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ORIGINAL ARTICLE

Effect of *Mentha longifolia* essential oil on *oqx*A efflux pump gene expression and biofilm formation in ciprofloxacin-resistant *Klebsiella pneumoniae* strains

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ABSTRACT

Background and Objectives: Today, medicinal plants and their derivatives are considered to reduce the prevalence of antibiotic resistance. The aim of this study was to investigate the effect of *Mentha longifolia* essential oil on *oqx*A efflux pump gene expression and biofilm formation in ciprofloxacin-resistant *Klebsiella pneumoniae* strains.

Materials and Methods: A total of 50 clinical strains of *K. pneumoniae* resistant to ciprofloxacin were studied. The minimum inhibitory concentration (MIC) of *M. longifolia* essential oil and its synergistic effect with ciprofloxacin were determined using the microbroth dilution method and the fractional inhibitory concentration (FIC) method. Minimum biofilm inhibition concentration (MBIC) of *M. longifolia* essential oil was detected. The effect of essential oils on the expression level of the *oqx*A gene was detected by Real-time PCR.

Results: *M. longifolia* essential oil showed inhibitory activity against ciprofloxacin-resistant strains of *K. pneumoniae*. When *M. longifolia* essential oil was combined with ciprofloxacin, the MIC was reduced 2-4 times. In 28% of the strains, *M. longifolia* with ciprofloxacin showed a synergistic effect. *M. longifolia* essential oil reduces the strength of biofilm formation and alters the biofilm phenotype. A significant decrease in *oqx*A gene expression was observed in all isolates after treatment with *M. longifolia* essential oil.

Conclusion: Based on the results of this study, it was observed that supplementing *M. longifolia* essential oil can help reduce ciprofloxacin resistance and inhibit biofilm formation in fluoroquinolone-resistant *K. pneumoniae* strains.

Keywords: *Klebsiella pneumoniae*; Ciprofloxacin; Biofilm; Efflux pump inhibitor

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen known for causing various infections, including pneumonia, bacteremia, and urinary tract infections, particularly in hospital settings. The pathogenicity of *K. pneumoniae* is heightened in individuals with compromised immune systems, prolonged hospital stays, and extensive antibiotic use (1, 2). Treating *K. pneumoniae* infections, especially those involving biofilm formation, is challenging due to the increased antibiotic resistance of biofilm-forming bacteria (3). Fluoroquinolone antibiotics like ciprofloxacin are commonly used to treat bacterial infections, includ-

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ing those caused by *K. pneumoniae*. However, resistance to these antibiotics is on the rise globally, often due to mutations in DNA gyrase and topoisomerase IV, quinolone-mediated resistance plasmids, and efflux pumps, such as the *oqx*AB efflux pump, which is prevalent in *K. pneumoniae* (4-10). While chemical inhibitors have been used to counter efflux pump activity, their toxic nature limits their widespread use (11, 12). Therefore, the exploration of natural inhibitors such as essential oils (EOs) from medicinal plants, like *Mentha longifolia*, has garnered interest. These EOs have shown antimicrobial activity and the ability to disrupt bacterial cell membranes and increase permeability (13-15). Since there has been no study on the effect of *M. longifolia* essential oil on biofilm and efflux pump gene expression formation in ciprofloxacin-resistant *K. pneumoniae* strains, this study aims to investigate the inhibitory effect of *M. longifolia* EO on the OqxAB efflux pump and biofilm formation in ciprofloxacin-resistant *K. pneumoniae* strains, considering the plant's abundance and availability in Iran, as well as its medicinal and edible uses.

MATERIALS AND METHODS

Isolation and identification of *K. pneumoniae* strains. In a cross-sectional study, From January to June 2021, 50 ciprofloxacin resistant *K. pneuomoniae* isolates were recovered from clinical specimens of hospitalized patients in Hamadan hospitals, Iran. *K. pneumoniae* isolates were confirmed by microbiological tests and polymerase chain reaction (PCR) in microbiology laboratory of Hamadan University of medical sciences. *K. pneumoniae* colonies observed as pink mucoid colonies on McConkey agar and were indole and MR negative, and VP, Simmons citrate, urea hydrolysis, and lysine decarboxylase positive in biochemical tests. PCR technique performed using species-specific primers for *ure*D gene (243 bp): *ure*DF: 5'-CCCGTTTTACCCGGAAGAAG-3' and *ure*DR: 5'-GGAAAGAAGATGGCATCCTGC-3' to amplify of *ure*D gene (15-17). PCR was conducted in a final reaction volume of 25 µl as follows: initial denaturation (3 minutes at 95°C) followed by 30 cycles of denaturation (30 seconds at 95°C), annealing (45 seconds at 45°C), extension (60 seconds at 72°C) and a final extension (60 seconds at 72°C) in a thermal cycler (Bio-Rad, Inc. USA). The final PCR products were electrophoresed on 1% agarose gel.

Antimicrobial susceptibility testing. Antimicrobial susceptibility to ciprofloxacin (CIP 5 µg/ml: Mast Company/UK) was detected by disk diffusion and microbroth dilution methods according CLSI guidelines (18).

Preparation of *Mentha longifolia* essential oil. The medicinal plant *M. longifolia* was harvested from the Alvand Mountains of Hamadan in the west of Iran, in April 2022. The leaves of *M. longifolia* were distilled by hydrolysis using a Clevenger-type apparatus to extract the essential oil. At last, the essential oil was acquired in the form of a pale yellow liquid. The essential oils were accurately weighed after being dehydrated with sodium sulfate, and they were kept in sealed bottles at 4°C in the dark until needed (19).

Antibacterial effect of *M. longifolia* essential oil by disc diffusion method. First, the antibacterial effect of *M. longifolia* essential oil (EO) on ciprofloxacin-resistant and standard *K. pneumoniae* (ATCC 10031) strains was investigated by disk diffusion method. A suspension of bacterial colonies with a concentration of 0.5 McFarland was prepared and cultured on Muller-Hinton agar using the lawn culture method. Then 30 microliters of *M. longifolia* was inoculated on a blank paper disk. A blank disk was also inoculated with 30 µl of Dimethyl Sulfoxide (DMSO). Then, the disks were placed on the prepared culture medium and after overnight incubation at 37°C, the effect of *M. longifolia* essential oil was studied by examining the inhibition zone around the colonies (19).

Antibacterial effects of *M. longifolia* essential oil by agar well diffusion. The Agar well diffusion assay was also used to measure EO's antibacterial activity. On sterile Muller–Hinton agar, 100 µl of ciprofloxacin-resistant *K. pneumoniae* and standard strains with a concentration of 0.5 McFarland were cultured. Wells in the agar (with a diameter of 8 millimeters) were created using a sterile Pasteur pipette, each well was filled with 100 milliliters of essential oil and DMSO (as a control). After incubating the plates at room temperature for one hour to facilitate the diffusion of essential oils into the agar wells, they were further incubated at 37°C for 24 hours to investigate the antibacterial activity of the essential oil, manifested by a distinct inhibitory zone surrounding the wells (20).

MIC and MBC detection of *M. longifolia* essential oil. The minimum inhibitory concentration (MIC) required to inhibit bacterial growth was determined using the microdilution method. The essential oils were dissolved in 10% DMSO at an initial concentration of 1000 µg/ml. The stock solutions of *M. longifolia* EO were diluted to achieve the following concentrations: 500, 250, 125, 62.5, 31.25, and 15.63 µg/mL. 95 µL of culture medium (Muller-Hinton broth), 5 µl of bacterial suspension (*K. pneumoniae*) with 0.5 McFarland dilution, and 100 µl of *M. longifolia* EO dilutions were added to each well, and then the microplates were incubated at 37°C for 24 h. Sub culturing sterile Muller-Hinton agar to wells that did not change color was used to determine the minimum bactericidal concentration (MBC). The plates were then left to incubate for 24 hours at 37°C (19, 21).

Synergistic effects *M. longifolia* essential oil and ciprofloxacin. Based on the standard protocol (CLSI), the minimum inhibitory concentration (MIC) of ciprofloxacin alone and in combination with EO was determined using the broth microdilution method in a 96-well microtiter plate for ciprofloxacin-resistant strains (22). In 96-well microplates, a dilution series of EO and ciprofloxacin (50 µl of antibiotic and 50 µl of essential oil) was prepared. The fractional inhibitory concentration (FIC) values for the two combined drugs (ciprofloxacin and essential oil) were obtained using the checkerboard method and the formula that follows: (MICAB/MICA) + (MICBA/MICB) is the formula for FIC. This was how the FIC index (FICI) was interpreted: synergistic effect less than 0.5, additive effect greater than or equal to 1.0, indifference effect between 1.0 and 4.0, and antagonistic effect less than 4.0 (19, 22).

Inhibitory effect of *M. longifolia* essential oil on biofilm formation. The microtiter plate method (MTP) using crystal violet, as previously mentioned, was used to carry out the biofilm formation assay (23). Using the microtiter plate method, the Minimum Biofilm Inhibitory Concentration (MBIC) of *M. longifolia* EO was also evaluated. All of the microplate's wells were first filled with 170 microliters of trypticase soy broth, followed by 20 microliters of bacterial suspension and then 10 microliters of various essential oil dilutions were added. The remaining procedures were identical to those of the biofilm formation assay test (24).

Detection of *oqxA* gene. The genomic DNAs were extracted from ciprofloxacin-resistant overnight *K. pneumoniae* strains by boiling method. Using the PCR all strains were examined for the presence of the efflux pump-encoded gene, including *oqx*A gene using specific primers: *oqx*AF (5′-CTCGGCGCGATGATGCT-3′) and *oqx*AR (5′-CACTCTTCACGGGAGACGA-3′) with products of 392 bp (25).

Real-time PCR. Changes in the expression level of the *oqx*A gene before and after *M. longifolia* was identified by cDNA amplification using qPCR method. A total of 11 ciprofloxacin-resistant *K. pneumoniae* strains containing the *oqx*A gene were cultured in Muller-Hinton broth, suspended in diluted *M. longifolia* EO at sub-MIC concentrations, and incubated for 24 hours at 37ºC following the guidelines provided by Ghafari et al. (22). Bacterial strains were prepared prior to RNA extraction total RNA was isolated using an RNA extraction kit (SinaClone, Iran) and then transcribed into cDNA using a cDNA synthesis kit (AddBio, Korea) following the manufacturer's protocol. The extracted cDNAs were stored at -20°C to use as DNA templates in the Real-time-PCR reaction.

Real-time quantification of cDNA was performed with the detection system (Roche, Germany) using the SYBR green PCR master mix. The optimized reactions were composed of a master mix (10X), 1 µl of each primer (10 pmol each), 2 µl of cDNA (100 µg/ml), and 6 µl of DEPC-treated water, making a total volume of 20 µl. The *ure*D gene primer was used as the internal control (7). Relative expression of the *oqx*A gene was calculated using the $2^{-\Delta\Delta Ct}$ method (26). Amplification proceeded as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 60 sec, annealing at 72°C for 30 sec, and melting curve at 60°C for 15 sec 94°C for 15 seconds.

Statistical analysis. The categorical variables were reported as percent, frequency, and continuous variables were reported as mean and standard deviation (SD). The Chi-square or Fisher's exact tests were used to test the association between categorical variables. The paired t-test was used to compare the expression level of *oqx*A before and after treatment. The statistical significance level was set at 0.05. Stata 14.2 (StataCorp, TX, US) was used for data analysis.

Ethics approval and consent to participate. All

the experiments in our study were conducted in accordance to the relevant guidelines and regulations or in accordance to the Declaration of Helsinki. The present study was ethically approved by the Hamadan University of Medical Sciences, Institutional Review Board (IR.UMSHA.REC.1400.863).

RESULTS

Antimicrobial effect of *M. longifolia* EO. The results of disk diffusion and well diffusion indicated that *M. longifolia* EO inhibits the standard strain and 50 ciprofloxacin-resistant *K. pneumoniae* clinical strains. Around the disk and the well containing *M. longifolia* EO, inhibition zones were observed.

MIC of *M. longifolia* EO and ciprofloxacin. The MICs of ciprofloxacin varied from 16 to 256 µg/ml. The MIC ranges for ciprofloxacin were as follows: 50 ciprofloxacin-resistant *K. pneumonia* strains contained concentrations of 32 (4%), 64 (18%), 128 (26%), and 256 (52%). By using the microbroth dilution method, the MIC of *M. longifolia* EO was found to range from 31.25 µg/ml to 500 µg/ml in *K. pneumoniae* clinical strains. The following were the EO MIC ranges for *M. longifolia*: 31.25 (2%), 62.5 (42%), 125 (26%), 250 (24%), and 500 µg/ml (6%). In 2%, 46%, 16 and 36% of isolates, *M. longifolia* EO had a minimum bactericidal concentration (MBC) of 62.5 µg/ml, 125 µg/ml, 250 µg/ml, and 500 µg/ml, respectively (Fig. 1).

Synergistic effects of ciprofloxacin in combination with *M. longifolia* EO. Additionally, the MIC of ciprofloxacin in combination with EO was examined and compared to the results of ciprofloxacin alone before adding EO. The findings demonstrated that the addition of EO decreased the ciprofloxacin MIC. The MIC of ciprofloxacin decreased by fourfold, threefold, and twofold in 46 isolates (92%), 14 isolates (28%), 12 isolates (24%), and 11 isolates (22%). There was no change in MIC in four isolates (8%). In 14 isolates (28%) of *K. pneumonia* the combination of ciprofloxacin and EO (FICI) demonstrated that EO had a synergistic effect. These strains were found to have FICI≤ 0.5. In these strains, the various FICI values included 0.5, 0.15, 0.22, 0.23, 0.35, 0.36, and 0.47. It should be noted that none of the isolates had an antagonistic effect. In 28% of the isolates, the essential oil had an additive effect (FICI 0.5 and 1), and in 44% of the isolates, there was no interaction or difference (FICI>1).

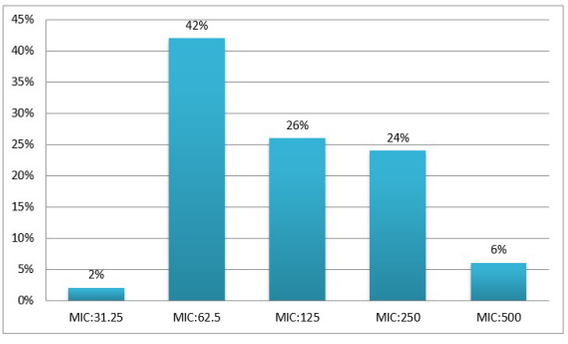

Fig. 1. MIC values of *M. longifolia* in 50 clinical *K. pneumoniae* strains

Effect of *M. longifolia* EO on biofilm formation strength. There was no biofilm formation in 18 of the 50 ciprofloxacin-resistant *K. pneumoniae* strains tested in the biofilm formation assay. 14 (28%), 12 (24%), and 6 (12%) isolates showed signs of the weak, moderate, and strong biofilm phenotype, respectively. The addition of *M. longifolia* EO resulted in a change in the biofilm phenotype. After exposure to *M. longifolia* EO, among the 6 isolates that were strong biofilm forming, one isolate showed no change, while 3 isolates transitioned from strong to medium biofilm, and 2 isolates shifted from strong to no biofilm formation. Among 12 isolates forming moderate biofilm after the effect of *M. longifolia* EO, in 7 isolates from moderate biofilm to weak biofilm and in 5 isolates from moderate biofilm to no biofilm formation was observed (Table 1). Statistical analysis results showed that there was a significant relationship between the reduction of biofilm and the effectiveness of *M. longifolia* EO ($p<0.001$).

Effect of *M. longifolia* EO on expression of *oqx*A gene. Based on the PCR results, the prevalence of *oqx*A in *K. pneumoniae* isolates was found to be 95%. Real-time PCR results showed that *oqx*A gene expression in *K. pneumoniae* was significantly reduced after treatment with *M. longifolia* EO ($P<0.001$). The results of the Real-time PCR confirmed the results of the checkerboard test, which showed an inhibitory effect of *M. longifolia* EO on *oqx*AB efflux pump expression, as this pump also contributes to the development of resistance to fluoroquinolones. After being treated with *M. longifolia* EO, the expression level of the gene *oqx*A decreased in 11 of the strains that were examined, though the rate of the decrease varied from strain to strain. From 1.19 to 5.88, various ratios were found (Fig. 2).

Table 1. Changes in the strength of biofilm formation after *M. longifolia* essential oil treatment in *K. pneumoniae* strains

Change in biofilm strength	Frequency	Reduction (fold)
Strong to no biofilm	2 isolates out of 6 isolates (33.3%)	4
Strong to moderate	3 isolates out of 6 isolates (50%)	2
Strong to strong	1 isolates out of 6 isolates (16.6%)	-
Moderate to weak	7 isolates out of 12 isolates (58.3%)	2
Moderate to no biofilm	5 isolates out of 12 isolates (41.6%)	3

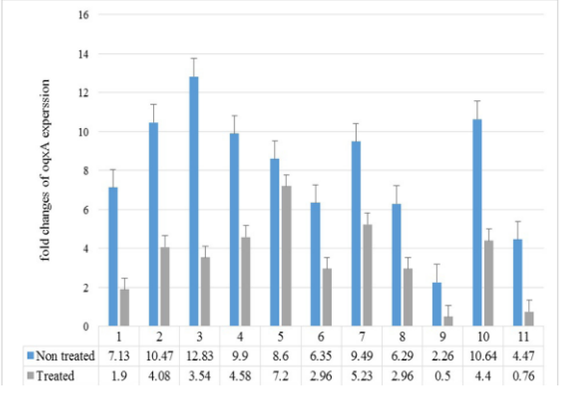

Fig. 2. Comparison of *oqx*A gene expression before and after exposure to *M. longifolia* essential oil in 50 ciprofloxacin-resistant *K. pneumoniae* strains

DISCUSSION

In this study, the antibacterial effect of *M. longifolia* EO against ciprofloxacin-resistant *K. pneumoniae* strains was demonstrated by disk diffusion and well diffusion. It was found that *M. longifolia* EO was able to reduce the MIC of ciprofloxacin up to fourfold in *K. pneumoniae* strains and in some strains, *M. longifolia* EO and ciprofloxacin have a synergistic effect. Furthermore, the results of the present study showed that *M. longifolia* EO could alter the strength of biofilm formation and also reduce the expression of the efflux pump gene. All of these results suggesting antibacterial effects of *M. longifolia* EO in different ways. In our previous study, high antibiotic resistance including resistance to ciprofloxacin was found (7). In the mentioned study, the MDR phenotype was observed in 65% and ciprofloxacin resistance in 89% of *K. pneumoniae* strains. In addition, 98% of the strains had the *oqx*B gene and 95% of the strains had the *oqx*A gene. A significant relationship was observed between the presence of the OqxAB pump genes and resistance to ciprofloxacin (7).

Essential oils or other plant derivatives' effects on fluoroquinolones, including ciprofloxacin, and their inhibitory effect on bacteria's efflux pumps have been the subject of several studies.

In Mahmoudi et al. research, *M. longifolia* EO's effects on clinical *Acinetobacter baumannii* isolates were examined in Iran. They measured the interaction between essential oil, ciprofloxacin, and imipenem antibiotics in their study. Their study's findings are in line with those of our study. In their study, only the frequency of adeABC efflux pump genes was determined and the effect of EO on the expression of the efflux pump was not investigated. The reduction in MIC indirectly suggested that *M. longifolia* EO may also affect efflux pumps. Contrary to our findings, there was no synergistic effect between ciprofloxacin and *M. longifolia* EO. This suggests that the type of plant or bacteria may have contributed to the findings (19).

Makvandi et al. in southern Iran investigated the antibacterial effect of *M. longifolia* EO against standard and clinical strains of *Shigella flexneri* and *Shigella sonnei* as well as the effect of *M. longifolia* on the diameter of the inhibitory zone around antibiotic disks (gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole, ampicillin). Their results showed that *M. longifolia* EO has a strong antibacterial effect against strains of *S. flexneri* and *S. sonni* and significantly increased the inhibitory effect (diameter of inhibitory zone) of the antibiotic. Their results, like the results of our study, show the antibacterial effect of *M. longifolia* EO against bacteria, including Gram-negative bacteria (27).

Seasotiya et al. conducted research in India to investigate the inhibitory and synergistic effects of 35 medicinal plant extracts and fluoroquinolones (ciprofloxacin and ofloxacin) on a range of gram-positive and gram-negative bacterial strains. The findings of that study indicated that plant extracts increased drug accumulation in bacteria while, on the other hand, decreasing the efflux of fluoroquinolones, which is in line with our results (28).

The inhibitory effect of essential oils on the expression of efflux pump genes was investigated in some studies, and the results showed that some essential oils reduced the expression of efflux pump genes in antibiotic resistant bacteria strains.

In a study by Islamieh et al. Real-Time PCR was used to see if *Satureja khuzistanica* EO reduced the expression of the efflux pump genes MexEF-OprN and MexXY-OprM in MDR *Pseudomonas aeruginosa* strains. In the presence of essential oil at sub-inhibitory concentrations (1.16 to 2), synergistic effects were observed. The effects of gentamicin and norfloxacin were up to eight times stronger. In accordance with our study's findings, treatment with *S. khuzistanica* EO resulted in a decrease in the expression of mexY and mexE genes (29). In a study conducted by Ghafari et al. with different essential oils, bacteria and efflux pumps, similar to the results of our study, plant essential oils had antibacterial properties and inhibition of efflux pumps. In the mentioned study, the effects of *Thymus daenensis* and *Origanum vulgare* essential oils on ciprofloxacin absorption in fluoroquinolone-resistant *Streptococcus pneumoniae* strains with increased expression of pmrA efflux pump were investigated. The results showed that *T. daenensis* and *O. vulgare* essential oils have antibacterial and efflux pump inhibitory effects in pneumococcal clinical isolates and the combination of these two essential oils with fluoroquinolone antibiotics may provide alternative ways to overcome fluoroquinolone-resistant pneumococci (22).

Compounds that inhibit or reduce biofilm formation can be useful in reducing antibiotic resistance because biofilm formation is thought to be one of the mechanisms by which bacteria become resistant to antibiotics. The effect of medicinal plant compounds, such as essential oil, on the development of bacterial biofilm has been the subject of some research. Martinez et al. investigated the antimicrobial and anti-biofilm properties of 15 essential oils (EOs) against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. The majority of the EOs tested exhibited antibiofilm activity against both strains. Among them, the Lippia origanoides thymol-carvacrol II chemotype EO demonstrated the most significant biofilm inhibitory and antibacterial effect on both strains, with a biofilm formation inhibition of 71% and 76% on *S. aureus* and *E. coli*, respectively (24).

In the Turkish study by Pazarci et al. the anti-biofilm activity of *M. longifolia* EO was investigated using standard strains of *E. faecalis, E. coli, S. aureus, Pseudomonas aeruginosa, K. pneumoniae,* and *Candida albicans* on the surfaces of steel and titanium orthopedic implant surfaces. The study found that at various concentrations of essential oil on titanium surfaces, the eradication of biofilm by microorganisms varied significantly. *P. aeruginosa* was identified as the most resistant strain to *M. longifolia* EO, while *S. aureus* and *C. albicans* were the most sensitive strains (30). Al-Shuneigat et al. conducted a study in Jordan to investigate the antibacterial and anti-biofilm effects of plant essential oils. Clinical strains of *Staphylococcus epidermidis* and *Proteus mirabilis, E. coli, S. aureus, P. aeruginosa,* and *K. pneumoniae* were found to have biofilm formation affected by *Thymus vulgaris* EO in their study. *T. vulgaris* essential oil's MIC and MBIC values demonstrated its potent antibacterial and antibiofilm activity. *P. aeruginosa*'s ability to bind to polyester surfaces was reduced by the sub-MIC inhibitory concentration of *T. vulgaris* EO (31).

CONCLUSION

This study revealed that *M. longifolia* essential oil can inhibit resistant *K. pneumoniae* strains by inhibiting the efflux pump, weakening biofilm formation, and decreasing antibiotic resistance. Antibiotics can be supplemented with *M. longifolia* oil EO to reduce bacteria's resistance to antibiotics. Additionally, the findings demonstrate that antibiotics and readily available oral and therapeutic plant compounds can be combined to treat bacterial infections more effectively and prevent the development of antibiotic resistance. The variety of bacterial strains, the variety of plant and its derivatives, and even the occupant of medicinal plants can all contribute to variations in studies results.

Based on the results of this and other similar studies, it is proposed: 1. Study on effects of available native medicinal plants and their derivatives on antibiotic-resistant bacteria, 2. Study on effects of medicinal plants in combination with antibiotics, 3. Study on effects inhibition and anti-biofilm formation of medicinal plants and their derivatives in vivo, 4. Using the results of studies conducted in the field of medicinal plants to prepare combination drugs or therapeutic supplements.

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