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Original Research Article

Harnessing baicalein to mitigate antibiotic resistance: resensitizing norfloxacin against urinary multidrug-resistant *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

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ABSTRACT

Background: According to the World Health Organization (WHO) Priority Pathogen List — 2024, multidrug-resistant pathogens will emerge and spread rapidly in the coming years. This has to be countered by the urgent development of novel antibiotics for their effective management. Restoring and enhancing the potency of existing antibiotics by combining with bioactive compounds is a novel strategy to be further studied. The purpose of our study is to determine the synergistic effect of one of the active ingredients from Chinese skullcap, baicalein with norfloxacin on multi-drug resistant uropathogens.
Methods: The present cross-sectional study was undertaken at School of Medical Education (SME), Kerala, India, during the period of November 2023 to November 2024. Based on the calculated FICI values, results were interpreted as Synergy (FICI≤0.5), Additive (0.5<FICI≤1), Indifferent (1<FICI≤4), Antagonistic (FICI>4). The data were analysed using Microsoft Excel software (Windows 2010). Chi-square test was performed using IBM statistical package for the social sciences (SPSS) Statistics 24 to evaluate significant differences (p value <0.05) among the parameters examined in this study.
Results: A total of 150 drug-resistant strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa* were isolated from urinary tract infections (UTIs). Resistance to norfloxacin was observed in 64% of *E. coli*, 68% of *K. pneumoniae*, and 74% of *P. aeruginosa* isolates. Notably, baicalein resensitized 56.3% of *E. coli*, 70.6% of *K. pneumoniae*, and 75.7% of *P. aeruginosa* strains to norfloxacin, as determined by the checkerboard assay and these findings were statistically significant.
Conclusions: This study emphasizes the need for ongoing surveillance of antimicrobial resistance in common urinary pathogens and also suggests that natural compounds like baicalein may resensitize existing antibiotics like norfloxacin against resistant strains, warranting further research into the underlying mechanisms and potential clinical applications.

Keywords: UTI, *E. coli, K. pneumoniae, P. aeruginosa*, Norfloxacin, Baicalein, Antimicrobial resistance

INTRODUCTION

Urinary tract infections (UTI) are prevalent in the community and were treated with either β-lactams, quinolones, or trimethoprim-sulfamethoxazole. However, the emergence of multidrug-resistant uropathogens often harbouring extended-spectrum β-lactamases (ESBL), as well as a variety of mutations has reduced susceptibility to

these drugs in the community settings.[1] Multidrug-resistant (MDR) and extensively drug resistant (XDR) strains display cross-resistance to a number of structurally unrelated antimicrobial agents.[2]

Exploring traditional medicinal plant extracts as therapeutic agents offers a promising approach to combat multidrug-resistant uropathogens. Baicalein (5,6,7-trihydroxyflavone) is one of the flavonoids isolated from the root of *Scutellaria baicalensis* Georgi, a well-known medicinal plant used in various ancient traditional medicines as well as a culinary ingredient. It is known to exhibit anti-bacterial, anti-inflammatory, anti-cancerous and antioxidant activities; and has, therefore, been widely used in traditional medicine. Notably, baicalein enhanced the efficacy of tetracycline and β-lactams against two clinically relevant MRSA isolates such as OM481 and OM584.[3] Baicalein offering potential therapeutic options by enhancing gentamicin's effectiveness against vancomycin-resistant Enterococci.[4] Significantly, baicalein showed synergistic effect against MRSA strains with NorA overexpression on combination with ciprofloxacin.[5]

E. coli, *K. pneumoniae*, and *P. aeruginosa* are common Gram-negative bacterial pathogens that frequently cause UTI. Norfloxacin, a quinoline carboxylic acid is a recommended empirical antimicrobial agent for the management of UTIs.[6] The emergence of MDR and XDR strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa* in UTIs threaten community health and necessitate effective management strategies. Therefore, innovative approaches are necessary to mitigate this challenge through either the discovery of novel antimicrobial agents, or synergistic combinations of phytochemicals with existing antibiotics. Our study aimed to effectively introduce a bioactive compound, Baicalein in the management of UTI, thereby we can refurbish resistant antimicrobials, and it will supplement the management of multidrug-resistant uropathogens.

METHODS

The present cross-sectional study was undertaken at School of Medical Education (SME), Kerala, India, during the period of November 2023 to November 2024.

Reagents and bioactive compound

Norfloxacin (Analytical standard ≥98%, TLC) and Baicalein (≥98%, HPLC) were purchased from Sigma-Aldrich, India and Sigma-Aldrich, USA respectively. Mueller Hinton Broth no. 2 control cations (CAMHB), Mueller Hinton agar, dimethyl sulfoxide (DMSO), sodium hydroxide pellets, 2,3,5-triphenyl tetrazolium chloride (TTC) solution 1% were purchased from HiMedia Laboratories Private Limited, India. 96 Well 'U' bottom polypropylene microtitre plates were purchased from Tarsons Products Limited, India.

Following antimicrobial agents were bought from HiMedia Laboratories Private Limited, India. Amikacin (30 µg), Ciprofloxacin (5 µg), Norfloxacin (10 µg), Imipenem (10 µg), Cefepime (30 µg), Piperacillin-tazobactam (100/10 µg), Aztreonam (30 µg), Gentamicin (10 µg), Amoxicillin-clavulanate (10/20 µg), Cefoxitin (30 µg), Cefuroxime (30 µg), Cefixime (5 µg), Ampicillin (10 µg), Tetracycline (30 µg), Ceftazidime (30 µg), Ceftazidime-clavulanate, Cefotaxime (30 µg), and Cefotaxime-clavulanate.

Collection and identification of urinary isolates of E. coli, K. pneumoniae and P. aeruginosa

A total of 150 bacterial isolates (50 each of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*) exhibiting significant bacteriuria ($\geq 10^5$ CFU/ml) were obtained from St. Mary's Hospital, Thodupuzha, Kerala, India. The isolates were identified through conventional biochemical methods. Quality control strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 were stored in laboratory until further experiments were performed. Antimicrobial susceptibility was performed by the Kirby-Bauer disk diffusion as prescribed by CLSI M02-A13 conditions and analysed using interpretive standards of CLSI M100-S34 and are categorized into MDR, XDR and non MDR groups based on CDC/ECDC guidelines. Bacterial isolates were categorized based on their antimicrobial resistance profiles. MDR isolates were defined as those exhibiting non-susceptibility to at least one agent in three or more antimicrobial categories. In contrast, isolates that were non-susceptible to at least one agent in all but two or fewer antimicrobial categories, remaining susceptible to only one or two categories, were classified as XDR.[7-9]

Determination of the minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent that inhibit visible growth of bacteria. Norfloxacin was mixed in 1/2 volume of water, and then 0.1 mol/l NaOH was added dropwise until it was completely dissolved and finished diluting the final stock solution by using water.[8] Baicalein was dissolved in 0.1% dimethyl sulfoxide (DMSO). Test inoculum was prepared in sterile CAMHB and incubated at 35°C. MIC of individual agents were determined for all the quality control strains as prescribed by CLSI M07-11th edition.[10] All experiments were performed in triplicate and the mean was used to ensure reproducibility. Growth in each well were detected by chromogenic method using TTC solution.

Synergy testing of Baicalein with Norfloxacin using checkerboard dilution method

Checkerboard assay is a two-dimensional, two-agent broth microdilution method performed in a 96 well U-bottom microtiter plate to evaluate combinations of antimicrobial

agents against microorganisms. MICs of baicalein and norfloxacin for test isolates were determined individually.

Concentrations ranging from four to eight times the expected MIC to at least 1/8 to 1/16 times the expected MIC were included in the final panel in order to observe the occurrence and magnitude of synergism or antagonism.

Diagrammatic representation of 96-well microtitre plate is given in Figure 1.

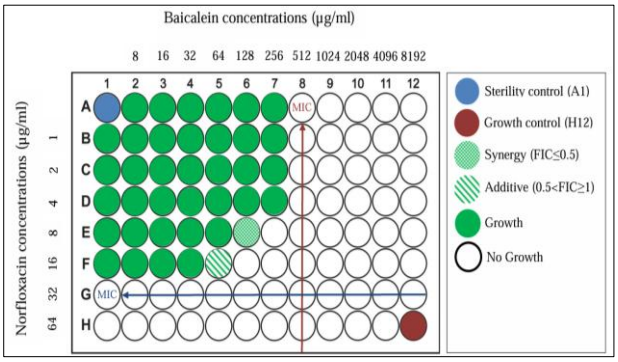

Figure 1: 50 µl of CAMHB was added to wells A2–A12 and B1–H1, with 100 µl in wells A1 and H12, and serial two-fold increasing concentrations of baicalein (50 µl) and norfloxacin (50 µl) were added column-wise and row-wise, respectively, excluding well H12.

Calculation of fractional inhibitory concentration index (FICI)

For each combination interaction, fractional inhibitory concentration (FIC) of each agent was calculated as follows.

$$FIC\ of\ Baicalein = \frac{MIC\ of\ Baicalein\ in\ combination}{MIC\ of\ Baicalein\ alone}$$

$$FIC\ of\ Norfloxacin = \frac{MIC\ of\ Norfloxacin\ in\ combination}{MIC\ of\ Norfloxacin\ alone}$$

$$FICI = FIC\ of\ Baicalein + FIC\ of\ Norfloxacin$$

Interpretation of results

Based on the calculated FICI values, results were interpreted as synergy (FICI≤0.5), additive (0.5<FICI≤1), indifferent (1<FICI≤4), and antagonistic (FICI>4).

Statistical analysis

The data were analysed using Microsoft Excel software (Windows 2010). Descriptive statistics were employed to summarize the data, and the Chi-square test was performed using IBM statistical package for the social sciences (SPSS) statistics 24 to evaluate significant differences (p value <0.05) among the parameters examined in this study.

RESULTS

A total of 150 bacterial isolates (50 each of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*) exhibiting significant bacteriuria (≥10^5 CFU/ml) were obtained from St. Mary's Hospital, Thodupuzha, Kerala, India during the study period spanning from November 2023 to November 2024.

Antibiotic susceptibility testing revealed varying degrees of sensitivity and resistance among *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates. In *E. coli*, gentamicin and cefoxitin exhibited high sensitivity rates of 70% (n=38), whereas ampicillin showed a high percentage of resistance (86%, n=43). Notably, 66% (n=33) of *E. coli* isolates were identified as ESBL producers. In *K. pneumoniae*, imipenem, meropenem, and aztreonam demonstrated high sensitivity rates of 96% (n=48). Conversely, high resistance rates were observed against norfloxacin (68%, n=34). Additionally, 14% (n=7) of *K. pneumoniae* isolates were identified as ESBL producers. In *P. aeruginosa*, Piperacillin/Tazobactam showed a high sensitivity rate of 76% (n=38). In contrast, norfloxacin exhibited a high percentage of resistance, with 74% (n=37) of isolates showing resistance. The comprehensive antimicrobial susceptibility profiles of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* are summarized in Figure 2.

The antimicrobial susceptibility profiles of the 50 isolates of each bacterial species were determined. The results indicated that, among the *E. coli* isolates, 30% (n=15) were classified as non-MDR, 66% (n=33) as MDR, and 4% (n=2) as XDR. Similarly, the *K. pneumoniae* isolates exhibited resistance patterns of 48% (n=24) non-MDR, 46% (n=23) MDR, and 6% (n=3) XDR. *P. aeruginosa* isolates demonstrated resistance patterns of 46% (n=23) non-MDR, 44% (n=22) MDR, and 10% (n=5) XDR, as depicted in Figure 3.

The susceptibility of urinary isolates to norfloxacin was evaluated, revealing that 36% (n=18) of *E. coli*, 32% (n=16) of *K. pneumoniae*, and 26% (n=13) of *P. aeruginosa* isolates were sensitive to norfloxacin. Conversely, 64% (n=32), 68% (n=34), and 74% (n=37) of the respective isolates exhibited resistance to norfloxacin.

The MICs of norfloxacin and baicalein were determined against the standard strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853. The results indicated that norfloxacin exhibited a uniform MIC of 4 µg/ml across all three strains. In contrast, baicalein displayed varying MICs: 512 µg/ml for *E. coli* and *P. aeruginosa*, and 1024 µg/ml for *K. pneumoniae*. When combined, norfloxacin and baicalein demonstrated synergistic effects, resulting in reduced MICs. The MICs of norfloxacin in combination with baicalein were uniformly detected as 1 µg/ml across all three standard strains. Conversely, the MICs of baicalein in combination with norfloxacin varied among the strains: 64 µg/ml for *E. coli*, 256 µg/ml for *K. pneumoniae*, and 128 µg/ml for *P. aeruginosa.*

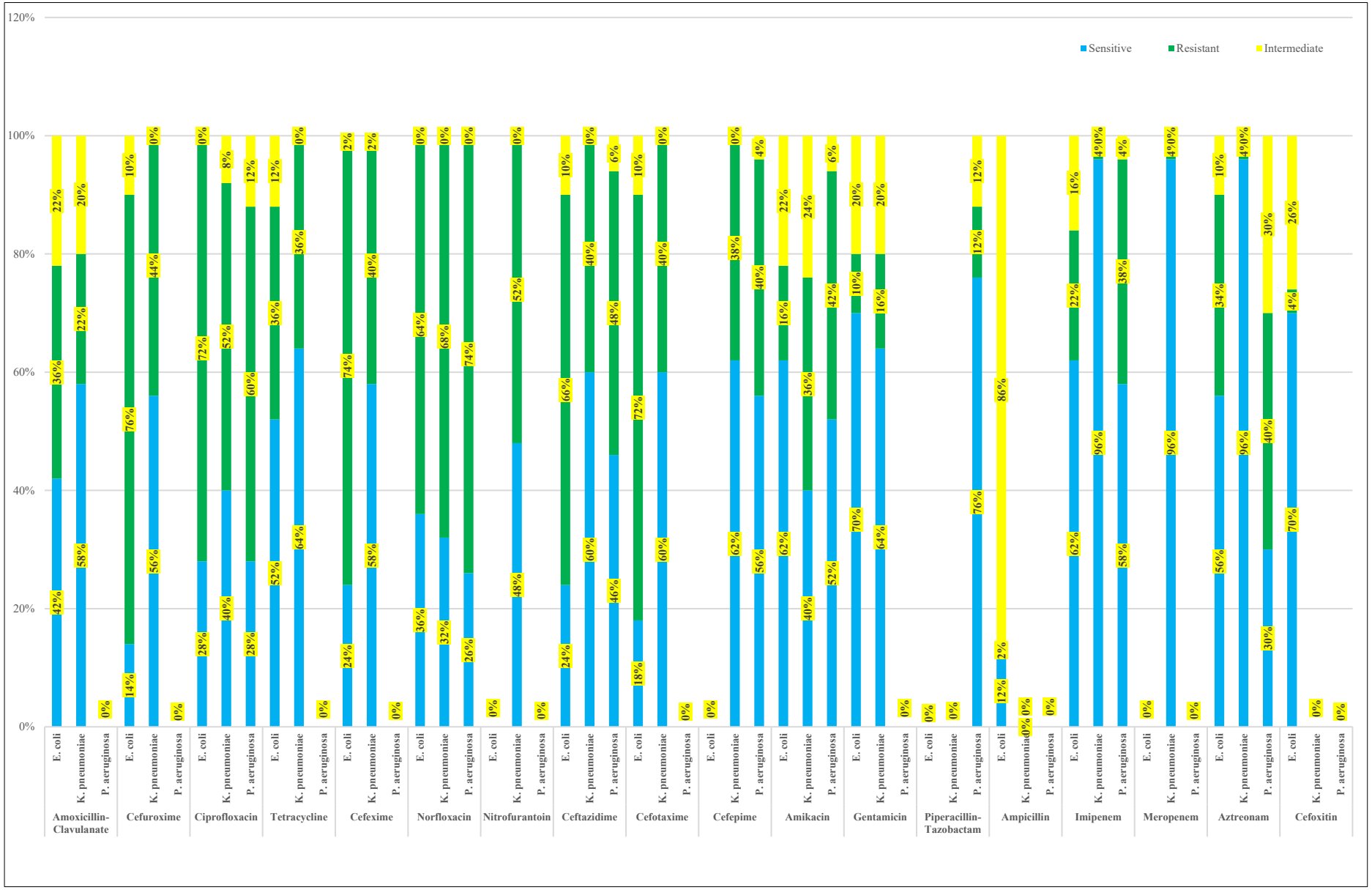

Figure 2: The comprehensive antimicrobial susceptibility profiles of *E. coli, K. pneumoniae*, and *P. aeruginosa*.

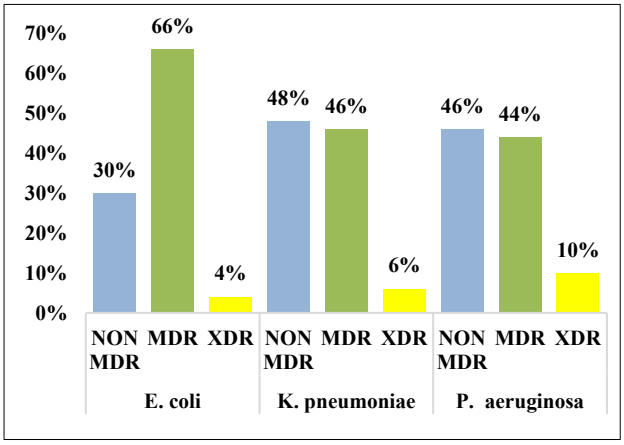

Figure 3: Distribution of non- MDR, MDR and XDR strains among isolates of *E. coli, K. pneumoniae* and *P. aeruginosa.*

Comparison of the combined antibacterial activity of norfloxacin and baicalein against E. coli, K. pneumoniae, and P. aeruginosa

Our investigation revealed pronounced synergistic effects between baicalein and norfloxacin against a substantial proportion of bacterial isolates, including 72% (n=36) of *E. coli*, 80% (n=40) of *K. pneumoniae*, and 82% (n=41) of *P. aeruginosa*, whereas additive interactions were observed in the remaining 28% (n=14), 20% (n=10), and 18% (n=9) of isolates, respectively as illustrated in Figure 4.

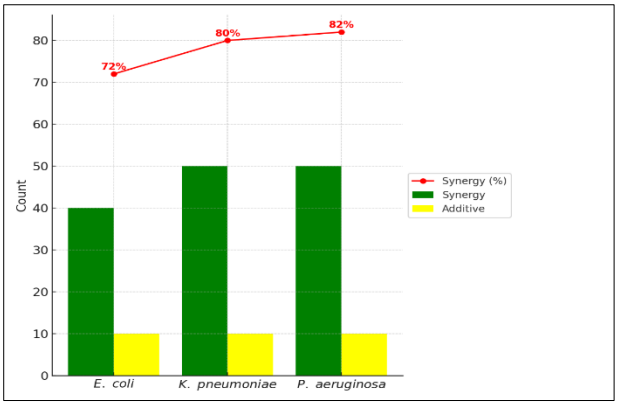

Figure 4: Comparison of the combined antibacterial activity of norfloxacin and baicalein against *E. coli, K. pneumoniae*, and *P. aeruginosa.*

A cross-tabulation analysis using the Pearson Chi-square test was performed to investigate potential correlations between the distribution of MDR strains of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* and the combined antibacterial activity of norfloxacin and baicalein. The results of the analysis revealed statistically significant associations between the variables for all three species, with Pearson Chi-square values of 8.44 (p=0.015), 9.41 (p=0.009), and 12.31 (p=0.002) for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, respectively, indicating significant associations between the variables**.**

The synergistic effects of baicalein and norfloxacin on MDR strains of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* were investigated. The results demonstrated that the combination of baicalein and norfloxacin resensitized a significant proportion of MDR isolates to norfloxacin, with synergy observed in 60.60% (n=20) of *E. coli*, 69.60% (n=16) of *K. pneumoniae*, and 72.70% (n=16) of *P. aeruginosa* isolates. Conversely, additive effects were observed in 39.40% (n=13), 30.40% (n=7), and 27.30% (n=6) of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates, respectively, as depicted in Figure 5.

Furthermore, the synergistic effects of baicalein and norfloxacin on XDR strains of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* were also evaluated. The results demonstrated that the combination of baicalein and norfloxacin exhibited synergy in a subset of XDR isolates, with 50% (n=1) of *E. coli*, 33.30% (n=1) of *K. pneumoniae*, and 40% (n=2) of *P. aeruginosa* XDR strains being resensitized to norfloxacin. Conversely, the remaining XDR strains, comprising 50% (n=1) of *E. coli*, 66.70% (n=2) of *K. pneumoniae*, and 60% (n=3) of *P. aeruginosa*, exhibited an additive effect. Notably, synergistic interactions between baicalein and norfloxacin were observed in all non-MDR isolates of *E. coli*, *K. pneumoniae* and *P. aeruginosa.*

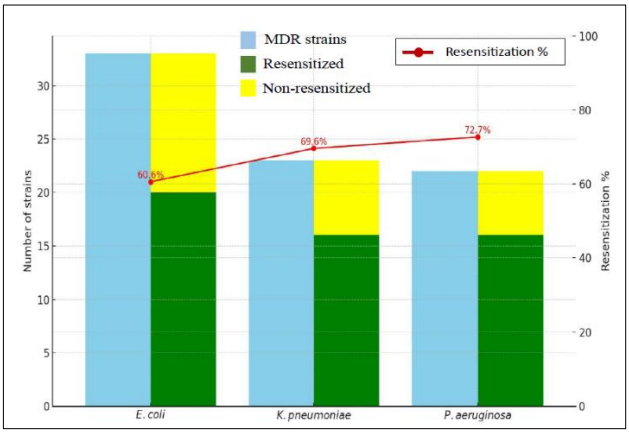

Figure 5: The synergistic effects of baicalein and norfloxacin on MDR strains of *E. coli, K. pneumoniae*, and *P. aeruginosa*.

A cross-tabulation analysis employing the Pearson Chi-square test was conducted to investigate the relationship between the norfloxacin sensitivity pattern of urinary isolates of *E. coli*, *K. pneumoniae* and *P. aeruginosa* and the combined antibacterial activity of norfloxacin and baicalein. The results showed statistically significant correlations, with Pearson Chi-square values of 10.94, 5.88, and 3.86 for *E. coli*, *K. pneumoniae* and *P. aeruginosa* respectively. The corresponding p values were 0.001, 0.015, and 0.05 indicating significant associations between the variables, as shown in Table 2.

Table 2: Comparison of Norfloxacin resistant strains of *E. coli, K. pneumoniae* and *P. aeruginosa* and combined antibacterial activity of norfloxacin and baicalein by Pearson Chi-Square test.

Norfloxacin resistant strains	Combined antibacterial activity of norfloxacin and baicalein		Total	Chi-square Tests		
	Synergy	Additive		Pearson Chi-square value	df	Asymp. sig. (2- sided)
E. coli	56.30% (n=18)	43.80% (n=14)	100% (n=32)	10.94	1	0.001
K. pneumoniae	70.60% (n=24)	29.40% (n=10)	100% (n=34)	5.88	1	0.015
P. aeruginosa	75.70% (n=28)	24.30% (n=9)	100% (n=37)	3.86	1	0.05

Furthermore, the combination of baicalein and norfloxacin resensitized a substantial proportion of norfloxacin-resistant strains to norfloxacin. Specifically, 56.30% (n=18) of *E. coli*, 70.60% (n=24) of *K. pneumoniae*, and 75.70% (n=28) of *P. aeruginosa* norfloxacin-resistant strains were resensitized to norfloxacin when combined with baicalein, indicating a synergistic effect and remaining 43.80% (n=14) of *E. coli,* 29.40% (n=10) of *K. pneumoniae* and 24.30% (n=9) of *P. aeruginosa* norfloxacin-resistant strains showed additive effects as illustrated in Figure 6. Notably, synergistic interactions between baicalein and norfloxacin were observed in all norfloxacin-sensitive strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa.*

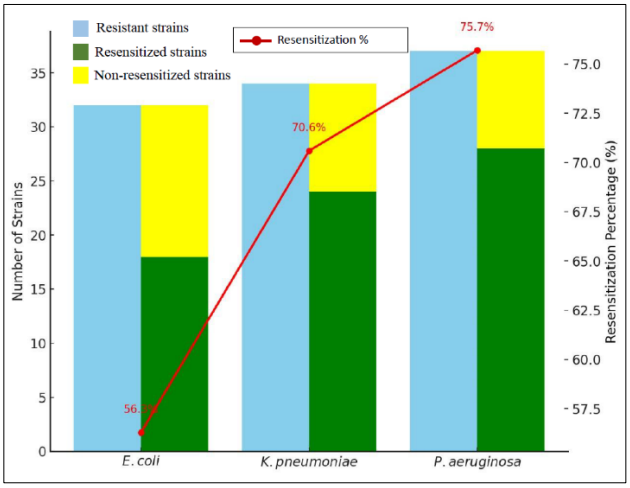

Figure 6: The synergistic effects of baicalein and norfloxacin on Norfloxacin resistant strains of *E. coli, K. pneumoniae*, and *P. aeruginosa*.

DISCUSSION

The present study provides critical insights into the prevalence of antimicrobial resistance among key bacterial pathogens implicated in UTIs. The investigation focused on three significant pathogens: *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, with a total of 150 urinary isolates analysed over a one-year period.

The antibiotic susceptibility testing revealed varied resistance patterns across the three bacterial species. Notably, *E. coli* showed high sensitivity to gentamicin (70%) which is in accordance with earlier studies from India, while ampicillin exhibited a striking resistance rate of 86%.[11,12] This aligns with global trends where *E. coli* has demonstrated increasing resistance to commonly used antibiotics. High sensitivity rates to carbapenems were observed for *K. pneumoniae* (96%), consistent with findings from a national multicentre study in India, and a study at a tertiary care centre, highlighting the importance of these antibiotics as a critical option for severe infections caused by resistant organisms.[13,14] In the case of *P. aeruginosa*, the sensitivity to Piperacillin/Tazobactam was relatively high at 76%, which is in parallel with study by Regha et al.[15] This underscores the need for continuous surveillance of antibiotic susceptibility patterns to guide empirical therapy effectively. The findings indicate a concerning trend in the emergence of ESBL-producing strains, particularly among *E. coli* isolates, with 66% identified as ESBL producers, aligning with similar research findings.[13] This high prevalence is particularly alarming, as ESBLs confer resistance to a broad range of beta-lactam antibiotics, thereby complicating the therapeutic management of infections caused by these pathogens. In contrast, only 14% of *K. pneumoniae* isolates were identified as ESBL producers, a result consistent with the study by Chander et al.[16] This discrepancy may reflect differing genetic backgrounds and resistance mechanisms between these species, as previously documented in the literature.

The classification of isolates into non-MDR, MDR, and XDR categories highlights a concerning prevalence of (MDR) strains. Specifically, 66% of *E. coli* isolates were categorized as MDR, with 4% identified as (XDR), consistent with earlier study.[17] Similarly, 46% of *K. pneumoniae* isolates were MDR, aligning with findings from another study, although the XDR rate was lower at 6%, as reported previously.[18,19] Notably, 44% of *P. aeruginosa* isolates were classified as MDR parallel with previous study, with 10% categorized as XDR, comparable to another study.[20,21]

The emergence of XDR strains represents a significant public health threat, as they exhibit resistance to most available antibiotics, critically limiting treatment options. In this study, the prevalence of norfloxacin-resistant *E. coli* (64%), *Klebsiella* (68%), and *Pseudomonas* (74%) was comparable to earlier studies respectively.[22-24]

This novel study, to the best of our knowledge, is the first to explore the synergistic antibacterial effects of baicalein and norfloxacin against the challenging resistant strains of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. The use of Pearson Chi-square tests provided robust statistical evidence supporting the associations between antibiotic susceptibility patterns and the combined antibacterial activity of norfloxacin and baicalein across all three species studied. The statistically significant p-values reinforce the relevance of these findings in understanding the dynamics between antibiotic resistance and potential therapeutic strategies. The results demonstrated significant synergy, particularly in resensitizing MDR strains to norfloxacin; for instance, synergy was observed in 60.60% of *E. coli* MDR isolates and even higher percentages for *K. pneumoniae* (69.60%) and *P. aeruginosa* (72.70%). These findings suggest that baicalein may serve as a valuable adjunctive therapy to overcome resistance mechanisms. The study also reported that the combination therapy effectively resensitized a substantial proportion of norfloxacin-resistant strains: 56.20% of resistant *E. coli*, 70.60% of resistant *K. pneumoniae*, and 75.70% of resistant *P. aeruginosa*.

Studies employing baicalein in combination with antibiotics have demonstrated synergistic effects against Gram-negative bacilli. Wang et al reported that baicalein resensitized doxycycline against MDR *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and a previously constructed XDR strain, *Acinetobacter baumannii* AB43Δcrispr-cas.[25] Similarly, Guran et al found that combining meropenem with baicalein exhibited significant synergism against XDR and pandrug-resistant (PDR) *A. baumannii*.[26] Cai et al demonstrated that baicalein enhanced the efficacy of cefotaxime against CTX-M-1 positive *K. pneumoniae*.[27] Moreover, other studies have reported the synergistic effects of baicalein against Gram-positive organisms. For instance, Chan et al observed that baicalein, when combined with ciprofloxacin, showed synergistic activity against *NorA*-overexpressing methicillin-resistant *Staphylococcus aureus* (MRSA).[5]

Despite the study demonstrated the potential of baicalein to resensitize norfloxacin-resistant Gram-negative uropathogens, it is not without limitations. The underlying mechanism of resensitization remains unclear. Additionally, the findings are limited to three common uropathogens with a relatively modest sample size of 50 strains each. A larger sample size with multiple species would enhance the statistical power and generalizability of the results. Further *in-vitro* studies using the combination of baicalein and norfloxacin by using time-kill curve analysis, are necessary to elucidate the precise kinetics of bacterial killing and the synergistic mechanisms underlying this combination. *In-vivo* animal studies are required to optimize the dosing regimen and assess the therapeutic efficacy and safety of this combination in a more complex biological system. This present study is particularly significant considering the rising rates of fluoroquinolone resistance globally, especially norfloxacin is used as an empirical therapeutic agent for UTIs. The observed synergistic effects between norfloxacin and baicalein may be attributed to a combination of mechanisms, including increased membrane permeability, efflux pump inhibition, modulation of resistance mechanisms, biofilm disruption, and antioxidant activity. Further investigations in this direction are essential to gain understanding of these interactions and their underlying mechanisms.

CONCLUSION

The findings of this study highlight the critical need for continuous monitoring of antimicrobial resistance patterns in common urinary pathogens such as *E. coli, K. pneumoniae, and P. aeruginosa*, as emphasized in the WHO 2024 Priority Pathogen List. The results demonstrate that bioactive compounds like baicalein can significantly enhance the efficacy of existing antibiotics, specifically norfloxacin, against resistant strains of these pathogens. This synergy not only offers a promising strategy for managing urinary tract infections caused by multidrug-resistant bacteria but also underscores the potential of integrating traditional medicinal compounds into contemporary treatment regimens.

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