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Evaluation of Limonene β-Amino Alcohol Derivatives for Synergistic Antibacterial activity against *Staphylococcus aureus*

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ABSTRACT

Limonene β-amino alcohols were synthesized and evaluated for their antimicrobial activity against *Staphylococcus aureus* Sp3, the ciprofloxacin resistance stain through the expression of the NorA efflux pump, and synergistic activity were evaluated by checkerboard and time-kill curves. The results showed that most of the synthesized compounds were found to exhibit potent activity. Synergistic investigation of combinations of the synthesized compounds with ciprofloxacin clearly suggested that compounds 6b and 6h exhibited enhanced synergistic effects (FICI of 0.25) which could provide novel therapeutic strategies for the treatment *S. aureus* infection.

Keywords: Limonene β-amino alcohols; Antimicrobial activity; Synergistic effect; Ciprofloxacin, Drug resistance

INTRODUCTION

Treatment of infectious diseases is one of the greatest challenges in overcoming wide spread epidemics and antibiotic resistance throughout the world. Searching for new antibiotics is one of the therapeutic approaches to combat the infectious diseases and antibiotic resistance. Antibiotic efflux by membrane transporters is an important mechanism of antibiotic resistance, and several efflux pumps in gram positive bacteria have been discovered. The NorA efflux pump is one of the most studied in *Staphylococcus aureus*. Using efflux pump inhibitors may be an effective strategy to reduce the Minimum Inhibitory Concentrations (MICs) associated with an antibiotic, and thus eliminate the antibiotic resistance strains and improve clinical treatment [1-5].

To search for NorA efflux pump inhibitors, secondary plant metabolites such as phenolics, alkaloids, saponins and terpenes are of consideration as in traditional medicines to treat various diseases. Limonene is one of the most common terpenes founded in the rind of citrus fruits such as lemons, limes, and oranges. Limonene has broad applications in foods, cosmetics, cleaning products, and natural insect repellents as it possesses broad-spectrum pharmacological activities such as anti-inflammatory, antioxidant, antimicrobial and antibacterial activities with safety and low toxicity [6-10].

Limonene is a promising antibacterial agent against multi-drug resistant bacterial pathogens. In addition, limonene derivatives are used as building blocks or as a chiral auxiliary in asymmetric synthesis [11-14]. Therefore, many limonene derivatives have been prepared and evaluated for their

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biological activity. Limonene β-amino alcohols through a regioselective limonene-oxide aminolysis by N-alkyl and N-aryl amines are an important class of organic compounds due to their common occurrence in nature and because they are versatile building blocks in the synthesis of a wide range of natural and synthetic products. For all cases founded in the literature, derivatives with nitrogen and an oxygen heteroatom were more active than limonene.

Ferrarini, et al., have synthesized several limonene β-amino alcohol derivatives at the cyclic double bond and evaluated its leishmanicidal activity against isolated parasites.

Compounds ((1S,2S,4R)-1-methyl-4-(prop-1-en-2-yl)-2-(propylamino)cyclohexan-1-ol) and ((1S,2S,4R)-1-methyl-2-(phenylamino)-4-(prop-1-en-2-yl)cyclohexan-1-ol) were found to inhibit Leishmania (Viannia) braziliensis promastigote species with LD50 values of 0.71 ± 0.095 µM and 0.408 ± 0.01 µM, respectively (**Figure 1**). Thus, limonene amino alcohol can be effectively exploited as an antibacterial agent against multi-drug resistant bacterial pathogens. To explore this, limonene amino alcohol derivatives were prepared, and the antibacterial activity against S. aureus Sp3 and the *S. aureus* efflux systems, including the synergistic effect in combination with ciprofloxacin, were investigated.

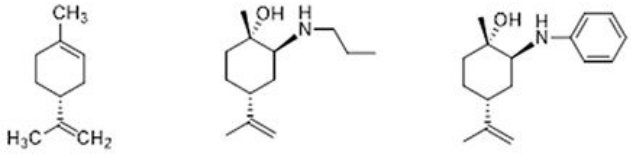

Figure 1: Chemical structure of limonene and its amino alcohol derivatives as potent antileishmanial agents.

MATERIALS AND METHODS

General Information

^{1}H and ^{13}C-NMR spectra were obtained with $CDCl_3$ solutions at 300 MHz for ^{1}H and 75 MHz for ^{13}C in a Bruker AVANCE 300 MHz nuclear magnetic resonance spectrometer with Tetramethylsilane (TMS) as an internal standard. The chemical shifts were presented in Parts Per Million (PPM, δ), the coupling constants (J) were reported in Hertz (Hz), and the signals were described as singlet (s), doublet (d), triplet (t) and multiplet (m). HR-MS was performed using a Hewlett packard 5973 mass spectrometer. All compounds were monitored using a TLC silica gel 60 F254 aluminium sheet. Column chromatography was performed using silica gel 60 (0.063 mm-0.200 mm) and visualized under UV light at 254 nm and 365 nm. Reagents and solvents were purchased from commercial suppliers.

Experimental Procedures

(1S,2S,4R)-2-bromo-1-methyl-4-(prop-1-en-2-yl)-cyclohexan-1-o (4): Limonene (1) (5.00 mL, 26.97 mmol) was dissolved in acetone (20 mL) and H_2O (4 mL) in a round bottom flask at room temperature. Then, N-Bromosuccinimide (NBS) (6.3 g, 0.034 mol) (6.0 eq.) was slowly added to the solution. The reaction mixture was stirred while being cooled in an ice bath for 1 h. Afterwards, the solution was extracted with CH_2Cl_2, and the organic layer was dried over anhyd. Na_2SO_4 and evaporated under reduced pressure. The crude product was purified using column chromatography (silica gel, 95:5 Hexane:EtOAc) to afford (4) as a yellow oil (3.77 g, 60%); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.22-1.41 (m, 1H,) 1.43 (s, 3H) 1.74 (s, 3H) 1.75-2.04 (m, 4H) 2.22-2.31 (m, 1H) 2.45-2.5 (m, 1H) 4.20 (t, J=2.7 Hz, 1H), 4.76 (d, J=6.5 Hz, 2H) ppm.

(1S, 4R, 6R)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0] heptane (5): Compound (4) (3.00 g, 12.88 mmol) was heated in 6M NaOH (9 mL) at 60°C for 2 h. Afterwards, the solution was extracted with CH_2Cl_2, and the organic layer was dried over anhyd. Na_2SO_4 and evaporated under reduced pressure. The crude product was purified using column chromatography (silica gel, 97:3 Hexane:EtOAc) to afford the title compound (5) as a yellow oil (1.05 g, 54 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.32 (s, 3H) 1.34-1.42 (m, 2H) 1.67 (s, 3H) 1.69-1.75 (m, 2H) 1.83-1.89 (m, 1H) 1.99-2.07 (m, 2H) 3.00 (d, J=5.3 Hz, 1H), 4.67 (s, 2H) ppm.

General Procedure for the Synthesis of Limonene Derivatives (6a-6h)

A mixture of limonene epoxide (5) (200 mg, 1.32 mmol), amine (0.4 mL, 3.78 mmol) and one drop of H_2O was refluxed for 24 h. Afterwards, the solution was extracted with CH_2Cl_2, and the organic layer was dried over anhyd. Na_2SO_4 and filtered. The filtrate was evaporated to dryness and the crude product was purified using preparative thin layer chromatography (silica gel, 50:50 Hexane:EtOAc) to yield the products (6a-6h).

(1S,2S,4R)-2-(butylamino)-1-methyl-4-(prop-1-en-2-yl)-cyclohexan-1-ol (6a)

Following the general procedure, limonene epoxide (5) was reacted with n-butylamine (0.4 mL, 3.78 mmol) to obtain the title compound (6a) as a yellow oil (0.14 g, 48 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.86-0.96 (t, J=9 Hz, 3H) 1.20 (s, 3H) 1.26-1.43 (m, 2H) 1.44-1.50 (m, 2H) 1.51-1.58 (m, 2H) 1.59-1.64 (m, 1H) 1.65-1.71 (m, 1H) 1.74 (s, 3H) 1.86-2.07 (m, 2H) 2.23 (br s,1H) 2.40-2.57 (m, 2H) 2.68-2.84 (m, 1H) 4.78 (s, 2H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) 14.0 (CH_3), 20.5 (CH_2), 21.7 (CH_3), 24.7 (CH_3), 26.0 (CH_2), 30.2 (CH_2), 32.4 (CH_2), 34.6 (CH_2), 38.0 (CH), 47.9 (CH_2), 61.9 (CH), 72.1 (C), 109.5 (CH_2) 148.4 (C) ppm.

(1S, 2S, 4R)-1-methyl-2-morpholino-4-(prop-1-en-2-yl)-cyclohexan-1-ol (6b)

Following the general procedure, limonene epoxide (5) was reacted with morpholine (0.4 mL, 3.78 mmol) to obtain the title compound (6b) as a yellow oil (99 mg, 33 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.22 (s, 3H) 1.47-1.58 (m, 4H) 1.75 (s, 3H) 1.90-1.99 (d, 1H) 2.06-2.14 (d, 1H) 2.46-2.57 (m, 4H) 2.64 (m, 2H) 3.71 (m, 4H) 4.85 (s, 1H) 4.95 (s, 1H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) 22.3 (CH_3), 22.5 (CH_3), 24.5 (CH_2), 24.9 (CH_2), 35.7 (CH_2), 38.9 (CH), 52.0 (CH_2), 67.4 (CH_2), 67.5 (CH), 72.8 (C), 111.1 (CH_2) 145.4 (C) ppm.

(1S,2S,4R)-1-methyl-2-(piperidin-1-yl)-4-(prop-1-en-2-yl)-cyclohexan-1-ol (6c)

Following the general procedure, limonene epoxide (5) was reacted with piper dine (0.4 mL, 3.78 mmol) to obtain the title compound (6c) as a yellow oil (93 mg, 30 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 3H) 1.33-1.47 (m, 2H) 1.48-1.70 (m, 8H) 1.74 (s, 3H) 1.86-2.00 (m, 1H) 2.01-2.15 (m, 1H) 2.34-2.52 (m, 4H) 2.62-2.79 (m, 2H) 4.85 (s, 1H) 4.93 (s, 1H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) 22.0 (CH_3), 22.5 (CH_3), 24.4 (CH_2), 24.6 (CH_2), 25.0 (CH_2), 26.7 (CH_2), 35.9 (CH_2), 39.1 (CH), 53.1 (CH_2), 67.9 (CH), 72.5 (C) 111.0 (CH_2) 145.5 (C) ppm.

(1S,2S,4R)-1-methyl-2-(phenylamino)-4-(prop-1-en-2-yl)-cyclohexan-1-ol (6d)

Following the general procedure, limonene epoxide (5) was reacted with aniline (0.4 mL, 3.78 mmol) to obtain the title compound (6d) as a yellow oil (75 mg, 23 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.30 (s, 3H) 1.42-1.90 (m, 3H) 1.92-2.05 (m, 2H) 2.13 (s, 1H) 3.57 (s, 1H) 4.78 (s, 1H) 6.61-6.72 (m, 3H) 7.14-7.32 (m, 2H); ^{13}C-NMR (75 MHz, CDC_{l3}) 21.3 (CH_3), 26.0 (CH_2), 26.8 (CH_3), 31.1 (CH_2), 34.5 (CH_2), 38.3 (CH), 57.2 (CH), 71.9 (C), 109.4 (CH_2), 113.4 (CH), 117.4 (CH) 129.3 (CH) 147.6 (C) 148.5 (C) ppm.

(1S,2S,4R)-2-(benzylamino)-1-methyl-4-(prop-1-en-2-yl)-cyclohexan-1-ol (6e)

Following the general procedure, limonene epoxide (5) was reacted with benzylamine (0.4 mL, 3.78 mmol) to obtain the title compound (6d) as a yellow oil (46 g, 14 %); ^{1}H-NMR (300 MHz, CDC_{l3}) δ 1.22 (s, 3H) 1.49-1.63 (m, 2H) 1.64-1.71 (m, 1H) 1.72 (s, 3H) 1.76-1.79 (m, 2H) 1.80-2.07 (m, 1H) 2.18-2.30 (m, 1H) 2.51-2.70 (m, 1H) 3.56-3.82 (m, 1H) 3.83-4.00 (m, 1H) 4.76 (s, 2H) 7.20-7.40 (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$) 21.5 (CH_3), 25.3 (CH_3), 26.0 (CH_2), 29.9 (CH_2), 34.5 (CH_2), 37.9 (CH), 52.0 (CH_2), 61.2 (CH), 72.1 (C), 109.4 (CH_2), 127.1 (CH), 128.2 (CH) 128.4 (CH) 140.3 (C) 148.6 (C) ppm.

(1S,2S,4R)-2-((2-(1H-indol-3-yl)ethyl)amino)-1-methyl-4-(prop-1-en-2-yl)-cyclohexan-1-ol (6f)

Following the general procedure, limonene epoxide (5) was reacted with tryptamine (0.6 g, 3.78 mmol) to obtain the title compound (6f) as a yellow oil (29 mg, 7 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.14 (s, 3H) 1.23-1.29 (m, 1H) 1.47-1.55 (m, 1H) 1.61-1.67 (m, 1H) 1.68 (s, 3H) 1.89-2.02 (m, 1H) 2.12-2.07 (m, 2H) 2.54-2.64 (m, 1H) 2.51-2.70 (m, 1H) 2.80-2.90 (m, 1H) 2.92-3.02 (m, 2H) 3.06-3.17 (m, 1H) 4.75 (s, 2H) 7.00-7.64 (m, 5H) 8.19 (br s, 1H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) 21.7 (CH_3), 24.4 (CH_3), 25.8 (CH_2), 25.9 (CH_2), 30.0 (CH_2), 34.7 (CH_2), 38.3 (CH), 48.2 (CH_2), 62.0 (CH), 72.2 (C) 109.6 (CH_2) 111.2 (CH) 113.7 (CH) 118.8 (CH) 119.2 (CH) 122.0 (CH) 136.4 (C) 148.2 (C) 161.3 (C) ppm; HRESI-MS: calcd for $C_{20}H_{28}NO_2$ $[M+H]^+$: 313.2274, found: 313.2273.

(1S, 2S, 4R)-2-((2-(5-methoxy-1H-indol-3-yl) ethyl) amino)-1-methyl-4-(prop-1-en-2-yl) cyclohexan-1-ol (6g)

Following the general procedure, limonene epoxide (5) was reacted with 5-methoxy tryptamine (0.7 g, 3.78 mmol) to obtain the title compound (6g) as a yellow oil (104 mg, 23%); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.12 (s, 3H) 1.20-1.32 (m, 2H) 1.43-1.54 (m, 2H) 1.68 (s, 3H) 1.88-2.07 (m, 2H) 2.09-2.19 (m, 1H) 2.50-2.60 (m, 1H) 2.72-2.86 (m, 1H) 2.87-2.99 (m, 2H) 3.00-3.16 (m, 1H) 3.84 (s, 3H) 4.74 (s, 2H) 6.77-7.27 (m, 5H) 8.32 (br s, 1H) ppm; ^{13}C-NMR (75 MHz, CDC_{l3}) 21.6 (CH_3), 24.8 (CH_3), 26.0 (CH_2), 29.7 (CH_2), 30.2 (CH_2), 34.6 (CH_2), 37.9 (CH), 48.2 (CH_2), 55.9 (CH_3), 61.9 (CH) 72.2 (C) 100.7 (CH) 109.4 (CH_2) 111.9 (CH) 112.1 (CH) 113.5 (CH) 122.8 (CH) 127.8 (C) 131.6 (C) 148.4 (C) 153.8 (C) ppm; HRESI-MS: Calcd for $C_{20}H_{30}N_2O_2$ $[M+H]^+$: 343.2380, found: 343.2379.

Methyl((1S,2S,5R)-2-hydroxy-2-methyl-5-(prop-1-en-2-yl)-cyclohexyl)-tryptophanate (6h)

Following the general procedure, limonene epoxide (5) was reacted with tryptophan methyl (0.8 g, 3.78 mmol) to obtain the title compound (6h) as a yellow oil (0.0358 g, 7 %); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.03 (s, 3H) 1.20-1.34 (m, 1H) 1.38-1.55 (m, 2H) 1.57-1.66 (m, 1H) 1.68 (s, 3H) 1.71-1.80 (m, 1H) 1.82-1.92 (m, 1H) 2.09-2.27 (m, 1H) 2.29-2.47 (m, 1H) 2.98-3.08 (m, 1H) 3.09-3.19 (m, 1H) 3.56-3.63 (m, 1H) 3.64 (s, 3H) 4.72 (s, 1H) 6.99-7.68 (m, 5H) 8.12 (br s, 1H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) 21.5 (CH_3), 24.9 (CH_3), 25.8 (CH_2), 29.9 (CH_2), 32.0 (CH_2), 34.2 (CH_2), 38.2 (CH), 51.9 (CH_3), 62.1 (CH), 62.9 (CH) 72.5 (C) 109.5 (CH_2) 111.2 (CH) 111.6 (CH) 118.8 (CH) 119.4 (CH) 122.1 (CH) 122.8 (CH) 127.5 (C) 136.1 (C) 148.3 (C) 176.6 (C) ppm; HRESI-MS: calcd for $C_{20}H_{20}N_2O_3$ $[M+H]^+$:371.2329, found: 371.2328.

Biological Activity Assays

Bacterial strains and drug: The *S. aureus* Sp3 used in the present study was isolated from a pus sample collected from a patient in Nakhon Pathom hospital. This strain exhibit resistance to ciprofloxacin through the expression of the NorA efflux pumps. The bacteria were cultured in Brain Heart Infusion agar (BHI, Himedia, India) at 37°C for 24 h prior to testing. Ciprofloxacin hydrochloride (CI) was dissolved and diluted in distilled water. This drug was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Antimicrobial susceptibility testing and factional inhibitory: The Minimum Inhibitory Concentrations (MICs) of ciprofloxacin and compounds (6a-6h) were determined using the two-fold dilution method according to the CLSI guidelines. A total of 100 µl *S. aureus* Sp3 (10^5 cfu/ml) was mixed with different concentrations of ciprofloxacin and compounds (6a-6h) (16 µg/ml, 32 µg/ml, 64 µg/ml and 128 µg/ml) in a 96 well plate and incubated at 37°C for 24 h. *S. aureus* Sp3 cultured with 0 µg/ml ciprofloxacin and compounds (6a-6h) were used as controls. To investigate the *in vitro* synergistic effects of ciprofloxacin and compounds (6a-6h) in combination against *S. aureus* Sp3, checkerboard microtiter plate assay was performed in Mueller-Hinton broth (MH, Himedia, india). The various concentrations of compounds (6a-h) (16, 32, 64 and 128 µg/ml) were applied to 100 µl *S. aureus* Sp3 (10^5 cfu/ml) in combination with ciprofloxacin (16 µg/ml, 32 µg/ml, 64 µg/ml and 128 µg/ml) in 96 well plates. The plates were incubated at 37°C for 24 h. The blank control was comprised of S. *aureus* Sp3 cells cultured in culture media only without ciprofloxacin or compounds (6a-h) treatment.

Each experiment was repeated three times. Synergy is observed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was the same for all components of the mixture. The Fractional Inhibitory Concentration Index (FICI) were calculated using: FICI=FIC A + FIC B, where FIC A is the MIC of drug A in the combination/ MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the FICI is ≤ 0.5, indifferent when the FICI is >0.5 to <2, and antagonistic when the FICI is ≥ 2.

Synergistic effect of limonene β-amino alcohol on ciprofloxacin activity by time kill curve assay: From the results of the checkerboard assay, the compounds which exhibited synergistic effects in combination with ciprofloxacin at the MIC of 32 µg/ml were selected for determination by time kill curve assay. The culture of 10 ml *S. aureus* Sp3 (10^5 cfu/ml) in MH broth with ciprofloxacin and the test compounds were added alone or in combination with the final concentration of 32 µg/ml. The cultures were incubated at 37° C on a shaking set at 120 rpm. One hundred microliters of the broth were collected at different time intervals from each culture, serially diluted in phosphate-buffered saline, and cultured on MH agar plates to obtain colony counts. Curves were constructed by plotting the log10 of cfu/ ml versus time. Synergy was defined as ≥ 2 log10 decreases in cfu of bacteria treated with the drug combination compared to the most active component of the test compound alone, as described previously [15-20].

RESULTS AND DISCUSSION

Chemistry

Limonene oxides were synthesized followed by the previous literature 22 through bromination and followed by peroxidation using N-Bromosuccinimide (NBS). The limonene epoxide was obtained as a major trans-isomer by column chromatography purification and the structure was confirmed by the previous report. Ring-opening of the trans-epoxide was attempted under various conditions, however only aminolysis by N-alkyl and N-aryl amines with 3 equivalents of water under reflux for 24 hours gave the limonene β-amino alcohol (6a-6h) in fair yields (**Figure 2**). All synthesized compounds were characterized *via* MS, ^{1}H-NMR, and ^{13}C-NMR.

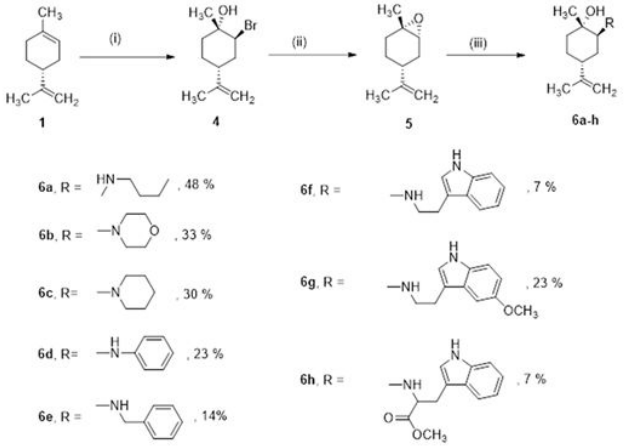

Figure 2: Synthesis of limonene β-amino alcohol derivatives.

Reagents and conditions: (i) NBS, H_2O, acetone, 0°C-25°C, 1 h, 60%; (ii) 6M NaOH, 60°C, 2 h, 54%; (iii) amines (1 eq), H_2O, reflux, 24 h.

Antimicrobial Activity and Factional Inhibitory Concentration Index (FICI)

All limonene β-amino alcohol derivatives (6a-6h) were evaluated for their antibacterial activity against *S. aureus* Sp3 in comparison with ciprofloxacin. Most compounds showed potent activity with the exception of compounds 6b and 6h with MIC>128 µg/mL, similar to ciprofloxacin. Then ciprofloxacin and compounds 6a-6h combinations were checked for synergistic activity. When the compounds 6a-6h was added, the MIC of ciprofloxacin decreased notably, demonstrating that the sensitivity of *S. aureus* Sp3 to ciprofloxacin was improved (**Table 1**). These may be effective efflux pump inhibitors as they disturbed the NorA function, restoring bacterial sensitivity to antibiotics. Among these, compounds 6b-6c and 6g-6h exhibited synergistic activity by the checkerboard assay (**Table 2**). When combined with ciprofloxacin, they inhibited *S. aureus* Sp3 at sub-MIC levels. There was a significant reduction in MICs of the compound combination showing strong synergy (FICI=0.28−0.50, **Table 1**).

Table 1: Synergistic studies of compounds 6a-6h with ciprofloxacin against *S. aureus* Sp3.

Compound	MIC of compound alone (µg/mL)	MIC of ciprofloxacin alone (µg/mL)	MIC of compounds in combination (µg/mL)	FICI	Outcome
6a	64	256	32:128	1	Indifference
6b	256	256	32:32:00	0.25	Synergy
6c	64	256	16:32	0.375	Synergy
6d	32	256	16:32	0.625	Indifference

6e	64	256	16:32	0.375	Synergy
6f	16	256	16:00	1	Indifference
6g	16	256	16:00	1	Indifference
6h	256	256	32:32:00	0.25	Synergy

Table 2: Synergistic activity of compounds 6a-6h with ciprofloxacin by check board assay.

Compound (µg/mL)	Ciprofloxacin (µg/mL)				
	0	**16**	**32**	**64**	**128**
6a	>128	64	64	64	32
6b	128	64	32	16	16
6c	64	32	16	16	16
6d	>128	32	16	16	16
6e	64	32	16	16	16
6f	32	16	16	16	16
6g	128	16	16	16	16
6h	>128	64	32	16	16

The Time kill Curve Assay

Compounds 6b and 6h with strong synergy (FICI) were selected to further study for their efficacy. The time kill kinetic studies were performed with CFUs of the *S. aureus* Sp3 and showed synergy at 2, 4, 6, 8, 10 and 24 h on Ciprofloxacin (CI) activity in combination with 6b (**Figure 2**, left) and with 6h (**Figure 2**, right). However, a complete bactericidal effect was not observed at 24 h of incubation. Ciprofloxacin with >2 times reduction in MICs were observed when used in combination with 6b and 6h. In this combination, compounds 6b and 6h inhibited *S. aureus* Sp3 at 32 µg/ml, which was >2 times lower than the MIC of compounds 6b and 6h alone and indicated an effective inhibitory drug resistance if used with ciprofloxacin (**Figure 3**).

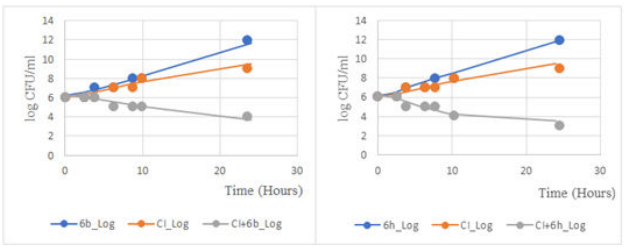

Figure 3: Time kill curves assay of compound 6b (left) and 6h (right) against *S. aureus* Sp3.

CONCLUSION

In conclusion, limonene β-amino alcohols have been developed and studied for the antimicrobial activity against *S. aureus* Sp3, the ciprofloxacin resistance stain through the expression of the NorA efflux pump. The result showed that most of the compounds were found to exhibit potent activity. Synergistic investigation of combinations of the synthesized compounds with ciprofloxacin clearly suggested that compound 6b and 6h exhibited enhanced synergistic effects through FICI values and time kill assay studies. Thus, the limonene β-amino alcohols could serve as good candidates for lead compounds for novel antimicrobial agents as well as in combination with ciprofloxacin. However, due to the complexity of the *S. aureus* efflux system, further study is required to elucidate the precise mechanism by which limonene β-amino alcohols inhibit the efflux system of *S. aureus*, and to develop novel therapeutic strategies and treatment of *S. aureus* infection.

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AUTHOR CONTRIBUTIONS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. WSP conceived and designed the study. BR and TT. performed the experiments and collected the data. WSP, WP and TT analyzed data. WSP wrote him manuscript. All authors read and approved the manuscript for publication.

CONFLICTS OF INTEREST

No conflict of interest associated with this work.

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