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Research Article

Synergistic Antibacterial Activity of 1-Methyl Ester-Nigericin and Methyl 5-(Hydroxymethyl) Furan-2-Carboxylate Against *Proteus* spp.

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Abstract

Background and Objective: Synergistic combinations of antimicrobial agents with different mechanisms of action are successful approaches for combating bacterial infections. This study aimed to evaluate the synergistic effect of 1-methyl ester-nigericin (**1**) and methyl 5-(hydroxymethyl) furan-2-carboxylate (**2**) against *Proteus* spp., isolates. **Materials and Methods:** The synergistic antimicrobial activity of the compounds was tested by the checkerboard method and time-kill curves. To estimate the interaction between the compounds, the Fractional Inhibitory Concentration Index (FICI) of the combination was calculated. The cytotoxic activity of the compounds in combination was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on LLC-MK2 cell lines. The reduction percentage of biofilms was obtained using the colourimetric method. **Results:** The MIC values for compounds **1** and **2** against test bacteria ranged from 39.06-78.12 μg mL^{-1} and from 78.12-156.25 μg mL^{-1}, respectively. The MIC was reduced to 1-8th as a result of the combination of compounds **1** and **2**. After 4-24 hrs of treatment with ½ MIC of compounds **1** and **2**, the killing rate (in CFU mL^{-1}) increased to a greater degree than observed with either test compound alone. The combination of compounds **1** and **2** showed a synergistic effect with FICI of 0.50 and 0.28. The synergistic combination of compounds **1** and **2** was effective on the biofilm reduction of *Proteus vulgaris* NP16 (85.72%) and NP47 (89.14%). **Conclusion:** This study recommends compounds **1** and **2** in combination as a potential alternative treatment agent for *Proteus* spp. infections.

Key words: 1-methyl ester-nigericin, antibacterial activity, biofilm elimination, cytotoxic activity, methyl 5-(hydroxymethyl) furan-2-carboxylate, *Proteus* spp., synergistic effect

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Proteus spp., is an opportunistic bacterial pathogen that, under favourable conditions, cause Urinary Tract Infections (UTIs), wound infections, meningitis in neonates or infants and rheumatoid arthritis[1]. Among various *Proteus* species, *Proteus mirabilis* and *Proteus vulgaris* are clinically significant and usually responsible for UTIs and wound infections. They are the 5th most common cause of nosocomial UTIs and sepsis in hospitalized individuals[2]. The spread of multidrug-resistant *P. mirabilis* and *P. vulgaris* isolates producing extended-spectrum β-lactamases is constantly increasing worldwide[3-11]. One virulence factor of *Proteus* spp., is biofilm formation: An attached structure with microbial cells and populations embedded in a polysaccharide layer. For example, the biofilm facilitates, survival, enabling better adaptation to conditions of the external environment and enhancing resistance to antibiotics and the host immune system[12].

Synergism is the act of combining 2 different compounds to enhance their activity. If such a combination results in a worsening effect, it is called antagonism. An effect less than synergistic effect but not antagonistic, is termed as additive or indifference[13]. In synergism, microbial inhibition is achieved at concentrations below that for each agent alone[14], hence, synergy is determined as a significantly greater activity provided by 2 agents combined than that provided by the sum of each agent alone[15,16]. In general, the clinical use of the combination of antibiotic therapy for bacterial infections can be divided into 2 categories[16]. In the 1st category, such therapy improves clinical outcomes of infections with strains susceptible to 1 or more individual antibiotics. The primary rationale for combining 2 agents is to enhance the activity of either agent by achieving a synergistic effect. The secondary rationale is to allow lower doses of either antibiotic to reduce toxicity. The third rationale is to use 2 antibiotics that might prevent the emergence of resistance to either antibiotic. The second category of antibiotic combination use has evolved during the last decade, during which certain clinical species have become resistant to all available antibacterial agents or all except a single agent. Combination therapy is occasionally recommended to prevent resistance from emerging during treatment[14,16].

Recently, 1-methyl ester-nigericin (**1**, MEN) and methyl 5-(hydroxymethyl) furan-2-carboxylate (**2**, MFC) were isolated from *Streptomyces hygroscopicus* BRM10, an endophyte in *Alpinia galanga*[17] and *Streptomyces zerumbet* W14, an endophyte in *Zingiber zerumbet*[18], respectively. Both compounds showed great antibacterial properties. Compound **1** is a carboxylic ionophore that can intercalate into intracellular organelle membranes and exchange protons for K^+ or Na^+[19]. Compound **2** is a furancarboxylate that leads to bacteriolysis by causing cell wall damage[20]. Synergistic combinations of antimicrobial agents with different mechanisms of action are successful approaches for combating bacterial infections. To the best of the authors' knowledge, furan and furanone derivatives are antibiofilm agents reported in the literature. For example, Liu *et al.*[21] synthesized 5-substituted 3,4-dihalo-5H-furan-2-one derivatives: 3,4-dibromo-5-(4-nitrofuran-2-yl(hydroxy)methyl) furan-2(5H)-one, 3,4-dibromo-5-(phenylmethylene)-furan-2(5H)-one and 3,4-dibromo-5-((4-hydrophenyl) methylene)-furan-2(5H)-one. These compounds showed remarkable effects of biofilm formation inhibition on *Pseudomonas aeruginosa*. A phytochemical compound, 7-O-b-D-(6'-O-malonyl)-glucopyranosyl-5-methoxy-1(3H)-isobenzofuranone, extracted from *Helichrysum* italicum, was found to inhibit *P. aeruginosa* biofilm formation[22]. Jakobsen *et al.*[23] reported quorum-sensing inhibitors, (Z)-5-(bromomethylene)furan-2(5H)-one, (Z)-4-bromo-5-(bromomethylene)furan-2(5H)-one, (R)-10-isothiocyanato-3-oxo-N-(2-oxotetrahydrofuran-3-yl) decanamide, etc., against *P. aeruginosa*. Thus, antibiofilm compounds with synergistic activity against *Proteus* spp., could be interesting antibiotic adjuvants to prevent or treat chronic infections.

This study aimed to evaluate the synergistic effect of compounds **1** and **2** on the growth inhibition and biofilm elimination of 2 reference strains of *P. mirabilis* and 2 clinical isolates of Multi Drug Resistant (MDR) *P. vulgaris*.

MATERIALS AND METHODS

Study area: The study was carried out at the Departments of Microbiology and Chemistry, Silpakorn University, Nakhon Pathom, Thailand, from January-July, 2021.

Cultivation of *Streptomyces* and product isolation: *S. hygroscopicus* BRM10 and *S. zerumbet* W14 were obtained as endophytes from *A. galanga* Swartz and *Z. zerumbet* (L.) Smith, respectively, using the surface sterilization technique. Bacteria were grown on an ISP-2 agar at 30°C for 14 days. The initial steps of antibiotic isolation and purification were described previously[17,18]. The purified compounds were subjected to investigation by nuclear magnetic resonance spectroscopy. The spectral data for these compounds identified them as MEN ($C_{41}H_{70}O_{11}$; **1**) and MFC ($C_7H_8O_4$; **2**), respectively (Fig. 1).

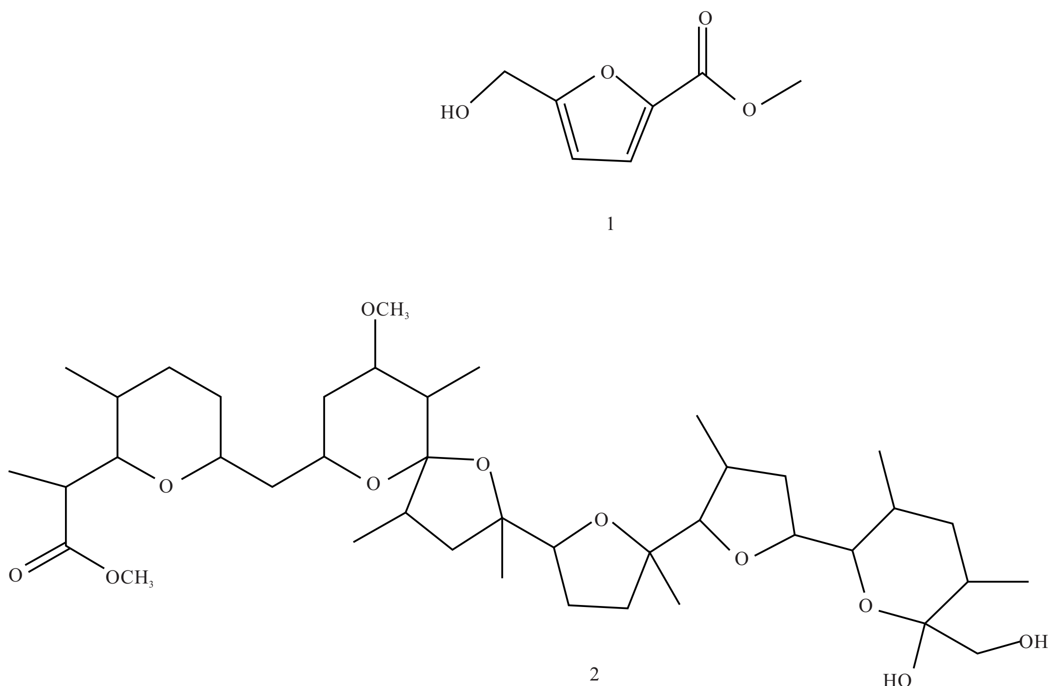

Fig. 1: Chemical structure of the test compounds
1-methyl ester-nigericin, MEN ($C_{41}H_{70}O_{11}$; 1) and methyl 5-(hydroxymethyl) furan-2-carboxylate; MFC ($C_7H_8O_4$; 2)

Preparation of test bacterial strains: Two clinical isolates of MDR *P. vulgaris* NP16 and NP47 were isolated from urine samples of UTI hospitalized patients in Nakhon Pathom Hospital, Thailand. The characterization of isolated strains was determined by biochemical tests. The antibiotic susceptibility test of clinical bacterial strains was done by the Kirby-Bauer disk diffusion method[24] as in the Clinical Laboratory Standard Institute (CLSI) guidelines[25]. The MDR strain was defined as a resistant bacterium to 3 or more antibiotic classes composed of aminoglycoside, b-lactam, quinolone and tetracycline. Standard strains of *P. mirabilis* TISTR100 and ATCC35659 were also used. Bacterial colonies were inoculated into Mueller Hinton (MH) broth (HiMedia, India) to turbidity comparable to 0.5 McFarland standard, equivalent to a bacterial count of ~10^6 CFU mL^{-1}.

Determination of minimum inhibitory concentrations (MICs): The antimicrobial activities of compounds 1 and 2 against clinical isolates of *P. vulgaris* and reference strains were determined by the micro broth dilution method using MH broth[26]. The MIC was recorded as the lowest concentration of the test samples, resulting in complete growth inhibition.

Synergistic antimicrobial assays: Synergistic combinations were investigated by the checkerboard method using clinical isolates of *P. vulgaris* and reference strains via MIC determination[27]. The concentrations of compounds 1 and 2 ranged from 2500-2.441 and from 1250-19.531 µg mL^{-1}, respectively. The antimicrobial activity of the compound combination was classified into one of the following categories: Synergy, indifferent, additive or antagonism. The Fractional Inhibitory Concentration (FIC) of each compound was calculated as the MIC of the compounds in combination divided by the MIC of the test compound alone. The interpretation of the antimicrobial activity of the compound combination was made according to the FIC index (SFICI), which is the sum of FICs of both compounds. FICI results were interpreted as follows: <0.5, synergy, 0.5 to 1, additive, 1 to 2, indifferent and >2, antagonism[28].

Time-kill curves: Time-kill curves were performed on successful synergistic combinations obtained by the checkerboard method. Flasks containing 100 mL MH broth and the compound combination were inoculated with a log-phase culture of test bacterial strains at a density of 1×10^5 CFU mL^{-1}. Individual components of each combination, either compound 1 or 2, were added to the control flask for comparing the effects of synergistic combinations to their individual effects on the bacterial growth curve, whereas no compound was added to the growth control flask. Flasks were incubated for 24 hrs at 37°C. One hundred microliters of the broth were collected at different time intervals from each flask,

serially diluted in phosphate-buffered saline and cultured on MH agar plates to obtain colony counts[29]. Curves were constructed by plotting the $\log_{10}$ of CFU mL^{-1} versus time. Synergy was defined as $\geq 2 \log_{10}$ decreases in CFU of bacteria treated with the drug combination compared to the most active component of the test compound alone as described previously[30].

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability: LLC-MK2 cells (rhesus monkey kidney cells) were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U mL^{-1}) and streptomycin sulfate (100 µg mL^{-1}) at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxicity studies were performed in a 96-well plate. The concentration of the compound combination, either compound 1 or 2, required to inhibit 50% of the growth of cell lines (IC_{50} values) was calculated by analyzing the relationship between concentrations and percent inhibitions. The therapeutic index of the compounds, the ratio of the dose that produced cytotoxicity to the dose that produced an effective response, was also calculated. The details of the procedures were described previously[31].

Biofilm elimination: MDR *P. vulgaris* NP16 and NP47 were grown in tryptic soy broth (TSB, HiMedia) for 18 hrs at 37°C at 150 rpm. Two hundred micro liters of bacterial culture were added in the wells of a polystyrene 96-well plate in three repetitions each. A sterility control was made of 200 µL TSB in at least three repetitions. The culture was incubated in a humid chamber at 37°C for 24 hrs. The content of each well was aspirated and each well was washed thrice with 250 µL sterile physiological saline and left to dry at 37°C for 15 min. Thereafter, 200 µL synergistic concentrations of compounds 1 and 2 in combination and compound 1 or 2 alone were added to each well but not to the control. After 4 hrs, the content was removed from wells and microtiter plate wells were washed 5 times with sterile distilled water to remove loosely associated bacteria. The plates were stained for 5 min with 200 µL of 2% crystal violet (Sigma-Aldrich, USA) per well. Excess stain was rinsed off by placing the plate under running tap water. The plates were air-dried and the bounded dye to the adherent cells was resolubilized with 160 µL of 33% (v/v) glacial acetic acid per well and incubated at 30°C for 15 min. The Optical Density (OD) of each well was measured at 492 nm using an enzyme-linked immunosorbent assay reader (Sunostik SPR-960, China). Finally, the reduction percentage of biofilms was obtained using the following formula:

$$\text{Reduction (\%)} = \frac{(C-B)-(T-B)}{(C-B)} \times 100$$

Where:

C = Mean OD of control wells
B = Mean OD of negative controls
T = Mean OD of test wells

Statistical analysis: Data were analyzed by analysis of variance followed by the Tukey-Kramer multiple comparison test. The $p<0.05$ was considered significant.

RESULTS

Antimicrobial activity of the compounds: Susceptibility studies showed that compounds 1 and 2 have antimicrobial activity against MDR *P. vulgaris* NP16 and NP47 and reference strains of *P. mirabilis* TISTR100 and ATCC35659 (Table 1). The clinical isolates of *P. vulgaris* NP16 with phenotypes resistant to ampicillin, streptomycin, nalidixic acid and tetracycline and *P. vulgaris* NP47 with phenotypes resistant to ampicillin, streptomycin, nalidixic acid, ciprofloxacin and tetracycline were inhibited by 12.5 µg mL^{-1} compound 1 and 50 µg mL^{-1} compound 2 by the disk diffusion method. Their MICs of compounds 1 and 2 ranged from 39.06-78.12 µg mL^{-1} and from 78.12-156.25 µg mL^{-1}, respectively. Although insignificant, the MICs of compounds 1 and 2 against *Proteus* spp., were lower for the reference strains of *P. mirabilis* than the clinical isolates of *P. vulgaris*.

Synergistic activity of the compound combination: Compounds 1 and 2 combinations were checked for synergistic activity and 2 different combinations showed strong synergistic activity (Table 1). When compounds 1 and 2 were combined, they inhibited *Proteus* spp., at sub-MIC levels. There was a significant reduction in MICs of the compound combination, explaining strong synergy (FICI = 0.28-0.50) in this combination. Time-kill curves showed synergy at 2, 4, 6, 8, 10 and 24 hrs in all test bacteria: *P. vulgaris* NP16 (Fig. 2a), *P. vulgaris* NP47 (Fig. 2b), *P. mirabilis* TISTR100 (Fig. 2c) and *P. mirabilis* ATCC35659 (Fig. 2d). A complete bactericidal effect was observed after 24 hrs of incubation. Compound 1 with 8-32 times reduction in MICs and compound 2 with 2-4 times reduction in MICs were observed when used in combination. In this combination, compound 1 inhibited *P. vulgaris* NP47 at 2.44 µg mL^{-1}, which was 32 times

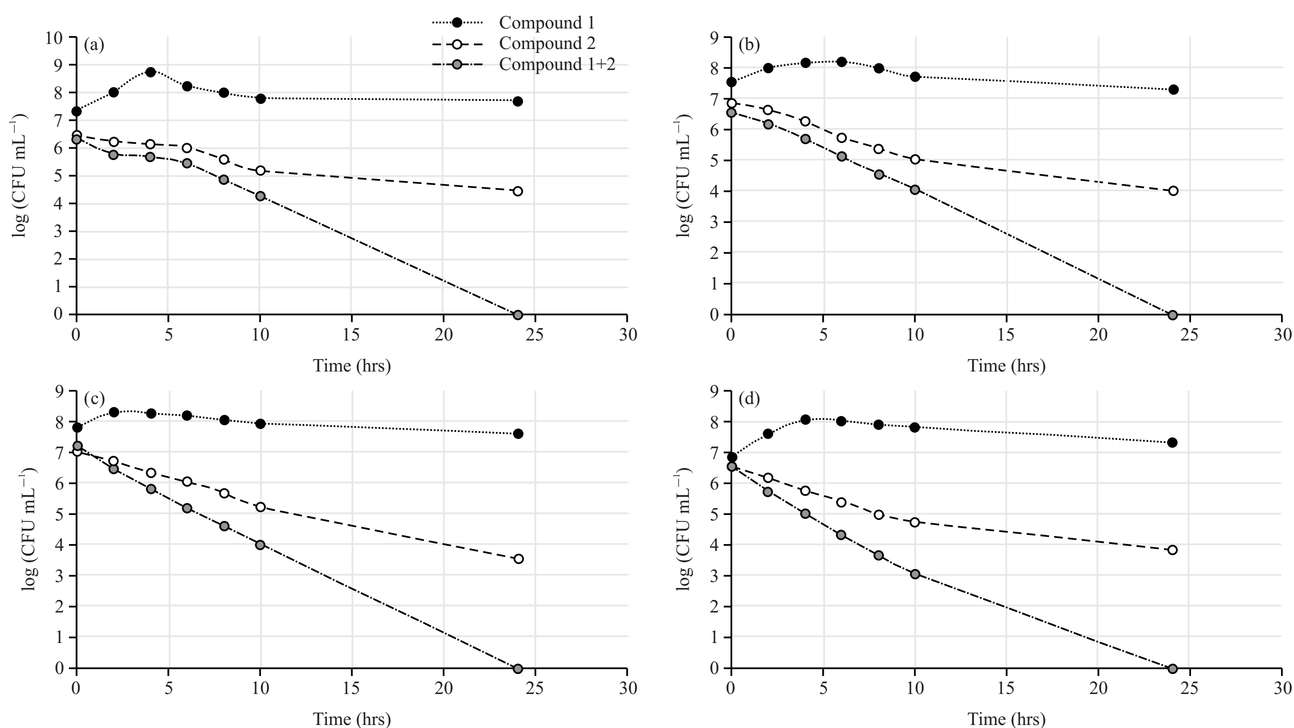

Fig. 2(a-d): Synergistic antimicrobial activity of the compound combination by time-kill curves, time-kill curves of MIC of compounds **1** and **2** alone and ½ MIC of compound **1** with ½ MIC of compound **2** against (a) *P. vulgaris* NP16, (b) *P. vulgaris* NP47, (c) *P. mirabilis* TISTR100 and (d) *P. mirabilis* ATCC35659

Bacteria were incubated with compound 1 alone, compound 2 alone, or compounds 1 and 2 in combination

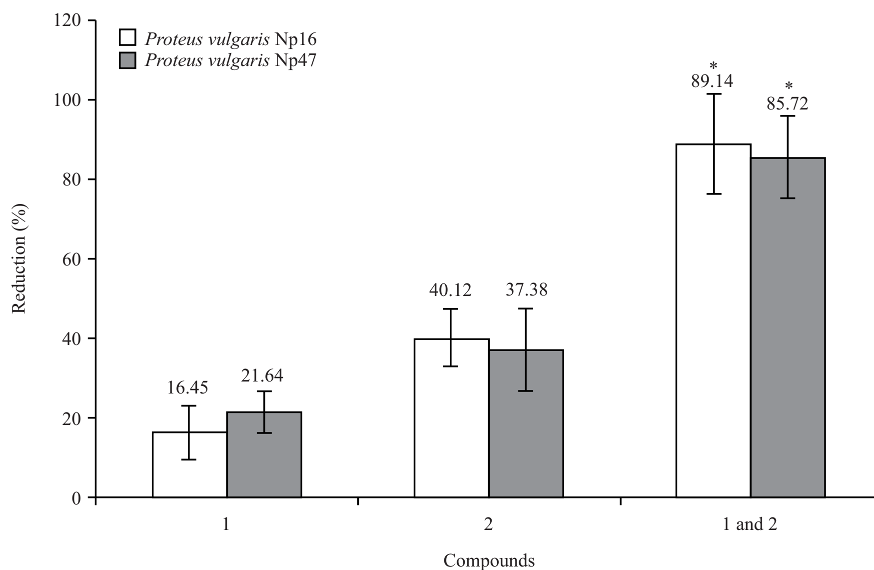

Fig. 3: Biofilm elimination of the test compound alone and in combination on *P. vulgaris* NP16 and NP47

Growing bacteria were incubated with compound alone or compounds in combination in a 96-well plate. After 4 hrs, the plate wells were washed and stained for 5 min with 2% crystal violet. The bounded dye to the adherent cells was resolubilized with 33% (v/v) glacial acetic acid at 30°C for 15 min. The optical density (OD) of each well was measured at 492 nm. The reduction percentage of biofilms was calculated and *p<0.05 versus the test compound alone

Table 1: Synergistic antimicrobial activity of compounds 1 and 2 in *Proteus* spp.

Bacteria	Resistance phenotype	MIC of compound 1 alone ($\mu g\ mL^{-1}$)	MIC of compound 2 alone ($\mu g\ mL^{-1}$)	MIC of compounds in combination 1:2 ($\mu g\ mL^{-1}$)	FICI	Outcome
P. vulgaris NP16	Amp, Str, Na, Te	78.12	156.25	4.88:39.06	0.28	Synergy
P. vulgaris NP47	Amp, Str, Na, Cip, Te	78.12	156.25	2.44:78.12	0.50	Synergy
P. mirabilis TISTR100	-	78.12	78.12	4.88:39.06	0.28	Synergy
P. mirabilis ATCC35659	-	39.06	78.12	4.88:39.06	0.28	Synergy

Resistance phenotypes: Amp: Ampicillin, Str: Streptomycin, Na: Nalidixic acid, Cip: Ciprofloxacin and Te: Tetracycline

Table 2: Cytotoxic activity (IC_{50} values) of the test compound alone and in combination against LLC-MK2 cells

Compounds	IC_{50} values[a] on LLC-MK2[b] ($\mu g\ mL^{-1}$)	SI[c]
1	15,321.00	196.12
2	969.52	6.20
1 and 2 in combination	903.71	393.53

[a]IC_{50} values: Concentration causing 50% growth inhibition, [b]LLC-MK2: Rhesus monkey kidney epithelial cell lines and [c]SI: A ratio that compares the IC_{50} concentration at which a compound becomes toxic and the MIC value at which a compound is effective

lower than the MIC of compound 1 alone and indicated an effective MDR if used with compound 2.

Cytotoxic activity of the compound combination: Table 2 presents the IC_{50} values of compounds 1 and 2 alone and combination for LLC-MK2 cells. The Safety Index (SI) was defined as the ratio of the concentration of the compound required for 50% cell kill to the MIC value. The main aim of this study was to evaluate the cytotoxic activity of the combinations of compounds 1 and 2 compared to those of the compounds alone. In the combination treatment, cells were treated with compounds 1 and 2 at a MIC ratio in combination (4.88:39.06) and 2 fold increasing concentrations. Cytotoxicity activity was investigated using the MTT assay. The IC_{50} values of the compound treatments are shown in Table 2. Between treatment compounds, compound 1 was found safer than compound 2, however, the highest SI value appeared in the combination treatment, suggesting that the combination treatment is safer than either test compound alone.

Biofilm elimination of compounds in combination: The elimination potential of the compounds in combination with bacterial biofilms is shown in Fig. 3. Approximately 85.72 and 89.14% of bacterial biofilms of *P. vulgaris* NP16 and NP47 were eliminated by the synergistic concentrations of compounds 1 and 2 in combination, respectively.

DISCUSSION

Bacterial resistance to conventional antibiotics combined with an increasing acknowledgment of the role of biofilms in chronic infections has led to a growing interest in new antimicrobial strategies that target the biofilm mode of growth. Many researchers have studied the synergistic effect of the compound combination that could be used as antimicrobial agents against infectious microorganisms and have employed novel dosing regimens and antimicrobials that would be advantageous for combating the therapeutic problems associated with MDR *Proteus* spp.[32-34]. Combination studies indicated synergies between MEN and MFC and the FICI aids in the quantification of the degree of synergy. The FICI demonstrated that the activity of MEN combined with MFC was $\leq$0.5, indicating a synergistic antimicrobial activity against *Proteus* spp., including the clinical isolates of MDR *P. vulgaris*. A previous study showed that MEN had antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* ATCC25932 and *Bacillus cereus* ATCC6633, with MIC values of 0.5 and 1.0 $\mu g\ mL^{-1}$, respectively[17]. In this study, the MIC values of MEN against *Proteus* spp., ranged from 39.06-78.12 $\mu g\ mL^{-1}$. For MFC, the antibacterial activity against Gram-positive bacteria *S. aureus* ATCC25932 and *B. cereus* ATCC7064 had MIC values of 1.00 and 4.00 $\mu g\ mL^{-1}$, respectively and the antibacterial activity against Gram-negative bacteria *Escherichia coli* ATCC10536, *Salmonella typhi* ATCC19430, *Serratia marcescens* ATCC8100 and *P. aeruginosa* ATCC27853 had the MIC values between 32 and 64 $\mu g\ mL^{-1}$, described in the previous report[20]. Although the antimicrobial activity of these compounds has been previously reported, their interaction in combination is unknown, which is prescribed to treat MDR bacterial infections, especially in MDR *Proteus* spp. infections that produce biofilm formation.

In general, bacterial biofilm formation generates at least 3 steps: Reversible adsorption, primary adhesion of microorganisms to the surface and colonization. The rates of these processes vary widely depending on environmental conditions and the type of microorganisms but adhesion and colonization stages are considered to be relatively slow compared to the 1st step of cell adsorption[35,36]. Biofilm formation is one of the important virulence factors of

pathogenic bacteria. Its structure preserves bacteria from the unfavourable influence of environmental conditions and facilitates the distribution of the nutritional agents[37]. Biofilm protects bacteria from the host's immune system response (hinders phagocytosis, chemotaxis and opsonization) and decreases antibiotic and antibody penetration[38]. After evaluating different concentrations of biofilm formation by pathogenic bacteria, Mahdavi *et al.*[36] concluded that the crystal violet-based method is a quick screening technique with high sensitivity. Kwiecinska-Piróg *et al.*[39] evaluated biofilm formation by *P. mirabilis* strains using 2 independent quantitative and qualitative methods with 2,3,5-triphenyl tetrazolium chloride and crystal violet application. *P. mirabilis* rods could form biofilm on the surfaces of both biomaterials applied, polystyrene and polyvinyl chloride (Nelaton catheters). The differences in the ability to form biofilm observed between *P. mirabilis* strains derived from the urine of catheterized and non-catheterized patients were not statistically significant. In this study, synergistic concentrations of compounds 1 and 2 in combination affected not only the growth inhibition of test bacteria but also biofilm elimination. In recent reports, some furan derivatives, for example, 3-methyl-2(5H)-furanone, showed inhibition of biofilm formation of *Klebsiella pneumoniae*[40] and 5-(aryl-2-methy lene)-3,4-dihalo-5H-furan-2-one compounds showed inhibitory activity on biofilm formation of *P. aeruginosa*[21].

As mentioned previously, MFC causes cell lysis due to its primary activity on the cell wall to which it binds[20]. Also, MEN induces cytoplasmic membrane interference damage and Na^+ and K^+ leakage, leading to the loss of membrane potential[19]. Synergistic combinations of these compounds with different mechanisms of action are successful approaches for combating bacterial infections. Moreover, this study showed the low cytotoxic potential of antibacterial compounds and suggested using the above described synergistic combinations for combating infections caused by *Proteus* spp. These compounds are reported to reduce cytotoxicity and also inhibit biofilm formation when used in combination.

CONCLUSION

This study highlighted the synergistic potential of compounds 1 and 2 in combination as antibacterial agents for combating infections caused by *Proteus* spp., with low cytotoxicity. The combination of these compounds showed MIC values lower than the MIC of the test compound alone. Moreover, these compounds increased the susceptibility of test bacteria, reduced cytotoxicity and inhibited the biofilm formation of these compounds when used in combination. These experimental findings encourage further studies with these and other antibiotic agents and *in vivo* animal experiments to validate these interesting observations before clinical testing can move forward. Investigations are underway to further characterize the interaction of the test compounds and antibiotics.

SIGNIFICANCE STATEMENT

This study discovered the combination of MEN and MFC inhibits the growth and the biofilm production of *Proteus* spp., moreover, this study showed the low cytotoxic potential of these compounds. This new combination of antimicrobial agents is surprisingly effective. These results suggested that using the combination of MEN and MFC maybe help in the treatment of *Proteus* spp. infection.

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