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# Antibacterial Activity of Some Non-steroidal Anti-inflammatory Drugs against *Proteus mirabilis*

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## Abstract

In some recent investigations, non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol demonstrated antibacterial action. This study aimed to evaluate the inhibitory effects of diclofenac, piroxicam, and paracetamol against multidrug-resistant *Proteus mirabilis.* The sensitivity of *Proteus* spp. was tested for nine antibiotics representing seven different families using an agar diffusion test. The isolates with multiple antibiotic resistances were selected to test the effect of NSAIDs and paracetamol on growth using a resazurin-based microplate broth dilution assay. The findings of the agar diffusion test revealed the highest level of antibiotic resistance for *Proteus* isolates was recorded against ceftazidime with a percentage of (91.53%) followed by amoxicillin-clavulanate and cefixime (81.36%) and (76.27%), respectively. The isolates were resistant to azithromycin at a rate of (69.49%). Furthermore, moderate resistance was observed among the isolates to levofloxacin (66.10%), gentamicin (55.93%), and aztreonam (47.46%). While the lowest resistance was reported (18.64%) against piperacillin-tazobactam and (8.47%) against imipenem, Imipenem had the greatest antibacterial activity (88%); also, NSAIDs showed distinct antibacterial activity against *Proteus mirabilis* in (2500, 5000 µg/ml) and above concentrations for piroxicam and diclofenac, respectively. Paracetamol failed to show antibacterial activity against *Proteus mirabilis.*

**Keywords**: NASIDs, *Proteus mirabilis,* multidrug resistance, antibacterial.

## 1.Introduction

*Proteus* spp. are gram-negative bacteria that belong to the *Morganellaceae* family within the *Enterobacterales* order [1]. These bacteria are part of the typical intestinal tract flora and are regarded as opportunistic pathogens capable of causing urinary tract infections, septicemia, and wound infections in humans [2, 3]. The *Proteus* genus is currently classified into eight species: *Proteus mirabilis, Proteus vulgaris, Proteus hauseri, Proteus penneri, Proteus columbae, Proteus incostans, Proteus cibarius*, and *Proteus terrae*. Among these, *P. mirabilis* is the most frequently identified species in human infections [4]. Multidrug-resistant (MDR) *Proteus* is a significant public health concern due to its potential for wide dissemination and the associated risks to public health, particularly those strains that produce Extended-Spectrum Beta-Lactamases (ESBLs) [5].

To prevent the spread of these bacteria and reduce the impact of infections caused by them, it is essential to promote appropriate antibiotic use and implement effective infection control measures.

Ongoing research into new treatments and prevention strategies is also crucial.to treat infections caused by antibiotic-resistant bacteria, numerous research groups have consistently documented the presence of important antimicrobial properties in a range of non-antibiotic compounds. It is essential to explore new approaches and strategies. Recent research has highlighted nonsteroidal anti-inflammatory drugs' potential antimicrobial and antibiofilm properties [6]. Furthermore, some studies have found that non-antibiotic compounds can exhibit synergistic effects when used together with antibacterial agents [7]. However, it is important to know that certain investigations have revealed antagonistic interactions between antibiotics and anti-inflammatory drugs, which can result in increased minimum inhibitory concentrations (MICs) of antibiotics and elevated biofilm formation in the presence of anti-inflammatory drugs [8, 9]. Some studies also reported that paracetamol has antibacterial activity, its efficacy depends on the concentration, with the risk of harming the intestinal flora at high concentrations [10].

## 2. Materials and Methods

### 2.1.Bacteria Isolation and Identification

Several standard morphological and biochemical tests were carried out to identify *Proteus* isolates collected from various hospitals in Baghdad from October 2022 to February 2023. The hospitals involved Baghdad Teaching Hospital, Ghazi Hariri Hospital, Al-Yarmouk Teaching Hospital, and Al-Kadhimiya Teaching Hospital. Bacterial isolates were initially identified based on their growth characteristics on culture media. Usually inoculated onto two media: MacConkey’s agar and blood agar plates. On MacConkey agar, the bacterial isolates appeared colorless and lactose non-fermented with a putrid odor. On blood agar, a swarming phenomenon was observed. Biochemical tests were also performed to aid in identification. These tests included oxidases and catalases. Additionally, the ability of the isolates to produce indole and urease was assessed, along with their capacity to utilize citrate as a carbon source. Then the identification was confirmed by the VITEK 2 system (bioMérieux) according to the manufacturer's specifications using the VITEK® 2 GN kit.

### 2.2.Preparation of a Stock Solution

Paracetamol (Integrated Laboratory, India), piroxicam (Pfizer, USA), and diclofenac (Acino Pharma, Switzerland) injection stock solutions were prepared at 20 mg/ml.

### 2.3.Resazurin solution

To prepare Resazurin dye, 337.5 mg of Resazurin powder was added to 50 ml of sterile distilled water in a sterile beaker. The solution was thoroughly mixed using a vortex to ensure uniformity and was prepared in a dark environment [11].

### 2.4.Antibiotic Susceptibility Testing

The Kirby-Bauer disk diffusion method was used to test antimicrobial susceptibility, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [12]. Four to five fresh bacterial colonies were suspended in saline and measured for turbidity by DensiCheck (aturbidity meter) to reach 0.5 McFarland (equivalent to $1.5x10^8$ CFU/ml). They were then spread on the surface of Muller-Hinton agar (MH) plates using sterile swabs. The tested antibiotic discs were obtained from Bioanalyze (Turkey) according to CLSI guidelines (Amoxicillin-Clavulanate 20/10µg, Piperacillin-Tazobactam 100/10µg, Ceftazidime 30µg, Cefixime 5µg, Imipenem 10µg, Gentamicin 10µg, Azithromycin 15µg, Levofloxacin 5µg,

Aztreonam 30µg). Following the bacterial inoculation on the MH agar plates, the antibiotic discs were placed on the plates' surface and incubated under ambient air conditions at 37 ºC for 18 h. The antibiotic inhibition zone was measured, and the distance between each zone was recorded. The CLSI interpretive criteria were used to classify the diameters of the zones of inhibition for antibiotics into resistant, intermediate, and sensitive categories. If isolates were resistant to at least one agent from three or more categories of antibiotics, they were categorized as multi-drug-resistant (MDR) [13].

### 2.5.Minimum Inhibitory Concentration

The minimal inhibitory concentration (MIC) of the medications used in this study was determined using the resazurin-based assay; this test was carried out in a 96-well microtiter plate using the broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI) [12]. the microtiter plate was incubated overnight at 37°C, then 5µL of the resazurin dye solution previously prepared was added to all wells, and incubated for 2–4 h to the observation of color change, the last well before the color change from blue to pink was recorded MIC (lowest concentration that does not show growth) [11].

## 3. Results and Discussion

A total of 460 specimens were collected from people suffering from various diseases attending several hospitals in Baghdad. The hospitals involved were Baghdad Teaching Hospital, Ghazi Hariri Hospital, Al-Yarmouk Teaching Hospital, and Al-Kadhimiya Teaching Hospital. These specimens consisted of 234 ear swabs, 63 burns, 74 wounds, and 89 ear swabs. All bacterial isolates belonging to *Proteus* spp. showed a swarming phenomenon on blood agar, and the colonies were pale, lactose non-fermented on MacConkey agar, as shown in **Figure 1** [14]. The biochemical tests showed that 59 isolates were negative for the oxidase test and positive for urease and catalase production, with variable citrate utilization. Out of all the isolates, only two showed a positive result for the indole test, which is used to distinguish between *P. vulgaris* and *P. mirabilis*. These two isolates were indole-positive for *P. vulgaris* but indole-negative for *P. mirabilis*. Due to its rapidity and accuracy, this identification was confirmed for the suspected *Proteus* isolates using the VITEK 2 system.

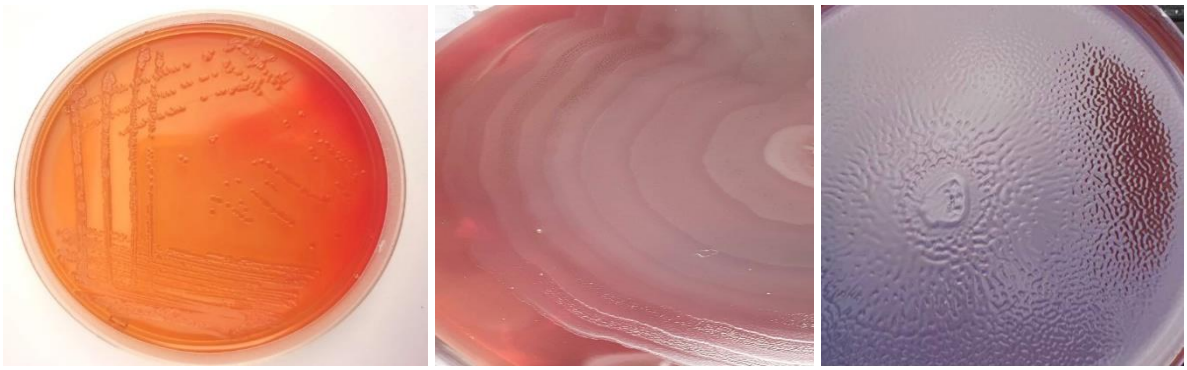


**Figure 1**. (A) lactose non-fermented by *proteus* spp. on MacConkey agar, (B and C) *Proteus* swarming phenomenon on blood agar.

The current results showed that the prevalence of *Proteus* spp. in clinical samples was (12.83%), this converges with its percentage in other studies [15, 16] and is higher than those of [17]

isolated (1%) and less than [18] obtained (23.75%). Variations in the number of *Proteus* spp. infections observed in different studies might be attributed to several factors, such as differences in sample size, hospital settings, medication histories of patients, and seasonal and yearly variations. For instance, [19] reported higher incidences of *Proteus* spp. infections during the summer compared to the spring, and [20] observed a significant increase in rates from 1.9% to 8.1% between 2011 and 2020. The prevalence percentage of *P. mirabilis* was higher than that of *P. vulgaris,* as shown in **Figure 2**. of the 59 isolates belonging to the *Proteus* genus, 57 (96.61%) were *P. mirabilis* and two (3.39%) were *P. vulgaris*. These results are consistent with a previous study conducted by [21], which found higher rates of *P. mirabilis* than *P. vulgaris* in urine samples. Similarly, [22] reported a higher incidence of *P. mirabilis* (90.40%) than *P. vulgaris* (1.90%) in clinical samples. This is an obvious result since the isolation of *P. vulgaris* is usually from immunocompromised people, such as patients with acquired immunodeficiency syndrome (AIDS), or cancer, people who have used antibiotics for a long time, and patients with long-term indwelling catheters [23].

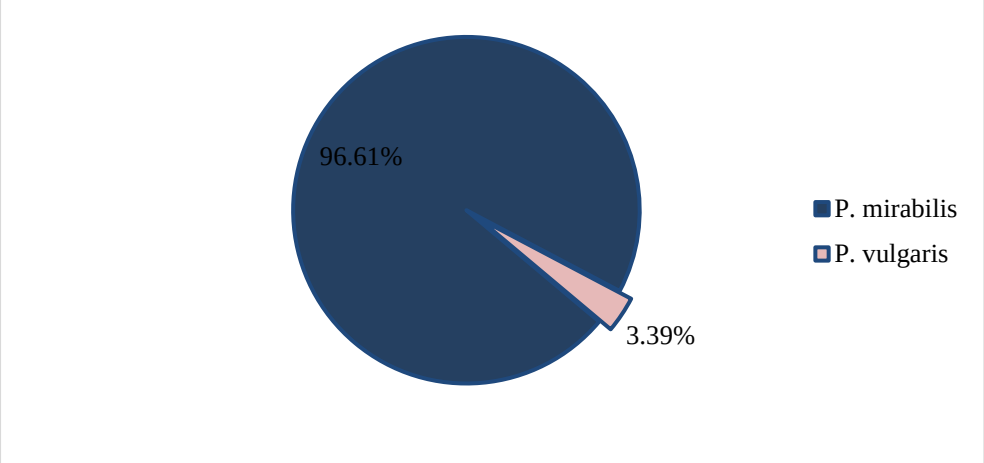


**Figure 2.** Percentages of the *Proteus* spp. Understudy.

### 3.1.Antibiotic Susceptibility of *Proteus* spp.

The susceptibility of 59 *Proteus* spp. to various antibiotics was assessed through the Kirby-Bauer disk diffusion method (**Figure 3**), following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [12].

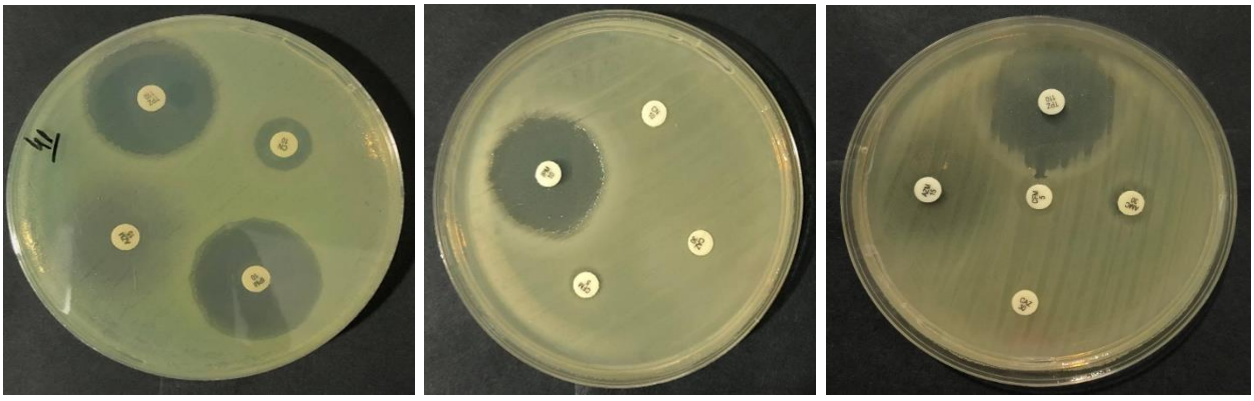

**Figure 3.** The Kirby-Bauer disk diffusion method was employed to determine the susceptibility of *Proteus* spp. to various antibiotics.

Results revealed, as shown in **Figure 4,** that the maximum antibiotic resistance for *Proteus* strains was recorded against beta-lactamase inhibitors to ceftazidime with a percentage (91.53%) followed by amoxicillin-clavulanate and cefixime with a resistant rate (81.36%) (76.27%), respectively. The isolates were resistant to azithromycin from the macrolides family at a rate of (69.49%). Furthermore, moderate resistance was observed among the isolates to levofloxacin (66.10%), gentamicin (55.93%), and aztreonam (47.46%). While the lowest resistance was reported (18.64%) against piperacillin-tazobactam and (8.47%) against imipenem.

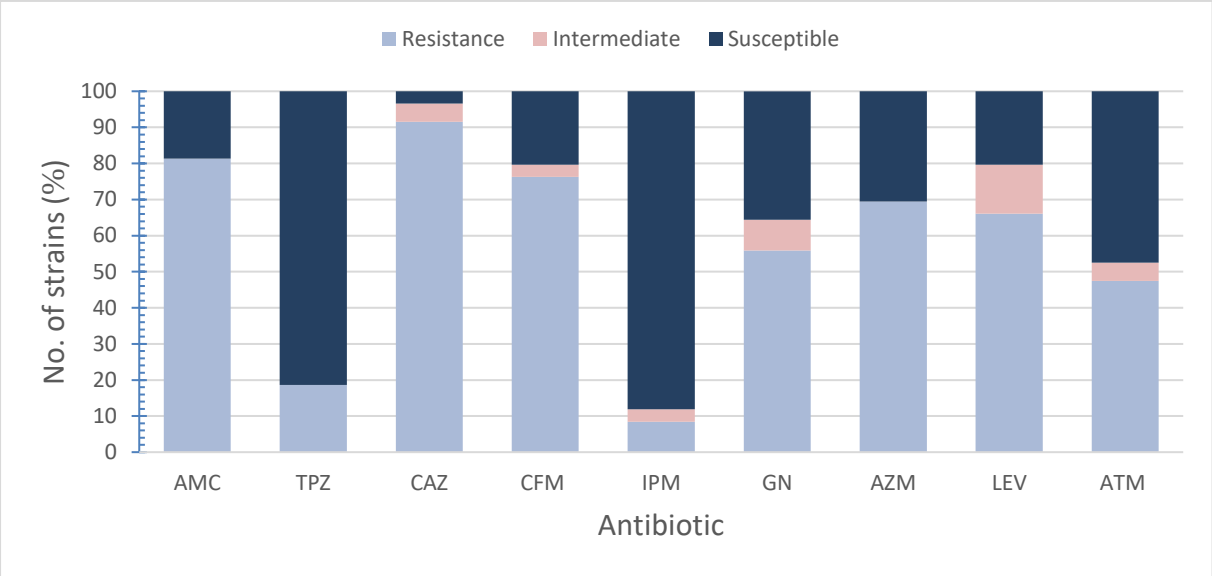


**Figure 4.** The percentage of antibiotic susceptibility profiles of Proteus spp. the percentage of strains is depicted on the y-axis, while on the x-axis of the graph, the challenge with antibiotics is displayed. AMC: Amoxicillin-Clavulanate, TPZ; Piperacillin-Tazobactam, CAZ; Ceftazidime, CFM: Cefixime, IPM: Imipenem, GN: Gentamicin, AZM: Azithromycin, LEV: Levofloxacin, ATM: Aztreonam.

Other researchers reported that the resistance of *Proteus* spp. against ceftazidime was more than 90% [24, 25]. This agrees with the results of this study. Ceftazidime belongs to the 3rd generation cephalosporins category. Usually, the production of Extended-Spectrum-Lactamases (ESBLs) leads to a high level of ceftazidime resistance. Moreover, acquired cephalosporinases in *Proteus* species provide high-level ceftazidime resistance and decreased aztreonam susceptibility [26]. Also, cefixime appeared with a high resistance rate, this is similar to the result of [27] where its resistance was (76.4%). The targeted use of third- and fourth-generation cephalosporins is beneficial to prevent or hinder the emergence and spread of antibiotic resistance in Gram-negative bacteria. The high prevalence of resistance to these cephalosporins is worrisome to physicians since they are considered key risk factors in the development and propagation of multidrug resistance in Gram-negative bacteria, which can lead to severe infections resulting in morbidity and mortality [28]. Combination β-lactams showed high resistance to amoxicillin-clavulanate, the outcomes are consistent with other studies where the percentage of resistance ranged between 75 and 85% [18, 29]. This may be attributable to the plenty of usage and frequent prescriptions by doctors recently. While (81.36%) of *Proteus* spp. were sensitive to piperacillin-tazobactam, this finding is in agreement with the results of another study conducted by [30]. The most effective antibiotic against *Proteus* spp. was imipenem, with a susceptible percentage of 88%. Nearly similar results were recorded in Iraq which mentioned imipenem as one of the most effective antibiotics against *Proteus* species [31, 32]. Carbapenems are still

utilized as a final line of defense in several countries to treat severe infections caused by *Enterobacterales* that produce ESBLs [33]. The broad-spectrum beta-lactamase enzymes that hydrolyze third- and fourth-generation cephalosporins are unable to hydrolyze carbapenems. The rapid spread of carbapenemase-mediated resistance within certain members of the *Enterobacteriaceae* family presents considerable public health concerns. Invasive infections caused by carbapenemase-producing *Enterobacterales* (CPE) have been associated with a high mortality rate, as evidenced by previous studies. Invasive infections caused by carbapenemase-producing *Enterobacterales* (CPE) are often associated with a high mortality rate, as indicated by previous research [34, 35]. [20] confirmed the constantly increasing trend in detecting *proteus* strains that are resistant to antibiotics at a rate ranging from 48.4% in 2011 to 74% in 2020. One of the most important reasons for the increase in antimicrobial resistance rates is medical professionals and the general public's incorrect and excessive use of antimicrobials [36, 37]. Establishing and implementing a reliable antibiotic surveillance system globally means ensuring the antimicrobial agent's ongoing use before its effectiveness is irrevocably compromised. In light of the dangers associated with repeated antibiotic use, current research has emphasized the potential benefits of non-antibiotic components of NSAIDs.

**3.2.Determine the minimum inhibitory concentration (MIC)**

The resazurin-based microplate broth dilution assay was used to investigate the inhibitory effects of paracetamol and NSAIDs (Piroxicam and Diclofenac) in the range of concentrations tested against MDR *P. mirabilis*. The assay is based on the ability of the indicator dye, resazurin, to change its color in response to the metabolic activity of live cells. The presence of bacterial growth changes the color of resazurin from blue to pink due to the reduction of the dye by bacterial metabolic activity. It is one of the highly standardized methods in antimicrobial testing; in contrast to the traditional assay, the color change may be seen visually; hence, no spectrophotometer is required in this assay [11]. Numerous investigations have demonstrated that certain NSAIDs possess significant antibacterial properties, with diclofenac sodium, in particular, displaying potent antibacterial effects against both gram-positive and gram-negative bacteria [7]. According to the outcomes of this study, the minimum inhibitory concentration of diclofenac (DCF) ranged from $5\times10^3$ to $1\times10^4$ µg /mL. Singh *et al.* demonstrated the MICs of DCF (2650 µg/mL) against *Proteus* spp. from animal sources [38]. Wide variations in the MICs for this drug have been seen in numerous investigations, which may be related to methodological elements such as the culture media utilized, the research methods, and the type of solvent to dissolve the drug, or it may be due to the differences in types of strains in different studies. The MIC for piroxicam (PXM) was 2500 µg/ml for all tested strains **(Figure 5)**. [39] mentioned that the MIC of PXC against *Escherichia coli* was 800 µg/ml, while against *Staphylococcus aureus* it was >2000 µg/ml. Another study did not test the raw drug but used sulfonate esters, a derivative of piroxicam, and found that it has potent efficacy against both gram-positive and gram-negative bacteria [40].

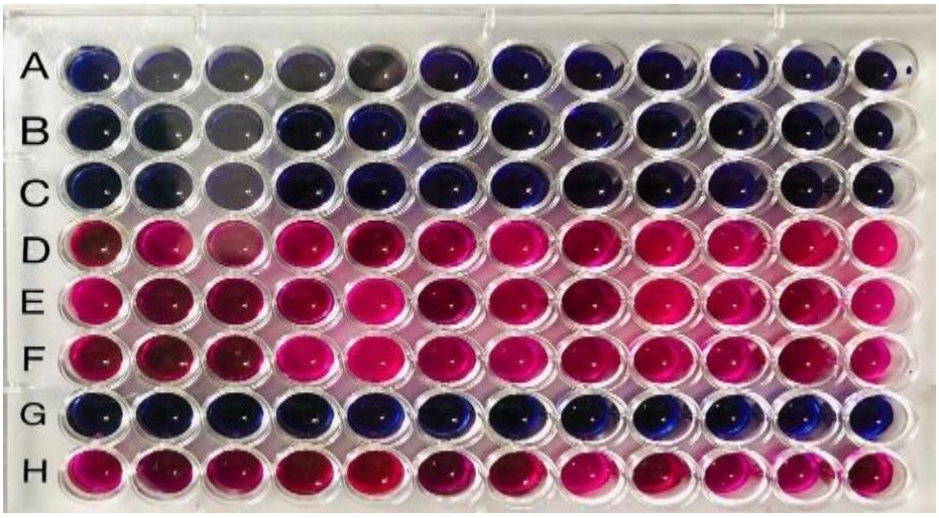


**Figure 5.** Piroxicam minimum inhibitory concentration of *Proteus mirabilis* by Resazurin-based method. Row G represents the negative control showing the natural color of resazurin (blue/purple). Row H, a positive control, changed to a reduced form (pink). Wells A – F of each raw contained piroxicam at $1\times10^4$ –312.5 µg /ml concentrations, respectively.

NSAIDs may exert their antibacterial effects by impacting the integrity of the bacterial cytoplasmic membrane, this influence may lead to changes in the physicochemical properties of the bacterial surface, increased cell permeability to propidium iodide, and the release of intracellular $K^+$ [41]. NSAIDs may also interfere with DNA synthesis, block DNA replication, and hinder bacterial membrane repair [39]. In concentrations of $1\times10^4$ µg/ml, paracetamol was found to be ineffective as an antimicrobial. These findings align with previous studies, which have reported that paracetamol either has little to no antimicrobial efficacy or only exhibits moderate effectiveness [38].

## 4. Conclusion

The exploration of new applications for existing drugs, particularly antimicrobials, is a growing field aimed at combating antibiotic resistance and creating substitutes for antibiotics. This study focused on assessing the antimicrobial properties of medications that are frequently used as supplements to antibiotics, such as paracetamol, piroxicam, and diclofenac sodium, against *Proteus mirabilis.* Diclofenac and piroxicam exhibited antimicrobial activity against the tested isolates of *Proteus mirabilis*, while paracetamol did not demonstrate any antibacterial activity at the tested concentrations.

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## Conflict of Interest

The authors declare that they have no conflicts of interest.

## Funding

There is no funding for the article.

## Ethical Clearance

The study was conducted after receiving agreement from the participants and ethical approval from the biology department's ethics committee at the University of Baghdad's College of Science (CSEC/0922/0088) on September 26, 2022. and this is consistent with the instructions of the Iraqi Ministry of Health and Environment.

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