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Effect the Natural Efflux Pump Inhibitor (Berberine) in Multidrug Resistant *Kleibsiella pneumoniae* Isolated from Urinary Tract Infections in Several Baghdad Hospitals

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ABSTRACT

Background: *Kleibsiella pneumoniae* in urinary tract infections (UTIs) have grown for years. Antibiotic-resistant microorganisms make infection control and treatment difficult for clinicians. Efflux pumps contribute to K. pneumoniae's multidrug resistance.

Objective: This study aimed to test Phenylalanine-Arginine β-Naphthylamide (PAβN) and Berberine as antibacterial agents against multidrug-resistant bacteria (UTI K. pneumonia isolates).

Subjects and Methods: Between December 2021 and April 2022, five Baghdad hospitals collected 260 urine samples from outpatients and inpatients with urinary tract infections, both genders, aged 15 to 72.

Results: We isolated 76 of the cultures (65.5%) and employed selective media, biochemical assays, and the VITEK2 system to identify bacteria as *K. pneumoniae*. Using the disc diffusion technique, it was demonstrated that clinical isolates of *K. pneumoniae* were moderately resistant to the majority of drugs. *K. pneumoniae* isolates were extremely resistant to Amoxicillin (96.1%), Trimethoprim (80.3%), Gentamicin, Amikacin and Meropenem (55.1%), and Ciprofloxacin (53.9%). The efflux pump inhibitor (PAβN) greatly boosted strain susceptibility to Ciprofloxacin. PAβN lowered MICs by 4–64-fold. The minimum inhibitory concentrations (MICs) of Berberine against ten multidrug-resistant *K. pneumoniae* isolates ranged from 3.9 to 500 µg/ml, indicating that Berberine affects *K. pneumoniae* growth at very low doses.

Conclusion: In this work, Berberine was found to be an efflux pump inhibitor (EPI) and antibacterial drug that could overcome bacterial resistance mechanisms.

Keywords: UTIs infections, Multidrug resistance, Berberine, *Kleibsiella pneumoniae.*

INTRODUCTION

Hospitals are struggling with *Kleibsiella pneumoniae*-related UTIs [(1)]. Due to the lack of treatments for this pathogen, its high multidrug resistance (MDR) causes clinical issues [(2, 3)].

K. pneumoniae, a Gram-negative, rod-shaped, capsulated Enterobacteriaceae bacterium; a common nosocomial pathogen in this species can cause urinary tract, respiratory, and blood infections [(4, 5)]. Gram-negative bacteria evade drugs via multidrug efflux pumps. Efflux systems make microorganisms more resistant [(6)]. *K. pneumoniae* use resistance-nodulation-division (RND) efflux pumps OqxAB and AcrAB to become antibiotic-resistant [(7)].

Blocking efflux pumps may function when antibiotics fail. EPIs and natural substrates allow drug accumulation inside the bacterial cell, where it can do the most benefit. Berberine and reserpine are plant-derived EPIs [(8)]. Considerably used synthetic efflux inhibitors to detect the efflux activity in *K . pneumoniae* are carbonyl cyanide-chlorophenylhydrazone (CCCP) and phenylalanine-arginine β-naphthylamide (PAβN) [(9)]. The present study aimed to investigate the efficacy of Berberine against clinical *K. pneumoniae* isolates and evaluate the role of this natural efflux pump inhibitor as an alternative therapeutic agent for multidrug resistant strains in patients with UTIs.

SUBJECTS AND METHODS

Isolation and identification of *K. pneumoniae*:

Between December 2021 and April 2022, this study was conducted at various hospitals located in Baghdad, Iraq. Patients suffering from burn diseases generated 76 isolates of *K. pneumoniae* out of a total of 260 urine samples. In order to isolate *K. pneumoniae*, blood agar, macConkey agar, and CHROM agar orientation were utilized as media. The typical bacteriological methods as well as biochemical testing with the VITEK 2 system were used in accordance with the guidelines provided by the manufacturer in order to determine the identities of these isolates (bioMerieux, France).

Antibiotic Susceptibility Test:

An antimicrobial disc diffusion technique test was carried out for the susceptibility evaluation. In a nutshell, an overnight culture of *K. pneumoniae* was grown on MacConkey agar, and it was afterwards suspended in Mueller-Hinton broth (Himedia). The turbidity of the suspension was adjusted to an equivalent of 0.5 McFarland so that it could be inoculated onto plates made of Mueller-Hinton agar (Himedia). In the course of this research, antibiotic discs bearing the following designations were utilized:

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Amikacin (AK), Gentamicin (CN), Imipenem (IPM), Meropenem (MEM), Ceftriaxone (CRO), Cefoxitin (CX), Ciprofloxacin (CIP), Levofloxacin (LEV), Amoxicillin/clavulanic acid (AMC), Trimethoprim (SXT) and Cefepime. After incubation period of one day at a temperature of 35 °C, the agar plates were examined. The inhibition zone was then calculated and interpreted by employing the percentage of susceptible, intermediate, or resistant isolates, as specified by the CLSI breakpoint interpretation criteria [(10)].

Minimum inhibitory concentrations (MICs) of *Kleibsiella pneumoniae* isolates:

Kleibsiella pneumoniae's MICs against the eight medications were calculated according to the criteria laid out by the clinical and laboratory standard institute (CLSI) [(10)]. The quality control strain utilized was *Escherichia coli* ATCC 25922.

Powdered antibiotics were reconstituted with the recommended solvent or sterile deionized water. Antibiotic efficacy was determined by serially diluting the drugs to concentrations of 256, 128, 64, 32, 16, 8, 4, and 2 g/mL. In each well of a 96-well microtiter plates, 100 mL of the antibiotic dilution and Müller-Hinton broth were added. Then, in order to obtain 5x 106 CFU/mL, the 0.5 McFarland sample was diluted by a factor of 1:20. By adding 0.01 milliliters of this suspension to the broth, the ultimate test concentration—roughly 5 x105 CFU/mL—was attained. The suitable density for the turbidity standard was determined using the absorbance measurements from a spectrophotometer.

The absorbance at 625 nm must fall between 0.08 and 0.13 in order to meet the criteria of 0.5 McFarland. The samples were stored in an incubator 37°C for the entire day. The lowest concentration of an antibiotic at which detectable bacterial growth is suppressed is known as the lowest or minimum inhibitory concentration (MIC). The lowest concentration at which an antibiotic can stop bacterial growth is at this level.

Treatment of the efflux pump inhibitors:

100 μg/mL PABN (BACHEM/USA) was tested for antibiotic susceptibility alterations. PABN and non-PABN antibiotic susceptibility were tested. After antibiotics and bacterial cell inoculum, the microplate wells received 2 μL of 5 mg/mL PABN stock. To examine the test's acridity and PABN's effect on bacterial growth, all bacteria were grown in Mueller Hinton broth with 100 μL/mL PABN. A 96-well round-bottom plate contained 3.9–500 g/ml Berberine HCL serially diluted 1:2 in Mueller Hinton Broth (MHB). The bacterial inoculum was made by culturing *K. pneumoniae* subcultures in LBB for 18-24 hours at 35 ± 2°C. After diluting the bacterial suspension to 1x108 CFU/mL (the McFarland turbidity equivalent of 0.5), which was verified by spectrophotometry at 0.08-0.1 at 625 nm, we diluted it 1:200 in MHB to 5x105 CFU/mL. The 96-well plates with diluted bacterial solution received serially diluted peptides.

Each well used 100 μL of chemical and 100 μL of diluted bacterial solution. Negative and positive growth controls were MHB alone or *K. pneumoniae* with MHB in the wells. After 24 hours at 37 °C, all wells received 20 μl of resazurin (0.015%) and were incubated for 2–4 hours to detect color change. After incubation, resazurin-blue columns exceeded the MIC. Incubation estimated the MIC as the lowest chemical concentration without bacterial growth.

Ethical considerations:

The research was sanctioned by Baghdad Hospital's Ethics Committee and the postgraduate Institute of Genetic Engineering and Biotechnology. A consent document was signed by all those involved. The World Medical Association's Declaration of Helsinki was strictly adhered to in all human subjects' studies.

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

Isolation and characterization of *K. pneumonia:* This study was conducted among patients suspected of UTI visiting 5 of Baghdad hospitals. A total of 260 non-repetitive MSU samples were collected from patients for urine culture. All these urine specimens were cultured on CHROM agar orientation medium, MacConkey agar and Blood agar plates for 24 hours at 37 °C. Only 116 (44.6%) samples showed significant growth. Seventy-six of positive cultures were identified as *K. pneumoniae* (65.5. %).

Most of the patients were of the females 50/76 (65.7%), while the percentage of the males was 26/76 (34.3 %). *K. pneumoniae* bacteria growing on MacConkey agar were bright pink colonies with a mucoid structure, which is a characteristic feature of these bacteria. By culture on blood agar media, all putative Kleibsiella colonies were big, glossy, mucoid, whitish-grey, and spherical with no hemolysis. Kleibsiella isolates formed metallic blue colonies on CHROM agar orientation at 37°C for 24 hours as shown in figure (1).

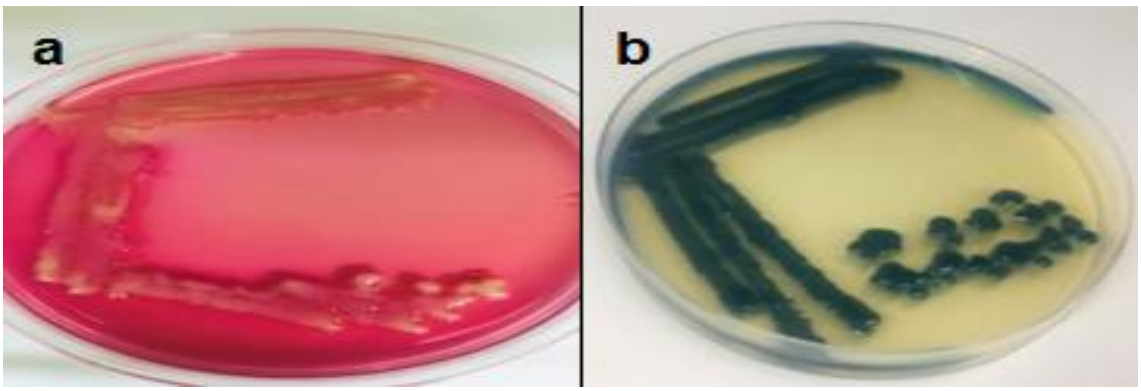

Figure (1): *Kleibsiella pneumoniae* on a) MacConkey agar plate and b) CHROM agar orientation medium

K. pneumoniae formed non-hemolytic grey-white, mucoid colonies on blood agar. All isolates were non-motile and had negative oxidase, indole, motility, and methyl red tests but positive urease, citrate utilization, catalase, and Voges-Proskauer tests. Glucose fermentation produced acid and gas, not H_2S. The VITEK 2 system was used for the identification of *Kleibsiella* spp. bacteria with accuracy and reliability of bacterial identification. This device diagnosed bacteria with the accuracy of 99%. After Identification, the number of *K. pneumoniae* isolates were 76. All 76 isolates previously identified as Kleibsiella spp. were shown to be *K. pneumoniae* after the results of the tests utilized in this system, which supported the results obtained from morphological, biochemical, and other testing.

Antibiotic Susceptibility test of *Kleibsiella pneumoniae* isolates:

All 76 *K. pneumoniae* isolates were tested for 12 antibiotics. This study found that Levofloxacin (59.2%), Meropenem (55.2%), and Ciprofloxacin (53.9%) had the highest antibiotic sensitivity against *K. pneumoniae*, while Amoxicillin (1.3%), Ceftriaxone (7.9%), and Trimethoprim (17.1%) all had the lowest. Amoxicillin (96.1%), Trimethoprim (80.3%), Gentamicin (59.2%), and Amikacin (58.9%) had the highest antibiotic resistance.

Most antibiotics tested in this investigation exhibited substantial resistance. 15 (19.7%). *K. pneumoniae* isolates were multi-drug resistant (MDR) as in (table 1).

Table (1): Antibiotic susceptibility results for 76 *K. pneumoniae* isolates against 12 antibiotics

Antibiotic (ug / disc)	Lev-5	CX-30	IPM-10	FEP-30	SXT-25	PIT-30	AK-30	Cip-10	MEM-10	CN-10	AMC-10	CRO--30
S	45 (59.2%)	33 (42.4%)	34 (44.7%)	33 (42.4%)	13 (17.1%)	38 (50.0%)	28 (36.8%)	41 (53.9%)	42 (55.2%)	22 (28.9%)	1 (1.3%)	6 (7.9%)
R	16 (21.1%)	24 (31.6%)	33 (42.4%)	40 (52.6%)	61 (80.3%)	30 (39.5%)	44 (58.9%)	29 (38.2%)	27 (35.5%)	45 (59.2%)	73 (96.1%)	66 (8.7%)
I	15 (19.7%)	19 (25.0%)	9 (11.8%)	3 (3.9%)	2 (2.6%)	8 (10.5%)	4 (5.3%)	6 (7.9%)	7 (9.2%)	9 (11.8%)	2 (2.6%)	4 (5.3%)
Chi-Square-χ^2	23.16 **	4.02 NS	15.98 **	30.81 **	78.50 **	19.25 **	32.33	25.23 **	24.59 **	26.51 **	135.9 **	98.99 **
P-value	0.0001	0.133	0.0003	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
** ($P \leq 0.01$).												

Minimum inhibitory concentrations (MICs) of *K. pneumoniae* clinical isolates in the presence and absence of efflux pump inhibitor (Phe-Arg-β- naphthylamide):

The minimum inhibitory concentrations (MICs) of eight antibiotics were measured using microdilution.

Antibiotic resistance was corroborated by microbiological indictment (MIC) data, which showed that most isolates were highly resistant to all antibiotics except Ciprofloxacin and Meropenem.

The MIC values for Amikacin, Gentamicin, and Ceftriaxone in highly resistant isolates were 265 g/ml, while the MIC values for Levofloxacin, Ciprofloxacin, and Meropenem were 2, 4, and 2 µg /ml respectively (Table 3).

Table (3): Minimum inhibitory concentration (MIC)

Antibiotic*	MIC** range (µg/mL)
AK	16 - 256
CN	4 – 256
IPM	2 – 128
MEM	Apr-32
LEV	2 – 64
CRO	4 – 256
FEP	16 – 128
CIP	4 – 64

The effect of Phe-arg-beta-naphthylamide (PAβN) on *K. pneumoniae* MICs was shown in the Table (4). The efflux pump inhibitor (PAβN) at 20 µg/ml dramatically reduced the effect of PAβN on fluoroquinolone (Ciprofloxacin and Levofloxacin) antibiotic MICs, reducing them by 4 to 64 fold. Amikacin MICs decreased by 4 to 32 fold and Gentamicin by 8 to 32 fold.

The efflux pump inhibitor reduced carbapenem MICs by 1–4 times. The antibiotics Imipenem, Cefepime, Ceftriaxone, and Meropenem reduced the inhibitor's action.

Table (4): Antimicrobial susceptibility in the presence and absence of Efflux Pump Inhibitor of K. pneumoniae

Isolate No.		LEV	CIP	IPM	MEM	CN	AK	CRO	FEP
K1	Alone	2	32	64	32	128	256	64	128
	+EPI	0.5	0. 5	32	16	32	16	16	32
K3	Alone	4	64	64	32	128	64	64	64
	+EPI	0.25	1	32	32	16	8	4	32
K4	Alone	64	64	32	8	256	128	256	32
	+EPI	4	4	8	4	32	16	16	8
K7	Alone	8	16	64	32	128	64	128	64
	+EPI	0.5	1	32	16	64	4	16	64
K8	Alone	32	64	128	16	64	16	32	16
	+EPI	4	4	128	8	16	0.5	16	1
K11	Alone	16	16	64	32	128	64	128	32
	+EPI	0.5	0.5	64	32	32	16	32	4
K12	Alone	16	4	4	8	16	64	4	16
	+EPI	0.25	0.25	2	4	8	2	1	4
K13	Alone	4	8	32	16	32	32	32	64
	+EPI	0.5	1	32	4	0.5	4	16	8
K14	Alone	32	4	2	4	4	16	64	16
	+EPI	8	0.25	2	2	1	4	16	16
K15	Alone	8	8	16	16	32	32	4	16
	+EPI	0.5	1	16	8	4	8	0.5	8
Fold of reduction In MIC+ EPI		4-64	8-64	1-4	1-4	2-64	4-32	2-16	1-16

MIC: Minimum inhibitory concentration; EPI: Efflux Pump Inhibitor (Phe-Arg-β- naphthylamide). Mipenem (IPM); Gentamicin (CN); Cefepime (FEB); Ceftriaxone (CRO); Amikacin (AK); Meropenem (MEM); Ciprofloxacin (CIP); Levofloxacin (LEV**).**

Minimum Inhibitory Concentrations (MICs) of *K. pneumoniae* clinical isolates in the presence of Berberine

Minimum inhibitory concentration (MIC) of Berberine was determined by Muller Hinton broth micro-dilution method in 96-well microplates. The results of the minimum inhibitory concentrations of antimicrobial Berberine HCL against ten isolates of *K. pneumoniae* revealed that there was a difference in MICs between the isolates, where some isolates, such as K1, K26 and K129, were affected by concentrations of 500 µg/ml only, while other isolates such as K2 and K3 were inhibited by lower concentrations (15.6- 500 µg/ml) (**Table 5 and Figure 2**).

The results demonstrated that all ten tested isolates were inhibited by the concentration of 500 µg/ml of Berberine and 7 isolates were inhibited by the concentration 250 µg/ml, while the low concentration 15.6 µg/ml showed inhibitor effect on 2 isolates (K2 and K3).

Table (5): The minimum inhibitory concentrations of Berberine against *K. pneumoniae* isolates at concentrations (3.9-500 µg/ml).

The Isolate code	Minimum Inhibitory Concentration (MIC) (µg/ml)							
	500	250	125	62. 5	31.25	15.6	7.81	3.9
K1	+	-	-	-	-	-	-	-
K2	+	+	+	+	+	+	-	-
K3	+	+	+	+	+	+	-	-
K6	+	+	-	-	-	-	-	-
K7	+	+	-	-	-	-	-	-
K23	+	+	-	-	-	-	-	-
K24	+	+	-	-	-	-	-	-
K26	+	-	-	-	-	-	-	-
K27	+	+	+	-	-	-	-	-
K129	+	-	-	-	-	-	-	-

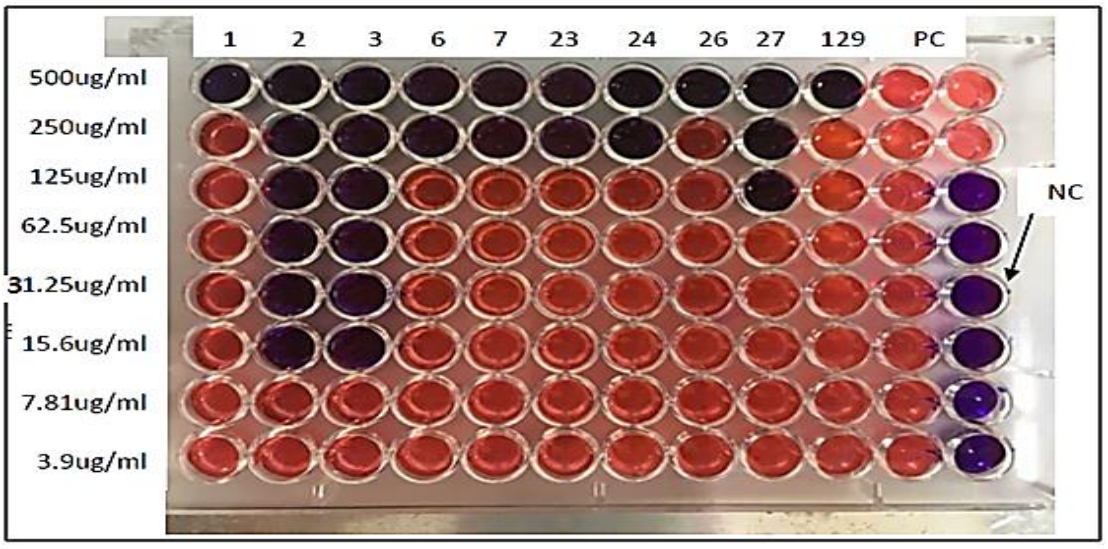

Figure (2): The minimum inhibitory concentrations (MICs) of Berberine at the concentrations (3.9-500 µg/ml) against *K.pneumoniae* Isolates by Microtiter Plate Assay with Resazurin dye

DISCUSSION

CHROMagar Orientation medium was used to isolate urinary tract infections. Each bacterial type has a unique color, so Escherichia coli colonies were pink-red. Aerobic Gram-negative bacteria can be detected on chromogenic agars due to their easy colony identification. Due to its excellent sensitivity, speed, and low false-positive rate, CHROM agar orientation medium is the medium of choice [(11)]. The usage of CHROM agar CO medium may be a cost-effective alternative to conventional techniques of culturing urine because it eliminates the need for supplemental reagents

and assays. Also, unlike Blood agar and MacConkey agar, it requires much less time in the microbiology lab, meaning it might be utilized to detect and report uropathogens [12, 13].

In a local study of *K. pneumoniae* resistance to 10 antibiotics, Ampicillin, Cefotaxim, and Piperacillin had 100% resistance, while Trimethoprim, Gentamicin, and Azithromicin had 80%, 54%, and 65% resistance, respectively. Imipenem, Chloramphenicol, and Ofloxacin had 4%, 12.5%, and 35% resistance, respectively [14]. Our results were consistent with **Vasaikar *et al.*** [15]. Observation conducted a systematic review and discovered that 29.7% of *K. pneumoniae* isolates from several South African hospitals were resistant to Ciprofloxacin, 51.0% were resistant to Gentamicin, and 70.8% were resistant to Trimethoprim. Antibiogram of *K. pneumoniae* isolates exhibited 19.09%, 21.81%, 10.0%, 9.09%, 44.54%, 25.45%, 11.81%, and 61.81% resistance to Ciprofloxacin, Norfloxacin, Gentamicin, Kanamycin, Cefotaxime, Trimethoprim, Chloramphenicol, and Colistin, respectively.

After evaluating the antibiotic resistance pattern of K. pneumoniae samples, 22 multi-drug resistant strains that were resistant to more than three antibiotics and fluoroquinolones were discovered [16]. But, **Wang and his co-workers [17]** showed that ceftriaxone and imipenem MIC values were >128 μg/mL in 29 and 28 resistant *K. pneumoniae* strains, respectively. Carbapenem medicines treat severe infections of drug-resistant Enterobacteriaceae, and *K. pneumoniae*. Increasing drug resistance and the spread of drug-resistant strains threaten public health [18]. Because of its in vitro broad-spectrum resistance to Gram-positive and Gram-negative microorganisms, the carbapenem antibiotic meropenem is increasingly being utilized to treat K. pneumoniae infections [19].

A prior study found that ciprofloxacin treatments significantly reduced the number of *K. pneumoniae* cells when compared to meropenem, and their respective MIC values were 0.03 and 0.06 g/ml [20]. According to the findings, meropenem demonstrated a lower level of efficacy in treating *K. pneumoniae* than ciprofloxacin did. It is possible that the short elimination half-life of meropenem is responsible for its lack of antibacterial action [21]. It was demonstrated that the addition of the PAβN reduced the MICs of the 2 classes of antibiotics fluoroquinolones and aminoglycosides. Antibiotic resistance is lessened because bacteria with active efflux pump systems have lower concentrations of the drugs inside their cells. Antibiotic-resistant bacteria can have their susceptibility to treatment restored by blocking their efflux pumps, suggesting that blocking these pumps may serve as a means of control. In the first stages of antibiotic resistance development, the activation of efflux pumps is linked to the fast-acting antibiotic mechanism. Antibiotic resistance among distinct classes may have its roots in the substrate-dependent efflux pump systems [22]. AcrAB/TolC strongly contributes to antibiotic resistance in *K. pneumoniae* strains, with 39% of bacteria exhibiting PaβN - modulated quinolone, chloramphenicol, and tetracycline resistance. In the presence of the efflux pump inhibitor PaβN, chloramphenicol accumulation significantly increased in these strains [23]. In agreement of the current study **Li *et al.*** [24]. Berberine's Hormesis Effect against *K. pneumoniae* was discovered to increase the minimal inhibitory concentrations of efflux-related medicines such rifampicin and azithromycin even at low concentrations. Applications against Gram-negative bacteria at low concentrations were also found to carry a danger.

Natural isoquinoline alkaloid berberine (BEB) is found in many different medicinal plants and has been shown to have antibacterial and antifungal actions both on its own and in combination with other medications [25]. Berberine has a synergistic effect by enhancing the bacterial inhibition of some antibiotics [26]. Berberine disrupts MRSA cell surface and alters saturated and unsaturated fatty acids, affecting membrane integrity in a dose-dependent manner [27]. Plants contain numerous bioactive compounds, including alkaloids, quinones, tannins, terpenoids, flavonoids, and polyphenols. Antibiotics are being discovered by studying secondary metabolites found in plants. They are functional and can act in a variety of ways [28]. Several other studies have confirmed that natural and synthetic EPIs are effective at inducing drug sensitivity against MDR strains of *K. pneumoniae* and other nosocomial pathogens with clinical relevance [23, 24, and 29]. However, a number of studies have shown that the use of magnetic fields and audible noises as a physical forces therapy against pathogenic bacteria can help in lowering the resistance of some pathogenic bacteria that caused a fatal infection. This is because magnetic fields and audible noises are both forms of physical forces [30].

CONCLUSION

Potent efflux pump inhibitors disable these pumps, making antibiotics more effective against more drug-resistant bacteria. Berberine and other efflux pump inhibitors could increase the efficiency of antibiotics at low doses to combat antibiotic resistance.

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