of multidrug-resistant (MDR) pathogens, specifically *Proteus* and *Aeromonas* species, and evaluates potential plant-based alternatives to combat these infections. The findings reveal concerning patterns of antimicrobial resistance, robust biofilm formation capabilities, and promising antimicrobial properties of plant extracts, particularly from *Piper umbellatum*. This investigation into the prevalence and distribution patterns of MDR *Proteus* and *Aeromonas* species across various clinical samples reveals distinct ecological niches for these pathogens. *Proteus* species were predominantly isolated from urine samples (59.1%), followed by stool (27.0%), hand swabs (9.6%), and blood samples (4.3%). This distribution strongly correlates with the established role of *Proteus* in urinary tract infections (UTIs), as supported by numerous clinical studies (Armbruster, *et al.* 2012; Jacobsen, *et al.* 2008; Różańska, *et al.* 2017).

In contrast, *Aeromonas* species exhibited a different distribution pattern, being mainly recovered from hand swabs (55.6%), and followed by urine (27.8%) and stool (16.7%), with no isolates detected in blood samples. These findings align with those reported by El-Hawary *et al.* (2024), who identified *Aeromonas* in both fish and human sources in Egypt, emphasizing their zoonotic potential and environmental adaptability.

The distinctive distribution patterns observed in this study reflect the specific pathogenic mechanisms and host tropism of these bacterial species, providing valuable epidemiological insights for clinical management strategies.

Biochemical profiling revealed significant biotype-specific variations with 43.8% of tests showing differences for *Proteus* species and 37.5% for *Aeromonas* species. Key differentiating tests included indole production, urease activity, saccharose fermentation, and ornithine decarboxylase for *Proteus*, while mannitol and rhamnose fermentation and the Voges-Proskauer reaction were critical for *Aeromonas* identification. These biochemical variabilities are consistent with previous studies, particularly the work of Palumbo *et al.* (2023), who emphasized the importance of combining biochemical and molecular identification techniques for accurate characterization of *Aeromonas* species from diarrheal patients. The observed biochemical heterogeneity among isolates from different sources underscores the phenotypic plasticity of these pathogens, which may contribute to their adaptability across diverse ecological niches and potentially influence their virulence and resistance profiles.

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The qualitative phytochemical screening of *Piper umbellatum* fractions revealed distinct tissue-specific flavonoid distribution, with consistent presence across all root extracts (F1–F5).

Limited occurrence in stem bark (notably in F4 and F5), and high variability in leaf fractions, particularly pronounced in F3 and absent in F5. This distribution reflects the biochemical diversity influenced by genetic and environmental factors. Quercetin was identified as the primary flavonoid contributing to antioxidant activity, evidenced by its abundance in leaf (F5), stem bark (F4 and F5), and root (F1) extracts. Kaempferol showed a weak correlation with antioxidant effects, suggesting alternative roles such as antimicrobial or anti-inflammatory activity (Khan *et al.*, 2024). Rutin exhibited moderate antioxidant synergy, especially in leaf fractions (Inngjerdingen *et al.*, 2017). The flavonoid profile of *P. umbellatum* parallels that of other ethnomedicinal plants with documented efficacy against multidrug-resistant pathogens, including ESBL-producing *E. coli* and *Klebsiella pneumoniae*, suggesting promising antimicrobial potential that warrants further quantitative and bioactivity-focused investigations (Khan *et al.*, 2024; Majeed *et al.*, 2024).

This analysis of biofilm formation revealed that 28% of *Proteus* isolates and 37.5% of *Aeromonas* isolates were strong biofilm producers, with moderate and weak producers accounting for 30% and 42% in *Proteus*, and 37.5% and 25% in *Aeromonas*, respectively. Notably, strong *Proteus* biofilm producers were primarily recovered from urine samples (71.4%), whereas strong *Aeromonas* biofilm producers were exclusively isolated from hand swabs (100%). This marked distribution pattern suggests a relationship between the source of isolation and biofilm-forming capacity, which may reflect adaptive responses to specific environmental pressures. These findings align with studies by Zhou *et al.* (2023) and Kim *et al.* (2024), who reported strong biofilm formation in approximately 30-40% of clinical MDR isolates. Furthermore, our observations are consistent with reports by Martínez-García *et al.* (2022) and Singh *et al.* (2025), which demonstrated that isolates from urine and skin, respectively, exhibit enhanced biofilm formation capabilities9. The biofilm-forming capacities of these pathogens are particularly concerning as biofilms contribute significantly to bacterial virulence, enhance survival against host immune responses, and reduce antibiotic efficacy, ultimately leading to persistent infections and treatment failures.

In light of the escalating antimicrobial resistance crisis, this study into plant-based alternatives yielded promising results. Phytochemical analysis of *Piper umbellatum* revealed abundant alkaloids, flavonoids, tannins, and phenolic acids across leaf, stem bark, and root fractions. These bioactive compounds have well-documented antioxidant and antimicrobial properties, as corroborated by recent studies (Zhang *et al.*, 2023; Oboh *et al.*, 2024; Miranda, *et al.*, 2024; Riaz, *et al.*, 2023; Kumar, *et al.*, 2024).

The antibiofilm activities of *P. umbellatum* fractions varied across plant parts, with the root fraction exhibiting particularly potent bactericidal activity against both MDR *Proteus* and *Aeromonas* isolates. This observation is consistent with reports that antimicrobial potency often correlates with phytochemical richness, as noted by Quralleh *et al.* (2018) and Okwu *et al.* (2019).

Observations in this study also demonstrated that *P. umbellatum* root fractions significantly reduced biofilm formation by interfering with early development stages, possibly through suppression of bacterial quorum sensing pathways or cell adhesion processes. These results align with previous studies on similar medicinal plants, including licorice root, sage, purple coneflower, and oregano (Sharma *et al.*, 2010; Wijesundara *et al.*, 2017), which have shown efficacy against various pathogens. The minimal cytotoxicity of these plant extracts on human cells, as reported in previous assays, further enhances their potential as complementary agents in managing MDR infections.

Conclusion

This study underscores the escalating threat of multidrug-resistant *Proteus* and *Aeromonas* species, characterized by high prevalence, distinct ecological niches, and robust biofilm formation, particularly in urine (59.1% *Proteus*) and hand swab (55.6% *Aeromonas*) isolates. Biochemical variability and strong resistance profiles highlight the need for advanced diagnostics and surveillance. *Piper umbellatum* extracts, rich in flavonoids like quercetin, exhibit potent antimicrobial and antibiofilm properties, offering promising alternatives against MDR pathogens. Integrating traditional microbiological approaches with ethnopharmacological innovations provides a robust framework to combat antimicrobial resistance, emphasizing the urgency of developing targeted therapies and stewardship programs.

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