Journal of Chemistry

New Compounds with Antimicrobial Activity

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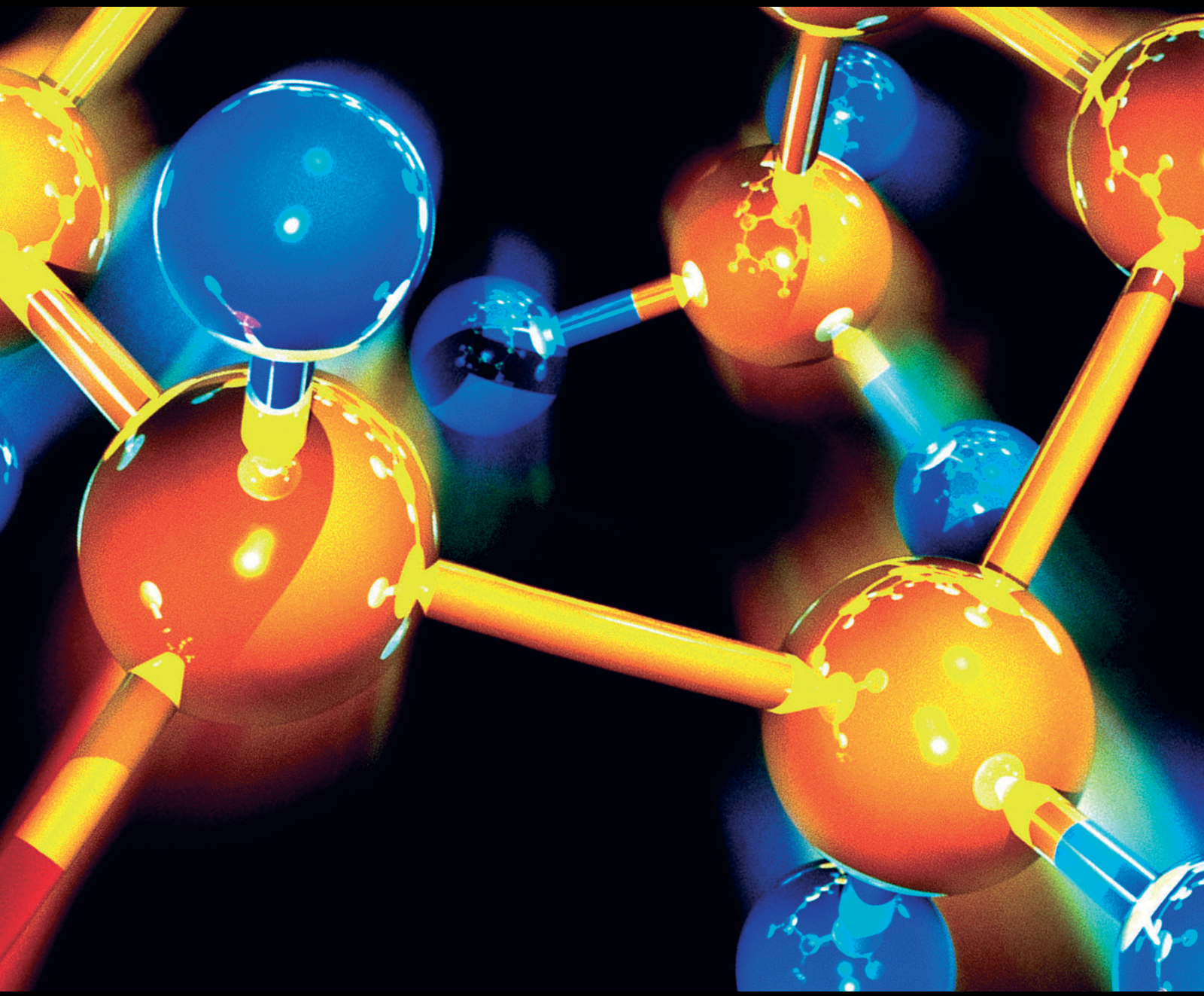

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Research Article

Synthesis and Evaluation of Antimicrobial Activities of Novel N-Substituted Indole Derivatives

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Received 28 June 2019; Accepted 20 March 2020; Published 14 April 2020

Guest Editor: Maria Grazia Bonomo

Indole motifs are one of the most significant scaffolds in the discovery of new drugs. We have described a synthesis of new N-substituted indole derivatives (**1**-**3**), and their *in vitro* antimicrobial activities were investigated. The synthesis of titled compounds has been demonstrated by utilizing commercially available starting materials. The antibacterial and antifungal activities were performed using new strains of bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* using the disc diffusion method. Notably, the compound 4-(1-(2-(1H-indol-1-yl) ethoxy) pentyl)-N,N-dimethyl aniline (**1**) was found to be most potent than the other analogues (**2** and **3**), which has shown higher inhibition than the standard drug chloramphenicol.

1. Introduction

The versatility of heterocycles has been known from the century since their direct involvement in natural products [1–4]. Particularly, the nitrogen-containing heterocycles (N-heterocycles) have proven ubiquitous structural features and pivotal role in medicinal chemistry [5–11]. Amongst the various N-heterocycles, indole motifs have received significant attention due to their presence in proteins, amino acids, bioactive alkaloids, and drugs (Figure 1) [12–23]. In this context, a large number of indole moieties have been investigated in the development of new efficient bioactive molecules with diverse pharmacological properties, such as antimicrobial, antiviral, anticancer, anti-inflammatory, inhibitors, and antioxidant [24–47]. Generally, indoles substituted at 2nd or 3rd position [48–50], are known to exhibit certain bioactivity. On the other hand, the importance of N1-substituted indole derivatives in marketed drug molecules, natural products, and marine organisms are at great extent [51–53]. Despite the structural novelties and valuable biological activities of N1-substituted indoles [54], it remains challenging due to the inertness of the nitrogen atom (-NH-) towards electrophilic reagents [55–61].

A literature survey reveals that the infections caused by bacteria, fungi, or microorganisms in tropical and subtropical regions could be controlled by designing new antimicrobial agents [62, 63]. In an attempt to design and synthesize new antimicrobial agents, herein, we reported the synthesis of N1-alkylated indole derivatives and investigation of their antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *and Candida albicans* (Figure 2).

2. Results and Discussion

As part of our studies to design new bioactive N-substituted indole derivatives, we envisaged that the utilization of commercially available 4-N,N-dimethylamino benzaldehyde might be suitable for the syntheses of target compounds **1**–**3**. The retrosynthetic analyses of N-substituted indoles (**1**-**3**) are outlined in Scheme 1. In order to find suitable synthons, firstly, the cleavage of C-O bond resulted to N-alkylated indole **7** and the corresponding benzylic alcohols **4**-**6**, which could be envisaged to form desired compounds (**1**–**3**) *via* O-alkylation reaction. Furthermore, a common intermediate **7** could be synthesized *via* N-alkylation reaction of commercially available indole (**9**) and 1,2-dibromoethane

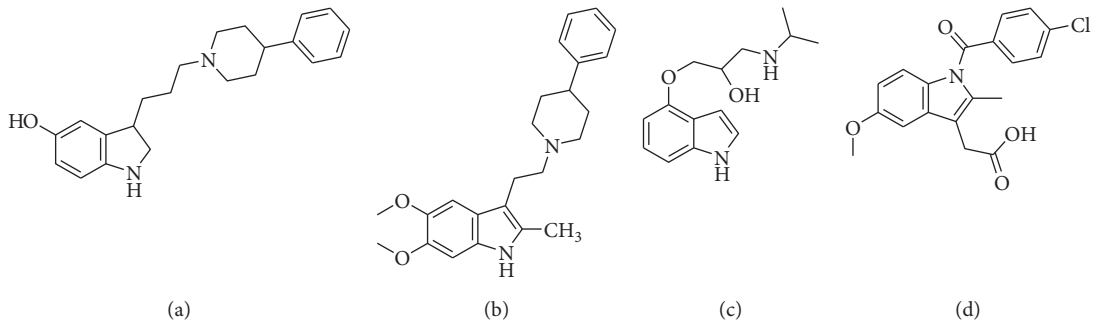


Figure 1: Representative examples of drugs with indole. (a) Roxindole schizophrenia. (b) Oxypertine antipsychotic drug. (c) Pindolol antihypertensive drug. (d) Indometacin anti-inflammatory drug.

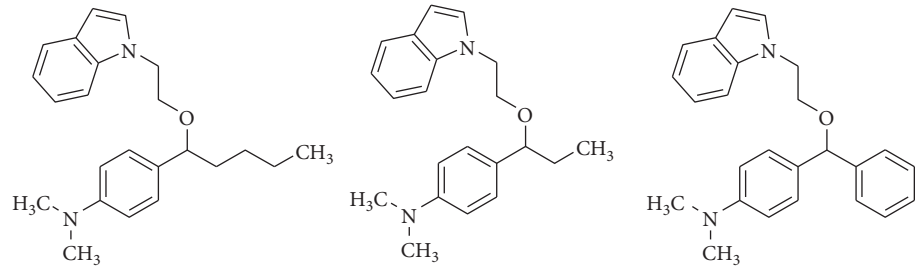
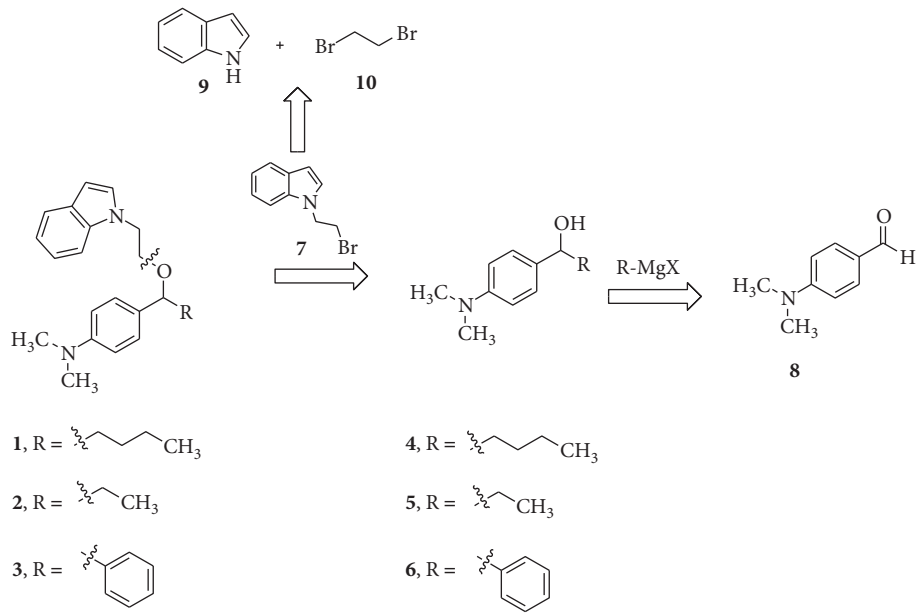


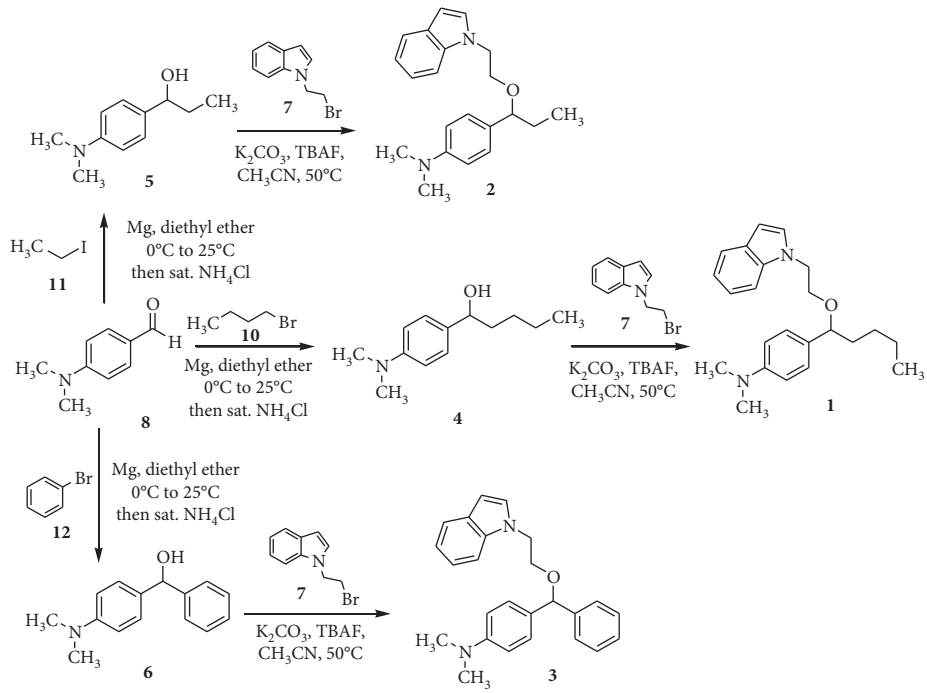
Figure 2: New bioactive indole derivatives.



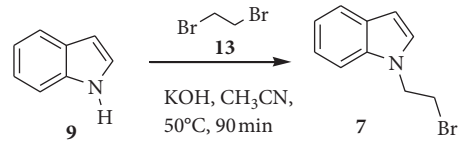
Scheme 1: Retrosynthesis of compounds **1–3**.

(**10**). Finally, the benzylic alcohols **4-6** could be obtained by performing Grignard reaction of 4-N,N-dimethylamino benzaldehyde (**8**) with the corresponding alkyl or aryl halide.

To validate our outlined approach, we have commenced our synthesis by performing Grignard reaction of 4-N,N-dimethylamino benzaldehyde (**8**) with *n*-BuMgBr, which was prepared by in situ reaction of *n*-BuBr (**10**) and Mg in diethyl ether solvent resulting in benzylic alcohol **4** at an excellent yield (Scheme 2). Similarly, the other benzylic alcohols **5** and **6** were successfully obtained by subjecting "Grignard reaction" of 4-N,N-dimethylamino benzaldehyde (**8**) with EtMgBr and PhMgBr, respectively. Next, we investigated the synthesis of N-alkyl indole (**7**) *via* N-alkylation, employing commercially available indole (**9**) with 1,2-dibromoethane (**13**) in the presence of potassium hydroxide as base and DMF as a solvent (Scheme 3) [64]. In



Scheme 2: Synthesis of target compounds **1–3**.



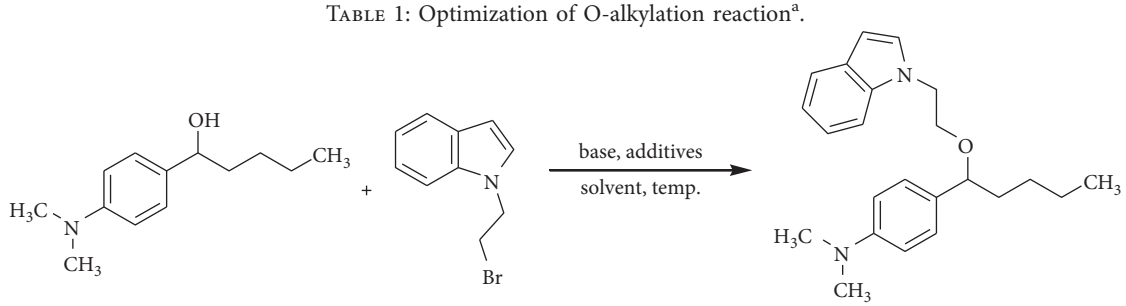
Scheme 3: Synthesis of N-alkylated indole **7**.

order to accomplish the synthesis of desired compounds **1–3**, we decided to facilitate O-alkylation of benzylic alcohol (**4**) with readily synthesized N-alkylated indole (**7**). To our surprise, the O-alkylation reactions reduced the yield and prolonged the reaction time. To overcome this problem, we began to optimize O-alkylation reaction under different conditions, and the results are presented in Table 1.

The O-alkylation between 1-(4-(dimethylamino)phenyl)pentan-1-ol (**4**) and N-alkylated indole (**7**) employing KOH as base under neat conditions resulted traces of O-alkylated product (**1**) along with the domination of unidentified side reactions (Table 1, entry 1). Screening of various solvents such as pyridine, acetonitrile, and dimethyl formamide provided slightly improved yields of O-alkylated product (**1**) (entries 3–6). However, substantial amount of starting material was recovered during the course of reaction. Subsequently, it was found that Chi and Kazemi exploited ionic-liquids and phase transfer catalyst in alkylation reaction [65, 66]. The situation improved dramatically, when we utilized the combination of K_2CO_3/TBAF in acetonitrile to provide the corresponding O-alkylated product (**1**) with 72% yield (entry 7). Thus, the optimized reaction conditions involved benzylic alcohol (**4**) (1.5 mmol), N-alkyl indole (**7**) (1 mmol), K_2CO_3 (1 mmol), TBAF (1 mmol), and acetonitrile (10 mL) heated at 50°C. Under the optimized conditions, we then explored O-alkylation of 1-(4-(dimethylamino)phenyl)propan-1-ol (**5**) and (4-(dimethylamino)phenyl) (phenyl)methanol (**6**) with N-alkyl indole (**7**) to provide the desired compounds **2** and **3** with good yields, respectively.

2.1. In Vitro Antimicrobial Activity. *In vitro* antifungal activities of the synthesized compounds **1–3** were evaluated utilizing *Staphylococcus aureus* and *Escherichia coli* and antifungal activity against *Candida albicans* using disc diffusion method (Table 2). The inhibition zone was measured in diameter. Bavistin and chloramphenicol were used as the standard drug to compare antifungal activity. In order to investigate antifungal activity, the inhibition against the test organisms and ethanol as positive control, and the effectiveness of the target compounds (**1–3**) was measured by calculating inhibition zone against the tested organisms. The zone of inhibition was compared with the standard drug after 72 h. Organisms *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) were subcultured into sterile nutrient broth. Then, aliquots of 50% and 100% of the sample solutions of target compounds, **1–3**, were

Table 1: Optimization of O-alkylation reaction[a].



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Entry	Base (mol%)	Additives (equiv.)	Solvent (mL)	Temperature (°C)	Yield of 1[b,c] (%)
1	KOH	—	Neat	25	Traces
2	Pyridine	—	Pyridine	80	nr
3	K_2CO_3	—	CH_3CN	80	25
4	K_2CO_3	—	DMF	100	20
5	K_2CO_3 (50)	TBAF	DMF	80	35
6	K_2CO_3 (50)	TBAF (1)	CH_3CN	50	50
7	K_2CO_3 (100)	TBAF (1)	CH_3CN	50	72[d]

[a]Reaction conditions: alcohol (1.5 mmol), indole-alkyl bromide (1 mmol), base, and solvent; [b]isolated yield; [c]characterized by IR and NMR; [d]further yield did not improve even when the 1.5 equiv. of base was utilized.

Table 2: Antimicrobial activities of target compound **1–3**.

Sr.No	Compounds	Zone of inhibitions (mm)					
		Escherichia coli		*Staphylococcus aureus*		*Candida albicans*	
		50%	100%	50%	100%	50%	100%
1	**1**	—	—	25	27	—	—
2	**2**	—	—	18	20	—	—
	3			15	20		
3	Chloramphenicol	2	3	18	20	—	—
4	Bavistin	—	—	—	—	20	30
5	Negative control	—	—	—	—	—	—

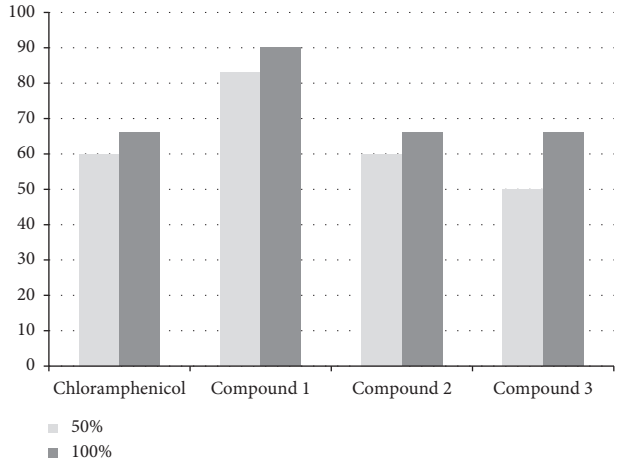

Figure 3: Comparison of % of bioactivity: chloramphenicol drug with compounds **1–3**.

pipetted to the discs in three replications each. The discs were impregnated with the sample solutions and then transferred to nutrient agar (NA) plate seeded with bacteria and incubated at 37°C for 24 h. Subsequently, the plates were examined for microbial growth of inhibition, and the inhibition zone diameter was measured to the nearest mm. All the tests were performed in triplicate. Obtained bioactivity results were compared with commercially available drugs, chloramphenicol and bavistin, and the results are presented in Figure 3.

3. Conclusion

We have reported the synthesis of indole derivatives using commercially available starting materials, and their antimicrobial activity was also investigated. The *in vitro* bioactivity was performed using *Staphylococcus aureus*, *Escherichia coli*, *and Candida albicans* as antibacterial and antifungal, respectively, using the disc diffusion method. The mean inhibition zone of commercially available drug chloramphenicol and bavistin was used as standard, and inhibition zone was calculated in mm. Compounds **1–3** tested for antifungal and antibacterial activity showed poor inhibition against Gram-negative bacteria *Escherichia coli*. On the other hand, compounds **1–3** showed good bioactivity towards pathogen *Staphylococcus aureus*, Gram-positive bacteria. Interestingly, it was observed that compound **1**, which incorporates butyl substitue nts, exhibited enhanced selectivity compared with analogues **2** and **3**. Further

extension of designing new indole derivatives is currently under development.

4. Experimental

4.1. General Experimental Procedure. FTIR spectra of the synthesized compounds were recorded on a Shimadzu 4000 instrument using KBr pellet within the range of 400 ± 10 to $4000 \pm 10\ cm^{-1}$. ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectra of compounds were recorded on Bruker Avance II-400 MHz NMR spectrometer in deuterated $CDCl_3$, MeOD and DMSO solvents using TMS as internal standard. Chemical shift values are expressed in δ (ppm).

4.1.1. Pharmacological Reagents. Reagents for biochemical assays such as potato dextrose agar (PDA, Micro master laboratories, India), nutrient agar (NA, Aldrich chemical company, Germany), bavistin (Domina Pharmaceuticals, India), chloramphenicol (Addis Pharmaceuticals S.C., Adigrat, Ethiopia) were used for the antimicrobial studies.

4.2. Synthesis of 1-(4-(dimethylamino)phenyl)pentan-1-ol (4). To an oven-dried three necked round bottom flask, Mg turnings (1.25 g, 5.14 mmol) was charged followed by the addition of iodine crystals (3 piece) and covered the flask with a $CaCl_2$ dry tube. To this, anhydrous diethyl ether (100 mL) was added using addition funnel. The whole reaction mixture was allowed to stir for 10 minutes. Then, bromobutane (**10**) (4.4 mL, 4.0 mmol) was introduced dropwise with the help of syringe. In order to initiate the reaction, the flask was warmed using hot water, and the addition of bromobutane was continued. It was observed that vigorous reaction between magnesium and bromobutane leads to the formation of Grignard reagent. The Grignard reagent in the flask appears grey in color. To the formed Grignard reagent (BuMgBr), 4-dimethylamino benzaldehyde (**8**) (5 g, 3.35 mmol) dissolved in 50 mL anhydrous DEE was introduced with the help of an addition funnel (dropwise). After the completion of the reaction (1 h), which was monitored by TLC, the mixture was quenched with saturated solutions of NH_4Cl (20 mL). The resulting mixture was transferred to a separatory funnel followed by the addition of ethyl acetate (50 mL), and the aqueous layer was removed, and the organic layer was dried with sodium sulphate. The solvent was removed using rotary evaporator, and compound **4** was obtained as the crude product. Then, further purification of the residue was performed using column chromatography using 20% ethyl acetate in petroleum ether as an eluent to give the pure 1-(4-(dimethylamino)phenyl)pentan-1-ol (**4**).

4.2.1. Appearance. Pale yellow oil yield = 72% (4.9 g); FT-IR (KBr, cm^{-1}). 3311(O-H str), 3072(C-H str, ring/cyclic), 2927(C-H str, acyclic), 1615(C=C str), 1464(C-C str); ^{1}H-NMR ($CDCl_3$/TMS) δ 0.92 (*t*, 3H, -CH_2CH_3), 1.21-1.29 (*m*, 2H,-$CH_2CH_2CH_3$), 1.40 (*q*, 2H, -CH_2-CH-OH), 3.06 (*s*, 6H, N-CH_3), 4.0 (*t*, 1H, OH), 4.58 (*t*, 1H, Ar-CH-OH), 6.76 (*d*, 1H,Ar-H), 6.76 (*d*, 1H,Ar-H), 7.25 (*d*, 1H,Ar-H); ^{13}C-NMR ($CDCl_3$/TMS) δ 76-78 ($CdCl_3$), δ 14.09, 22.69, 28.19, 38.47, 40.78, 74.47, 112.45, 126.97, 132.99, 150.19.

4.3. Synthesis of 1-(4-(dimethylamino)phenyl)propan-1-ol (5) via Grignard Reaction. The synthesis 1-(4-(dimethylamino) phenyl)propan-1-ol (**5**) was also achieved by Grignard reaction. This reaction was carried out similar to the aforementioned process. A 500 mL oven-dried round bottom flask was charged with Mg (1.25 g, 51.42 mmol) and iodine crystal (0.2 g) followed by the addition of anhydrous diethyl ether (100 mL) and stirred for 5 min. To this solution, ethyl iodide (**6**) (3.23 mL, 40.17 mmol) was added slowly with the help of a syringe. Upon addition of ethyl iodide, the reaction mixture color was changed from brown to grey color, which indicates the formation of Grignard reagent. To this Grignard reagent, 4-N,N-dimethylamino benzaldehyde (**8**) (5 g, 33.51 mmol) dissolved in anhydrous DEE was added with the help of the addition funnel. The resulting reaction mixture was stirred at room temperature for 2 h. The reaction was monitored by TLC 20% of ethyl acetate in petroleum ether. After completion of the reaction, saturated NH_4Cl (15 mL) was added to the reaction mixture. The combined organic layers was separated, washed, and dried over anhydrous Na_2SO_4. The crude product was obtained by removing organic solvent evaporated using the rotatory evaporator. Furthermore, the purification of crude product was performed using column chromatography: silica gel (100–200 mesh) and 30% ethyl acetate in petroleum ether as eluents to obtain pure 1-(4-(dimethylamino)phenyl)propan-1-ol (**5**).

Yield (80%), FR-IR (KBr) stretch(str) cm^{-1}: 1522.83, 1615.41 (str, C=C-Ar), 2873.98 (str, -CH_3), 2930.89 (str, -CH_2), 2959.82 (str, N(CH_3)$_2$), 3075.55(str, C-H), 3096.77, 3141.13 (str C-H-Ar), 3357.16 (str, O-H)^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ = 1.01 (t, 3H, CH_3), 1.62–1.96 (m, 2H, CH_2), 2.2 (brs, 1H, OH), 2.84 (s, 6H, N(CH_3)$_2$), 4.63 (t, 1H, -CH), 6.52 (d, 2H, Ar-H), 6.97 (d, 2H, Ar-H); ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ = 07.87 (1C, -CH_3), 31.18 (1C,-CH_2), 40.67 (2C, N(CH_3)$_2$), 79.32 (1C, -C-OH), 114.87 (2C, Ar-C), 128.11 (2C, Ar-C), 129.17 (1C,Ar-C), 146.12 (1C, Ar-C).

4.4. Synthesis of (4-(dimethyl Amino) Phenyl) (Phenyl) Methanol (6). To a three-necked round bottom flask, oven-dried Mg 1.17 g (4.87 mmol) and few crystals of iodine were taken and maintained inert atmosphere. To this flask, anhydrous diethyl ether (100 mL) was added with continuous stirring. The solution turned into brown color, and the flask was warmed using hot water. Then, bromobenzene (**12**) (6.5 mL, 6.2 mmol) was added dropwise maintaining the flask in ice cold water while the addition of bromobenzene was continued. The color of the reaction mixture turned to grey due to the formation of Grignard reagent phenyl magnesium bromide (PhMgBr). To this reaction mixture, 4-dimethylamino benzaldehyde (**8**) (5 g, 3.35 mmol) dissolved in 40 mL of anhydrous DEE was added drop wise. After completion of reaction, which was monitored by TLC, the

reaction mixture was quenched with saturated solution of ammonium chloride (20 mL). The resulting organic layer was then transferred to the separatory funnel, and the collected organic layer was dried over sodium sulphate. The solvent was removed using rotary evaporator, and the crude residue was further purified by column chromatography. The column chromatography was performed using 20% ethyl acetate in petroleum ether as the eluent to give pure (4-(dimethyl amino) phenyl) (phenyl) methanol in 67% yield (5.15 g) as a pale yellow solid.

FT-IR (KBr, cm^{-1}): 3454(O-H str), 2923–2955(C-H str), 1615(C=C str); ^{1}H-NMR ($CDCl_3$/TMS): δ 2.90 (*s*, 6H, N-CH_3), 5.72 (*s*, 1H, $(Ar)_2$-CH-OH), 6.67 (*d*, 1H, OH), 7.16–7.38 (*d*, Ar-H); ^{13}C-NMR ($CDCl_3$/TMS): δ 40.12, 79.67, 114.22, 125.87, 129.12, 129.82, 130.11, 130.80, 131.13, 140.06, 146.67.

4.5. Synthesis of 1-(2-bromoethyl)-1H-indole (7). A 100 mL round bottom flask was charged with potassium hydroxide (3.83 g, 68.3 mmol) and tetrabutylammonium fluoride (0.14 g, 0.54 mmol). Then, indole (**9**) (2 g, 17.1 mmol), which was dissolved in anhydrous DMF (25 mL), was added slowly to the above mixture with stirring. Then, the reaction mixture was heated at 50 C for 1.5 h and then cooled to 0 C. To this cooled reaction mixture 1,2-dibromoethane (**10**) (1.5 mL, 17.1 mmol) was added slowly with the help of syringe. Furthermore, the reaction mixture was allowed to stir for 30 min at 0 C and again heated at 50 C for 2 h. The reaction was monitored by TLC. After completion of reaction, the reaction mixture was poured in 70 mL water and extracted with dichloromethane (3×50 mL). The combined organic layers were washed with brine, and collected organic layers were dried over sodium sulphate. The solvent was removed using rotary evaporator, and the crude residue was subjected to column chromatography. The column chromatography was performed using silica gel and eluent combinations of petroleum ether/ethyl acetate (9 : 1) to obtain pure 1-(2-bromoethyl)-1H-indole in 54% yield (2.3 g) as pale yellow oil.

^{1}H-NMR ($CDCl_3$ + MeOD)/TMS): δ 3.33 (*t*, 2H, -CH_2-Br), 4.31 (*t*, 2H, N-CH_2-), 6.61 (*d*, 1H, pyrrole - H), 7.12 (*d*, 1H, benzene - H), 7.23 (*d*, 1H, pyrrole - H), 7.45 (*t*, 1H, benzene - H), 7.60 (*d*, 1H, benzene - H); ^{13}C-NMR ($CDCl_3$ + MeOD)/TMS): δ 80.9 ($CDCl_3$), 52.6 (MeOD), 25.4, 100.2, 108.7, 113.4, 124.5, 126.5, 127.2, 133.5, 139.4, 139.6.

4.6. Synthesis of 4-(1-(2-(1H-indol-1-yl) Ethoxy) pentyl)-N,N-dimethyl Aniline (1). A compound 1 was synthesized using *O*-alkylation reaction by combining intermediate **4** with **7**. To a round bottom flask Grignard product, secondary alcohol (**4**) (0.31 g, 1.5 mmol) was charged followed by the addition of phase transfer catalyst TBAF (0.31, 1.0 mmol) and acetonitrile (10 mL). This reaction mixture was stirred at room temperature, and then K_2CO_3 (0.14 g, 1 mmol) was added slowly and allowed whole reaction mixture to heat at 50 C. To this hot reaction mixture, N-alkylated product 1-(2-bromoethyl)-1H-indole (**7**) (0.224 g, 1 mmol) was added slowly. The reaction mixture was stirred further, and the progress of the reaction was monitored by TLC. After cooling the reaction mixture at room temperature, the reaction mixture was poured in 30 mL water and then extracted with 50 mL ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed using rotary evaporator.

The residue was purified by column chromatography and air pressure using a 1 : 12 (v/v) mixture of ethyl acetate and petroleum ether as eluting solution to afford 4-(1-(2-(1H-indol-1-yl) ethoxy) pentyl)-N, N-dimethyl aniline (**1**) in 72% yield (0.28 g) as yellow color oil.

^{1}H-NMR (DMSO/TMS): δ 1.30 (*t*, 3H, -CH_2CH_3), 1.65–1.81 (*m*, 4H, -$CH_3CH_2CH_2$-), 2.50 (*t*, 2H, O-CH-CH_2-), 3.14 (*s*, 6H, -N-$(CH_3)_2$), 4.76 (*t*, 2H, O-CH_2-), 4.94 (*t*, 2H, N-CH_2-), 5.36 (*t*, 1H, -O-CH-Ar), 6.37 (d, 1H,,pyrrole), 6.62 (*d*, 1, 1H, Ph.-H), 6.62 (*d*, 1H, Ar-H), 6.95, (t, 1H, Ar-H), 7.39 (d, 1H, Ph - H), 7.46 (*d*, 1H, pyrrole), 7.54 (*t*, 1H, Ar-H), 7.62 (*d*, Ar-H, 1H), 7.89 (*d*, 1H, Ar-H); ^{13}C-NMR (DMSO/TMS): δ 14.19, 23.27, 28.59, 35.62, 39.03, 40.80, 74.53, 77.68, 96.38, 105.29, 109.80, 113.01, 121.08, 121.43, 122.99, 127.15, 129.86, 130.22, 133.61, 149.93.

4.7. Synthesis of 4-((2-(1H-indol-1-yl) Ethoxy) (Phenyl) methyl)-N,N-dimethyl Aniline (2). The synthesis of target compound **2** was carried out by using the same procedure and conditions, which was described for the synthesis of compound **1**. To a round bottom flask Grignard product, secondary alcohol **6** (0.34 g, 1.5 mmol) was charged followed by the addition of phase transfer catalyst TBAF (0.316, 1.0 mmol) and acetonitrile (10 mL). The reaction mixture was stirred at room temperature, and then K_2CO_3 (0.14 g, 1 mmol) was added slowly and allowed whole reaction mixture to heat at 50°C. To this hot reaction mixture, N-alkylated product 1-(2-bromoethyl)-1H-indole (**7**) (0.224 g, 1.0 mmol) was added slowly. The reaction mixture was stirred further, and the progress of the reaction was monitored by TLC. After cooling the reaction mixture at room temperature, the reaction mixture was poured in 30 mL water and then extracted with 50 mL ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed using rotary evaporator. The residue was purified by column chromatography with air pressure using a 1 : 12 (v/v) mixture of ethyl acetate and petroleum ether as eluent to afford 4-((2-(1H-indol-1-yl) ethoxy) (phenyl) methyl)-N, N-dimethyl aniline (**2**) in 64% yield (0.25 g).

^{1}H-NMR (DMSO/TMS): δ 2.95 (d, 6H, -N-$(CH_3)_2$), 4.63 (*t*, 2H, -O-CH_2-), 5.07 (*t*, 2H, -N-CH_2), 5.66 (*s*, 1H, -O-CH-), 6.54 (*d*, 1H, Ar-H), 7.13 (*t*, 1H, Ar-H), 7.28 (*d*, 1H, Ar-H), 7.32 (*d*, 1H, pyrrole), 7.60 (*t*, 1H, Ar-H),7.68 (*d*, 1H, Ar-H); ^{13}C-NMR (DMSO/TMS): δ 40.59, 58.21, 96.48, 109.54, 110.52, 112.41, 120.71, 121.05, 123.42, 126.54, 127.73, 128.04, 129.30, 131.20, 132.66, 135.38, 139.23, 145.71, 149.73, 153.36.

4.8. Synthesis of 4-((2-(1H-indol-1-yl) Ethoxy) (Phenyl) methyl)-N,N-dimethyl Aniline (3). The synthesis of target compound **3** was carried out by using same procedure and

conditions, which was described for the synthesis of compound **1**.

The synthesis of target compound **2** was carried out by using same procedure and conditions, which was described for the synthesis of compound **1**. To a round bottom flask Grignard product, secondary alcohol **5** (0.34 g, 1.5 mmol) was charged followed by the addition of phase transfer catalyst TBAF (0.316, 1.0 mmol) and acetonitrile (10 mL). The reaction mixture was stirred at room temperature, and then K_2CO_3 (0.14 g, 1 mmol) was added slowly and allowed whole reaction mixture to heat at 50°C. To this hot reaction mixture, N-alkylated product 1-(2-bromoethyl)-1H-indole (**3**) (0.224 g, 1.0 mmol) was added slowly. The reaction mixture was stirred further, and the progress of the reaction was monitored by TLC. After cooling the reaction mixture at room temperature, the reaction mixture was poured in 30 mL water and then extracted with 50 mL ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed using rotary evaporator. The residue was purified by column chromatography with air pressure using a 1 : 12 (v/v) mixture of ethyl acetate and petroleum ether as eluent to afford 4-((2-(1H-indol-1-yl) ethoxy) (phenyl) methyl)-N, N-dimethyl aniline (**2**) in 64% yield (0.25 g).

^{1}H-NMR (DMSO/TMS): δ 2.95 (d, 6H, -N-$(CH_3)_2$), 4.63 (*t*, 2H, -O-CH_2-), 5.07 (*t*, 2H, -N-CH_2), 5.66 (*s*, 1H, -O-CH-), 6.54 (*d*, 1H, Ar-H), 7.13 (*t*, 1H, Ar-H), 7.28 (*d*, 1H, Ar-H), 7.32 (*d*, 1H, pyrrole), 7.60 (*t*, 1H, Ar-H),7.68 (*d*, 1H, Ar-H); ^{13}C-NMR (DMSO/TMS): δ 40.59, 58.21, 96.48, 109.54, 110.52, 112.41, 120.71, 121.05, 123.42, 126.54, 127.73, 128.04, 129.30, 131.20, 132.66, 135.38, 139.23, 145.71, 149.73, 153.36.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the PG Research grant of Mekelle University. The authors are thankful to Department of Chemistry and College of Natural and Computation Science for providing research facilities to complete this work.

Supplementary Materials

This section includes FT-IR spectrum, 1H-NMR spectrum, 13C-NMR spectrum, and DEPT-135 spectrum of 1-(4-(dimethylamino)phenyl)pentan-1-ol (4); FT-IR spectrum and 1H-NMR spectrum of (4-(dimethyl amino)phenyl)(-phenyl) methanol (6); 1H-NMR spectrum of and 13C-NMR spectrum of 1-(2-bromoethyl)-1H-indole (6); 1H-NMR spectrum, 13C-NMR spectrum, and DEPT-135 spectrum of 4-(1-(2-(1H-indol-1-yl)ethoxy) pentyl)-N,N-dimethyl aniline (1); 1H-NMR spectrum and 13C-NMR spectrum of 4-((2-(1H-indol-1-yl)ethoxy) (phenyl) methyl)-N,N-dimethyl aniline (3). (*Supplementary Materials*)

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Hindawi
Journal of Chemistry
Volume 2020, Article ID 5073508, 8 pages
https://doi.org/10.1155/2020/5073508

Research Article

Antimicrobial Characterization of Erythorbyl Laurate for Practical Applications in Food and Cosmetics

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Received 2 August 2019; Accepted 27 January 2020; Published 19 February 2020

Guest Editor: Maria Grazia Bonomo

In this study, antimicrobial spectrum of erythorbyl laurate (EL) against bacteria and fungi, leading to food-borne and infectious skin diseases, was evaluated for its practical applications in food and cosmetics. Furthermore, the influences of environmental factors including pH, oxidative stress, and dispersion medium on antimicrobial activity of EL were investigated. The three Gram-positive bacteria and the three molds were susceptible to 3.0 mM EL, while the yeast was susceptible to 6.0 mM EL. It was demonstrated EL retained antibacterial activity against *Staphylococcus aureus* after being oxidized while the antibacterial activity against the three Gram-positive bacteria including *S. aureus*, *Bacillus cereus*, and *Listeria monocytogenes* was significantly enhanced as decrease of pH from 7.0 to 5.0. Moreover, EL exhibited bactericidal effects against both Gram-positive and Gram-negative bacteria in an oil-in-water emulsion. Treatment of 5.0 mM EL for 4 h reduced 5.29 ± 0.24, 6.01 ± 0.18, 5.95 ± 0.13, and 6.24 ± 0.30 log CFU/mL against *S. aureus*, *L. monocytogenes*, *Pseudomonas aeruginosa*, and *Escherichia coli*, respectively. In a multipassage resistance selection study, it was observed minimum inhibitory concentrations of EL against *S. aureus* were not increased over 20 passages, indicating EL might not develop drug resistance of bacteria. This study suggests EL has a potential to be applied as the multifunctional additive in food and cosmetics.

1. Introduction

Lipid oxidation of food and cosmetics has been considered as a major hazard for consumer health [1]. Moreover, microbial contamination influences the physical and chemical properties of food and cosmetics [2–4]. Therefore, preservatives have been employed to control the lipid oxidation and the microbial contamination in food and cosmetic industry [5]. Previously, erythorbyl laurate (EL) was suggested as a novel multifunctional emulsifier with antioxidative and antibacterial activity to control the lipid oxidation and microbial contamination with a single compound [6].

A previous study investigated EL had antibacterial activity against Gram-positive food-borne pathogens including *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of EL against these Gram-positive bacteria were determined, and the antibacterial mechanism was thought to be disruption of the bacterial cell membrane [7]. In addition, it was investigated EL exhibited more effective antioxidative activity than that of erythorbic acid in oil-in-water (O/W) emulsion system since its antioxidative moiety is located on the interface of droplets where lipid oxidation mostly occurs [8]. However, no study has examined the antibacterial properties of EL in an O/W emulsion, which is thought to be the best system for application of EL.

Food and cosmetics are made at various pH values, which affect the antimicrobial activity of preservatives, caused by structural changes [9]. In addition, structure of EL might be changed once it begins to act as an antioxidant. It was reported that ranalexin, an antimicrobial peptide, retained its antimicrobial activity after being oxidized, while

catechins with antioxidative activity exhibited antimicrobial effects when functioning as an antioxidant [10, 11].

Antimicrobial spectrum of an antimicrobial agent is one of the crucial considerations for practical applications since microbial contamination of food and cosmetics can be caused by bacteria, yeasts, and molds [12]. Moreover, increasing concerns about bacterial resistance to antibiotics have prompted the requirement to search for alternative antibacterial agents for clinical application, as well as use in food and cosmetics. Since there are no reports of bacteria developing resistance to fatty acids, EL may not induce bacterial resistance [13, 14].

Fundamental investigation on antibacterial properties of EL with regard to antibacterial activity and mode of action was carried out in the previous study [7], but no study has been performed to bring detailed knowledge of antimicrobial properties of EL for the practical applications. Therefore, the aim of this study is comprehensive evaluation of antimicrobial properties of EL for the practical applications in food and cosmetics. The antimicrobial spectrum of EL against bacteria, yeasts, and molds was investigated, and resistance study was performed to figure out bacterial resistance against EL. Furthermore, the effects of structural changes in EL due to protonation by pH variation and oxidation on its antibacterial activity were evaluated. Finally, the antibacterial activity of EL in an O/W emulsion system was investigated to assess its applicability to emulsion-based foods and cosmetics.

2. Materials and Methods

2.1. Materials. Immobilized lipase, from *Candida antarctica* (triacylglycerol hydrolase, EC 3.1.1.3; Novozym 435) with a catalytic activity of 7,000 PLU/g (the activity of PLU refers to the millimoles of propyl laurate synthesized per min at 60°C), was purchased from Novozymes (Bagsvaerd, Denmark). Erythorbic acid (≥99.0%), lauric acid (≥99.0%), amphotericin B, and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monolaurin (>98.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Tween-20 was purchased from Ilshinwells (Cheongju, Korea), and sunflower oil was purchased from Ottogi (Seoul, Korea). All other chemicals were of analytical grade.

2.2. Production of Erythorbyl Laurate. EL was produced in gas-solid-liquid multiphase system [15]. The esterification between erythorbic acid and lauric acid was catalyzed by the immobilized lipase (Novozym 435) for 95 h. The total volume was 450 mL, and the erythorbic acid (0.97 mol) to lauric acid (1.93 mol) molar ratio was 1 : 2. The enzyme amount was 840 PLU/mL (0.12 g/mL), and nitrogen gas was bubbled at a flow rate of 6.0 L/min. The lauric acid was preincubated at 60°C for 20 min on the reactor to be melted.

Quantitative analysis of the EL was conducted using high-performance liquid chromatography (HPLC; LC-2002; JASCO, Tokyo, Japan) with a silica-based column (5.0 μm, 4.6 × 150 mm; Luna C18; Phenomenex, Torrance, CA, USA) and an ultraviolet (UV) detector (UV-2075; JASCO). The mobile phase was acetonitrile/water/acetic acid (90 : 5:5, v/v/v) at a 1.0 mL/min flow rate. Peaks in the HPLC chromatograms were confirmed according to the retention time of an EL standard. The EL concentration was determined using the standard curve of EL, generated by integrating the peak area at 265 nm using Borwin (ver. 1.21; JASCO).

2.3. Antimicrobial Susceptibility Test

2.3.1. Bacteria, Fungi, and Media. Bacteria and fungi for susceptibility testing were selected from pathogens causing infectious skin diseases and food-borne diseases. These included three Gram-positive bacteria (*Propionibacterium acnes* ATCC 6919, *Streptococcus pyogenes* ATCC 19615, and *Clostridium perfringens* ATCC 13124), three molds (*Trichophyton mentagrophytes* ATCC 18748, *Rhizopus oryzae* ATCC 10404, and *Aspergillus nidulans* ATCC 10074), and one yeast (*Candida albicans* KCTC 7678).

S. pyogenes ATCC 19615 was cultured in tryptic soy agar (TSA) at 37°C for 24 h in an atmosphere of 5.0% CO_2. *C. perfringens* ATCC 13124 and *P. acnes* ATCC 6919 were cultured in TSA at 37°C in an anaerobic atmosphere for 1 and 5 days, respectively. All molds were cultured in potato dextrose agar (PDA) at 25°C for 5 days, and the yeast was cultured in yeast malt agar (YMA) at 25°C for 2 days.

2.3.2. Disk Diffusion Assay. The antimicrobial susceptibility to EL was assessed using disk diffusion assays with reference to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Inocula of Gram-positive bacteria and yeast were made by direct colony suspension in tryptic soy broth (TSB) and yeast mannitol broth (YMB), respectively, equivalent to 0.5 McFarland suspension, and molds were inoculated (5×10^4 spores/mL) in potato dextrose broth (PDB). The bacterial and fungal suspensions were inoculated on the surfaces of agar plates. Gram-positive bacteria, molds, and yeast were inoculated on the surfaces of TSA, PDA, and YMA, respectively. EL, a negative control (TSB, PDB, and YMB with 2.0% dimethyl sulfoxide), and antibiotics (10 ppm ampicillin and 250 ppm amphotericin B) were prepared. Paper disks (8.0 mm thickness) were laid on the surface of the agar, and 40.0 μL samples were dispensed on the disk; the plate was then dried for 5 min.

The plates inoculated with *S. pyogenes* ATCC 19615 and *C. perfringens* ATCC 13124 were incubated at 37°C, anaerobically for 1 day, and that with *P. acnes* ATCC 6919 was incubated for 5 days. The plates inoculated with molds were incubated at 25°C for 2 days and *T. mentagrophytes* ATCC 18748 was incubated for 7 days; the yeast was incubated at 25°C for 2 days, and the clear zones were then analyzed [16–18].

2.4. Structural Changes and Antimicrobial Properties of EL

2.4.1. Structural Changes in EL. The degree of protonation of EL according to the pH was predicted using Chemicalize.org. EL was dissolved in 2.0% dimethyl sulfoxide in 100 mM universal buffer at pH 5.0, 6.0, or 7.0 to protonate the EL.

At neutral and alkaline pH, ascorbic acid is highly unstable due to its much faster conversion to dehydroascorbic acid. Dehydroascorbic acid also degrades more rapidly at alkaline pH (7.0–8.0) than at acid pH (3.0–5.0) [19]. Similarly, EL might also be oxidized and degraded at alkaline pH. Therefore, EL was added to 5.0% dimethyl sulfoxide in 50 mM Tris-HCl buffer at pH 8.0 for oxidation. The degree of oxidation was presented as the mean of decrement in the reduced form of EL measured by HPLC in triplicate.

2.4.2. Evaluation of Antimicrobial Activity. To evaluate the antimicrobial activity of EL according to the degree of protonation, a 100 μL inoculum of *S. aureus* ATCC 12692, *L. monocytogenes* ATCC 19115, or *B. cereus* ATCC 10876, equivalent to 0.5 McFarland suspension, was added to 100 μL universal buffer containing EL. The colony-forming units (CFUs) in a 100 μL aliquot were enumerated by preparing serial dilutions in 100 mM universal buffer at pH 5.0, 6.0, and 7.0; plating was performed in triplicate for each dilution with TSA plates, for 1 h after incubation at 37°C for 1 h. Then, each plate was incubated at 37°C for 18 h.

The antimicrobial activity of EL according to the degree of oxidation was evaluated using the MIC test after storage for 0, 6, 15, 24, and 48 h at 40°C. The reduced form of EL was quantified by HPLC in triplicate. For the MIC test, Tris-HCl buffer containing EL was added to 1.25% dimethyl sulfoxide in TSB. *S. aureus* ATCC 12692 was tested to determine the MIC of the oxidized form of EL, and the bacterial inoculum was diluted in 2.00% dimethyl sulfoxide in TSB as 0.5 McFarland standard. The MIC was determined using the broth microdilution assay. Serial dilutions of each desired concentration of oxidized EL were prepared in sterile TSB to a final volume of 100 μL in 96-well microplates. Then, each well was inoculated with 100 μL of the test organisms in TSB. The MIC was obtained as the lowest concentration at which the test compound inhibited bacterial growth after incubation for 12 h at 37°C [20, 21].

2.5. Multipassage Resistance Selection Study. An inoculum was prepared as 0.5 McFarland suspension after incubating *S. aureus* ATCC 12692 in TSB at 37°C for 18 h. Serial passages were performed every 18 h in TSB (2.0% dimethyl sulfoxide). The strain was treated with a 2-fold dilution series of EL in 200 μL broth in 96-well plates. For each subsequent passage, a 2 μL aliquot was taken from the wells with concentrations below the MIC that matched the turbidity of the growth control and was used to inoculate the dilution series for the next passage. Every passage was performed until 20 consecutive passages were completed [22].

2.6. Antimicrobial Assay in O/W Emulsion System

2.6.1. Preparation of the Emulsion and Inoculum. An O/W emulsion containing EL was formulated using sunflower oil, Tween-20, EL, and sterilized water. The concentrations of sunflower oil (5.0%, w/w) and Tween-20 (0.5%, w/w) were fixed for all emulsion formulations. The emulsion was prepared by mixing the oil and Tween-20 with or without EL (5.6 mM) followed by the addition of sterilized water. The emulsion was then sonicated with Sonomasher (ULH-700S; Ulso High-Tech Co., Cheongwon, Korea) at 4°C for 20 min at 210 W using pulses of 10 s on followed by 30 s off [23].

Bacteria related to food-borne and infectious skin diseases were selected. The three Gram-positive bacteria tested were *S. aureus* ATCC 12692, *L. monocytogenes* ATCC 19115, and *B. cereus* ATCC 10876; the two Gram-negative bacteria tested were *E. coli* ATCC 35150 and *P. aeruginosa* ATCC 10145. These bacteria were incubated in TSB at 37°C for 18 h. Then, cultures of the bacteria were diluted in 10 mM phosphate-buffered saline (PBS) at pH 7.4 as a 0.5 McFarland standard.

2.6.2. Bactericidal Assay. The MBC was determined as the standard criterion for evaluating the antimicrobial effect with some modifications, to determine the effective concentration of EL in the O/W emulsion [24]. Serial dilutions of each desired concentration of emulsion containing EL were prepared in an emulsion without EL to a final volume of 180 μL in 96-well microplates. Then, each well was inoculated with 20 μL of the inoculum. After incubation at 37°C for 12 h, the MBC of the emulsion containing EL against each bacterium was determined as the lowest concentration producing a 99.9% reduction of the viable bacteria count in the subcultured wells. The serially diluted subcultured well contents were spread on TSA plates, and the colonies were counted after incubation at 37°C for 24 h.

2.6.3. Time-Killing Assay. The time-killing assay was performed to study the concentration and time-dependent killing effect [25]. For the O/W emulsion containing EL, a 200 μL inoculum was added to 1,800 μL of O/W emulsion containing EL. After inoculation, the solutions were incubated at 37°C with shaking (220 rpm). After incubation for 0, 1, 2, 3, or 4 h, a 100 μL aliquot was removed and serially diluted. The diluted samples were inoculated on TSA plates and incubated for 24 h at 37°C. The number of survivors (CFU/mL) was determined by counting the colonies, and time-killing curves were constructed by plotting the log CFU/mL versus time. The limit of detection in the assay was 10 CFU/mL (1 log CFU/mL). The experiments were conducted in triplicate.

2.7. Statistical Analysis. The statistical analysis was performed using SAS software (SAS Institute, Cary, NC, USA). Experiments were conducted in triplicate. Mean separations were evaluated using Duncan's multiple-range test. A p value <0.05 was taken to indicate a significant difference.

3. Results and Discussion

3.1. Antimicrobial Spectrum of EL. The three bacteria, the three molds, and the one yeast, causing food-borne diseases

and infectious skin diseases, were selected for the evaluation of the antimicrobial spectrum of EL (Table S1 in Supplementary data) [26–31].

In the disk diffusion assay, EL showed antimicrobial activity against the three Gram-positive bacteria, one yeast, and three molds. *P. acnes*, *S. pyogenes*, *C. perfringens*, *A. nidulans*, *R. oryzae*, and *T. mentagrophytes* were susceptible to 3 mM EL and *C. albicans* to 6 mM EL (Table 1). These results demonstrated that EL can be applied to food and cosmetics to control the molds, yeast, and Gram-positive bacteria that lead to food-borne diseases and infectious skin diseases.

3.2. Effects of Structural Change in EL on the Antimicrobial Activity

3.2.1. Effect of pH. Three Gram-positive bacteria were used to examine whether the degree of protonation of EL altered its antimicrobial activity (Table 2). When *S. aureus* ATCC 12692 was treated with 0.5 mM EL, no significant reduction in viable cells was seen at pH 7.0. By contrast, 4.11 ± 0.28 and more than 6.89 ± 0.20 log CFU reductions in viable cells were observed at pH 6.0 and 5.0, respectively. A similar result was obtained for *L. monocytogenes* ATCC 19115. There was no significant reduction in viable cells at pH 7.0, but reductions of 7.02 ± 0.18 and 7.04 ± 0.20 log CFU were seen at pH 6.0 and 5.0, respectively. For *B. cereus* ATCC 10876, there was no significant difference in log CFU reduction by pH variation. The results might be caused by surviving spores of *B. cereus*, spore-forming bacteria, germinated [32].

At acidic pH, the chemical structure of EL changes with the protonated form of EL increasing. The percentage of the protonated form of EL at pH 5.0, 6.0, and 7.0 was 22.03, 2.75, and 0.28%, respectively, because EL has a strongly acidic pK_a of 4.5 according to the prediction (Figure S1 in Supplementary Data).

Protonation of the head group of EL with decreasing pH should reduce the electrostatic repulsion between EL and the outer membrane of bacteria.

Other antimicrobial peptides with antibacterial mechanisms involving damage to the bacterial cell membrane also have greater antibacterial activity at acidic pH. Under acidic conditions, the anionicity of the peptides was decreased by the protonation of aspartic acid and glutamic acid residues of which pK_a values are 3.65 and 4.25, respectively. The reduced anionicity of the peptide enhances its ability to interact with negatively charged phospholipids on the cytoplasmic membranes of Gram-positive bacteria, increasing its antibacterial activity [9]. Therefore, the reduced anionicity of the head group of EL caused by protonation at acidic pH enhanced its antibacterial activity against Gram-positive bacteria.

It was reported that pH values of human skin and most of the foods are all in the pH 4.2–6.5 [33]. Therefore, it was demonstrated EL is adequate to be applied in food and cosmetics, since the antimicrobial activity of EL can be enhanced in acidic conditions than neutral conditions.

3.2.2. Effect of Oxidation of EL. The MIC of EL against *S. aureus* ATCC 12692 did not increase significantly with oxidation (Figure 1). During oxidation, 58.99% (mol/mol) of EL was oxidized, whereas its MIC did not increase significantly.

Ascorbic acid is unstable in alkaline solution, which leads to the formation of dehydro-1-ascorbic acid on exposure to active oxygen species or UV light [34]. During ascorbic acid oxidation, dehydroascorbic acid is formed by the removal of two hydrogen atoms and two electrons from ascorbic acid [35]. In addition, the lactone ring of dehydroascorbic acid induces its irreversible conversion to 2,3-diketo-1-gluconic acid by degradation in alkaline solution [36]. Therefore, in this study, the form of the EL head group was predicted to change during the oxidation of EL (Figure S2 in Supplementary Data).

Although the structure of EL changed with oxidation, degree of oxidation did not affect the antibacterial activity of EL. The reason why EL retained the antibacterial activity after being oxidized could be explained in terms of hydrophilicity. It was reported hydrophilic/hydrophobic balance between the head and tail group of fatty acid esters is one of the crucial factors determining their antibacterial activity [37]. In addition, it is necessary hydrophilicity of the head group of lauric acid esters is above a certain level to possess antibacterial activity.

Hence, hydrophilicity of EL and oxidized form of EL were assessed by calculation of octanol-water partition coefficient (log P) with the atom/fragment contribution method [38]. As a result, the log p values of EL and oxidized form of EL were calculated as -0.686 and -1.753, respectively. The values indicated that the hydrophilicity of EL was not decreased after being oxidized, and the hydrophilicity of the oxidized form of EL is above the certain level to possess antibacterial activity. Therefore, EL can retain the antibacterial activity after it functions as an antioxidant in food and cosmetics.

3.3. Antimicrobial Activity of EL in O/W Emulsion.

The MBCs of EL in the O/W emulsions against the Gram-positive bacteria *L. monocytogenes* and *S. aureus* were 2 mM and less than 0.50 mM, respectively. In comparison, the MBCs of EL in the O/W emulsions against the Gram-negative bacteria *E. coli and P. aeruginosa* were both 2.00 mM (Figure S3 in Supplementary Data). The previous study on the antibacterial activity of EL against bacteria found that Gram-negative bacteria were not susceptible to EL in the aqueous phase [7]. However, the result showed that EL in the O/W emulsion had antimicrobial activity against both Gram-positive and Gram-negative bacteria. Even though not fully understood, the difference in the antibacterial spectrum of EL between in the O/W emulsions and the aqueous phase could be related to negatively charged characteristics of EL as described in Section 3.2.1. The outer membrane of Gram-negative bacteria consists of lipopolysaccharide (LPS) with negatively charged phosphate groups

Table 1: Susceptibility of various pathogens to erythorbyl laurate.

Classification	Pathogen	3 mM EL	6 mM EL
Bacteria	*Propionibacterium acnes* ATCC 6919	+	+
	Streptococcus pyogenes ATCC 19615	+	+
	Clostridium perfringens ATCC 13124	+	+
Mold	*Aspergillus nidulans* ATCC 10074	+	+
	Rhizopus oryzae ATCC 10404	+	+
	Trichophyton mentagrophytes ATCC 18748	+	+
Yeast	*Candida albicans* ATCC 11006	NT*	+

*NT = not tested.

Table 2: Effect of pH on the antimicrobial activity of erythorbyl laurate.

Bacteria	pH	Log CFU/mL[a,b]		
		Control culture	0.5 mM EL-treated culture	1 mM EL-treated culture
S. aureus ATCC 12692	5	7.89 ± 0.20^{Aa}	$<1.00^{Ab}$	$<1.00^{Ab}$
	6	9.88 ± 0.03^{Ba}	5.77 ± 0.30^{Bb}	5.47 ± 0.10^{Bb}
	7	9.63 ± 0.12^{Ba}	7.78 ± 0.18^{Cb}	7.06 ± 0.43^{Cc}
L. monocytogenes ATCC 19115	5	8.04 ± 0.20^{Aa}	$<1.00^{Ab}$	$<1.00^{Ab}$
	6	8.02 ± 0.18^{Aa}	$<1.00^{Ab}$	$<1.00^{Ab}$
	7	8.04 ± 0.13^{Aa}	7.04 ± 0.06^{Bb}	4.84 ± 0.16^{Bc}
B. cereus ATCC 10876	5	6.99 ± 0.07^{Aa}	2.65 ± 0.02^{Ab}	2.63 ± 0.01^{Ab}
	6	7.05 ± 0.05^{Aa}	2.64 ± 0.08^{Ab}	2.64 ± 0.10^{Ab}
	7	6.85 ± 0.20^{Aa}	2.61 ± 0.05^{Ab}	2.56 ± 0.06^{Ab}

[a]Values in the same column followed by different uppercase letters are significantly different ($p < 0.05$). [b]Values in the same row followed by different lowercase letters are significantly different ($p < 0.05$).

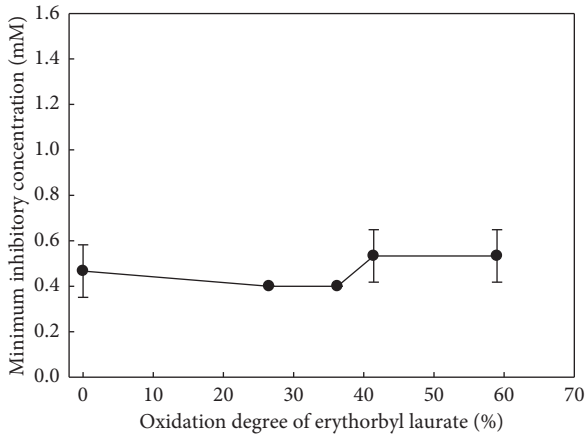

Figure 1: Effect of the oxidation of erythorbyl laurate on its minimum inhibitory concentration against *S. aureus* ATCC 12692.

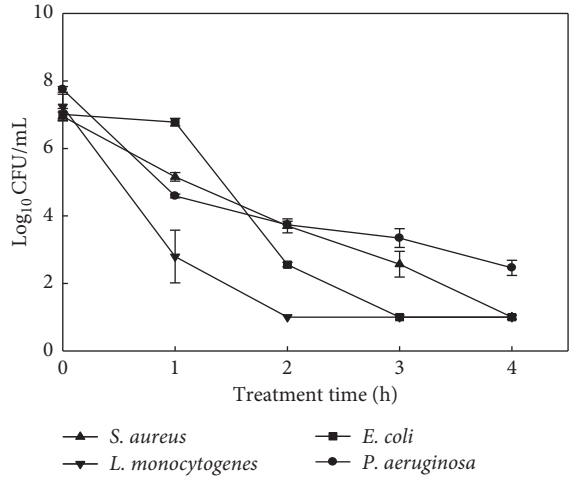

Figure 2: Time-killing curves of 5.0 mM erythorbyl laurate in oil-in-water emulsion against Gram-positive and Gram-negative bacteria.

[39]. In the aqueous phase, the electrostatic repulsion between EL and outer membrane (especially LPS) of the Gram-negative bacteria would harshly prevent EL interacting with the outer membrane. In contrast, the use of a nonionic surfactant (Tween-20) to stabilize the O/W emulsion might reduce the net negative charge of EL to overcome the electrostatic repulsion. As a result, in the O/W emulsions, EL can interact with the cell membranes of Gram-negative bacteria and consequently showed the antibacterial effect.

The time-killing assay quantified and confirmed the bactericidal activity of 5.0 mM EL in the O/W emulsion against two Gram-positive bacteria (*S. aureus* ATCC 12692 and *L. monocytogenes* ATCC 19115) and two Gram-negative bacteria (*E. coli* ATCC 35150 and *P. aeruginosa* ATCC 10145) (Figure 2). After treatment for 4 h, the numbers of *P. aeruginosa*, *E. coli*, *S. aureus*, and *L. monocytogenes* were reduced by 5.29 ± 0.24, 6.01 ± 0.18, 5.95 ± 0.13, and 6.24 ± 0.30 log CFU/mL, respectively.

3.4. Multipassage Resistance Selection Study. Resistance of microorganisms to EL can be selected for multipassage resistance selection, which enables strains to acquire greater resistance to antibiotics [40]. The initial MICs of EL and

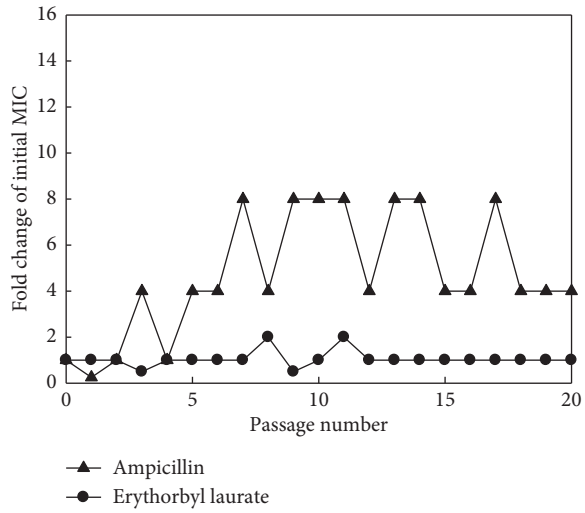

Figure 3: Changes in the minimum inhibitory concentrations of EL and ampicillin against *S. aureus* ATCC 12692 during 20 serial passages. Each line shows the result of one of three representative independent cultures passaged with each antimicrobial compound.

ampicillin for *S. aureus* ATCC 12692 were 286.76 and 0.125 μg/mL, respectively. A strain with reduced susceptibility to ampicillin was generated after 20 serial passages and was 4-fold less susceptible. Equal numbers of independent cultures were passaged with EL for 20 passages. No significant (>2-fold) increase in the MIC of EL was observed (Figure 3). Therefore, it was impossible to isolate bacteria that acquired resistance to EL.

As a β-lactam antibiotic, ampicillin is an irreversible inhibitor of the transpeptidase needed to make the bacterial cell wall. Generally, *S. aureus* acquires resistance to ampicillin by producing penicillinase and triggering the resistance pathway [41]. In comparison, evolution of bacteria acquiring resistance to free fatty acid was less problematic than conventional antibiotics [42]. Lauric acid and esters of fatty acids such as monocaprin kill microorganisms by disrupting bacterial cell membranes; hence, the emergence of resistance is unlikely [43]. Therefore, it was expected EL did not develop resistance since the antibacterial mechanism of EL is based on the damages to the bacterial cell membrane physically.

4. Conclusions

EL showed antimicrobial activity against Gram-positive bacteria, yeasts, and molds that cause food-borne diseases and infectious skin diseases. The protonation of EL in acidic conditions increased its antibacterial activity, while the oxidation of EL did not alter its antibacterial activity. EL exhibited bactericidal effects against both Gram-positive and Gram-negative bacteria in O/W emulsion system. A multipassage resistance study implied EL might not develop the drug resistance of bacteria. This study demonstrates EL is a promising additive to control microbial contamination in food and cosmetics, especially emulsion-based products.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Jin-Woo Kim, Hyunjong Yu, and Kyung-Min Park are co-first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jin-Woo Kim, Hyunjong Yu, and Kyung-Min Park contributed equally to this study.

Acknowledgments

This work was supported by "Cooperative Research Program for Agriculture Science and Technology Development (Project no. PJ01488801)," Rural Development Administration, Republic of Korea, and in part by R&D Program for Forest Science Technology (2019146C10-1921-AB02) provided by Korea Forest Service (Korea Forestry Promotion Institute), Republic of Korea.

Supplementary Materials

Table S1: criteria to select microorganisms for the susceptibility tests based on potential diseases related to food and cosmetics. Figure S1: predicted chemical structures of erythorbyl laurate in various pH values. Figure S2: predicted chemical structures of erythorbyl laurate according to the oxidation. Figure S3: bacterial suspensions spread on TSA plates after the treatment with various concentrations of erythorbyl laurate, showing the bactericidal activity of erythorbyl laurate in the O/W emulsions. (*Supplementary Materials*)

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Hindawi
Journal of Chemistry
Volume 2020, Article ID 6578579, 13 pages
https://doi.org/10.1155/2020/6578579

Review Article

Antibacterial Therapeutic Agents Composed of Functional Biological Molecules

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Received 29 July 2019; Accepted 6 January 2020; Published 29 January 2020

Guest Editor: Maria Grazia Bonomo

Antibacterial agents are a group of materials that selectively destroy bacteria by interfering with bacterial growth or survival. With the emergence of resistance phenomenon of bacterial pathogens to current antibiotics, new drugs are frequently entering into the market along with the existing drugs, and the alternative compounds with antibacterial functions are being explored. Due to the advantages of their inherent biochemical and biophysical properties including precise targeting ability, biocompatibility, biodegradability, long blood circulation time, and low cytotoxicity, biomolecules such as peptides, carbohydrates, and nucleic acids have huge potential for the antimicrobial application and have been extensively studied in recent years. In this review, antimicrobial therapeutic agents composed of three kinds of functional biological molecules were summarized. In addition, the research progress of antibacterial mechanism, chemical modification, and nanoparticle coupling of those biomolecules were also discussed.

1. Introduction

Bacteria that cause bacterial infections and disease are called pathogenic bacteria. Antimicrobial therapeutic agents are a group of materials that fight against pathogenic bacteria by killing or reducing the metabolic activity of bacteria. Traditionally, small molecule compounds are the most commonly used agents during the course of antibacterial therapy [1, 2]. Heterocyclic compounds are organic molecules, which include guanidines derived from carbazoles and azoles and have shown good antibacterial effect [3, 4]. Metal ions of heterocyclic compounds play a major role in the antibacterial activities [5, 6]. For example, the silver N-heterocyclic carbene complexes have good activity against a broad spectrum of bacteria [7]. However, antibiotic resistance becomes an escalating world-wide problem in recent years, and this happens because the diminishing molecules can be produced when the usual strategies was used to screen the libraries of compounds or chemically modify existing antibiotics. Particularly, Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria because the outer membrane of Gram-negative bacteria is often hidden by a slime layer, which in turn hides the antigens of the cell. The unique structure of the outer membrane of Gram-negative bacteria prevents certain drugs and antibiotics from entering into the cell, which means these bacteria have increased resistance to drugs and are more dangerous as disease-causing organisms [8, 9]. Therefore, the alternative antibacterial therapy strategies are being explored [10, 11].

Biological molecules are the basic substances that make up life [12]. Antibacterial agents composed of functional biological molecules have been extensively studied in recent years. These new types of antimicrobial therapeutic agents have the advantages of low cytotoxicity and side effects and often show environmental friendliness [13]. Chitosan, for example, is a natural biopolysaccharide and can also be prepared by deacetylation of chitin [14, 15]. One advantage

of chitosan over other polysaccharides is that its molecular structure is easy to be modified, especially at the C-2 position, which provides derivatives with different properties. In addition, the strong electrostatic action and the flexibility of the sugar chain make it easy to diffuse into the tissue fluid owing to the surface energy properties.

Antimicrobial peptides (AMPs) are ubiquitous in almost all organisms which can protect the host as the primary first line of defense against invading pathogens [16, 17]. AMPs have the characteristic functions of amphiphilicity and flexible conformational transitions which make the AMPs have the broad-spectrum antimicrobial activity [18]. The unique features and structures of the AMPs make them become particularly interesting compounds for developing new antimicrobial therapies. With the prevalence of antisense antimicrobial therapeutics and application of DNA aptamer [19, 20], nucleic acids are also considered to be the promising agents in antibacterial treatment. In this review, the current status of new antimicrobial therapeutic agents composed of functional biomolecules (nucleic acids, antimicrobial peptides, and chitosans) was explored. In addition, the chemical and nanoparticle modification of biomolecules and antibacterial mechanisms are also discussed.

2. Functional Nucleic Acid

2.1. Antisense Antimicrobial Therapeutic Agents. The synthetic short single-stranded oligomers can bind to complementary mRNA and inhibit translation or promote degradation of the targeted mRNA in microorganisms. The specific nucleic acid-based antimicrobial therapy confers an advantage over broad-spectrum antibiotics by avoiding unintended effects on commensal bacteria [19]. The reduction of drug discovery time is achieved by a rapid design and synthesis of oligomers based on target RNA sequence in microorganisms, and it also provides a flexible and rational approach to drug development.

Generally, four types of chemically modified nucleic acid analog have been developed. Phosphorothioate oligodeoxynucleotides (S-oligos) are analogs of nucleic acids, in which one of the nonbridging oxygen atoms on the phosphate linkage is replaced by a sulfur atom. The stability of the modified oligonucleotide is dramatically increased when exposed with nucleases [21]. The targeted mRNA can be digested by RNase H (ribonuclease H) when it is trapped with the complementary S-oligos [22]. Fomivirsen is the only commercially available antisense therapeutic agent for the treatment of cytomegalovirus-induced retinitis, and it was approved by USFDA (the United States Food and Drug Administration) in 1998 [23]. Locked nucleic acids (LNAs) and bridged nucleic acids (BNAs) are oxyphosphorothioate analogs that are modified with the extra bridge connecting the $2'$ oxygen and $4'$ carbon [24]. LNAs and BNAs are also stable to nuclease and may work by targeting mRNA and degrading it with RNase H [25]. Peptide nucleic acids (PNAs) are analogs of DNA in which the original sugar-phosphate backbones are replaced with electrically neutral pseudopeptide linkage [26, 27]. From the time they were invented in the early 1990s, these new types of DNA mimics have been shown to be capable of invading and opening up duplex structures of DNA effectively through forming new triplex or duplex assemblies with one of the target duplex strands. Owing to its unique mode of action, PNAs have been widely utilized to modulate gene expression and to perform diagnostic functions [28, 29]. PNAs are uncharged, which in part accounts for their high affinity for RNA. Phosphorodiamidate morpholino-oligomers (PMOs) are also analogs of DNA, in which the ribose is replaced by a morpholine ring and one of the nonbridging oxygen atoms on the phosphate linkage is displaced by a dimethyl amine [30]. PMOs are net neutral in charge and have the characteristic of water solubility and resistance to nucleases. It is believed that PMOs act by sterically blocking initiation of translation and do not activate RNase H degradation [31, 32].

Antisense antimicrobial therapeutic agents could be used against potentially available targets to any gene with a known base sequence in any bacterium in theory. This strategy significantly reduces the time required for discovery of a new antimicrobial. Synthetic nucleic acid oligomers have been synthesized and used as antimicrobial agents over thirty years ago [33]. The substantial improvements have been made until the attachment of cell-penetrating peptides (CPPs) with oligonucleotides was applied [34]. This happens because the biomacromolecule cannot penetrate the cell walls of bacteria, and delivery of synthetic oligonucleotides into the bacterial cytoplasm requires the attachment of another compound that can penetrate the bacterial cell wall. It is known that *ftsZ* in *S. aureus* is required for cell division, which was designed to be a target for a peptide-conjugated PNA (PPNA) [35]. Wesolowski et al. also reported that a CPP-PMO complex targets *E. coli gyrA* that is a conserved gene multiple bacterial species [36]. The sensitivities of a variety of both Gram-positive and Gram-negative bacterial strains to the CPP-PMO were tested, and the results showed that CPP-PMO reduced the viability of in four kinds of bacterial strains and CPP-PNA targeted the gyrase in another one. The expression of mRNA of *gyrA* was reduced, and the bacterium multiplication was inhibited. Furthermore, the CPP-PNA was synergistic with various current antibiotics [37].

2.2. Aptamer. Aptamer is an oligonucleotide selected from the library of nucleic acid molecules by a screening technique called SELEX (systematic evolution of ligands by exponential enrichment) *in vitro* [38]. Aptamers have been widely studied as antibacterial and anticancer agents because of the advantages of specific and accurate targeting, low cytotoxicity, easy preparation, and short research and development period [39]. Aptamer usually has a short sequence of 15–60 nucleotides. The intrastrand base pairing within aptamer can form stable secondary structures that has the special ability to bind targets with high affinity and specificity. In addition, aptamer can be extensively modified by chemical synthesis [40]. In the aptamer-based antibacterial agent development, limitations of traditional antibiotic selection such as accumulation in cells and the size and

charge of candidate drugs could be broken [41]. The diversity of single-stranded nucleic acid structures and spatial conformations provides the specific binding ability with various target molecules, and the highly specific binding forces may be caused by van der Waals forces, hydrogen bonding, electrostatic interactions, and shape matching [42]. Aptamers exert antimicrobial effects through completely different mechanisms compared to traditional antibiotics. It has been reported that a aptamer has the ability to combine surface antigens with passively neutralizing pathogens and then inhibit bacterial growth [43–45]. Aptamers can also target a particular factor, a key protein, for example, and then block the key biochemical process with actively neutralizing pathogens [46].

It had demonstrated that the binding of TiO_2 particles to the aptamer (*E. coli*-specific ssDNA) enhances the inactivation effect to of *E. coli* [47]. As shown in Figure 1, the close contact of the functionalized particles with *E. coli* was achieved by the strong binding effect between the aptamer and its target on the surface of *E. coli*, which can effectively induce reactive oxygen and be rapidly transferred to the cells. The aptamer-nanoparticle complexes may become a new generation of drug delivery systems with a higher specificity and effectiveness [48, 49].

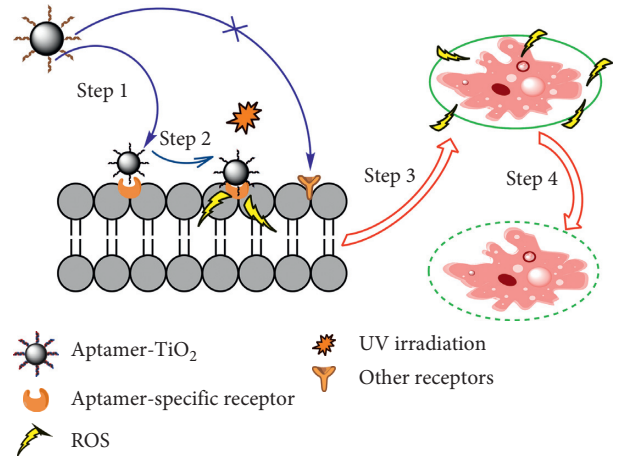

Figure 1: Schematic diagram of antibacterial mechanism of aptamer-TiO_2. Step 1: TiO_2 particles conjugated with aptamer bind on the cellular surface. The aptamer specifically recognizes its receptor on the bacterial surface to make the TiO_2 particles closely contact with the cell membrane. Step 2: TiO_2 is excited under UV irradiation to generate electrons on its surface and produce reactive oxygen species (ROS). Steps 3 and 4: ROS causes oxidative damage to bacteria by disrupting the cell wall or damaging DNA/RNA and proteins.

2.3. Topoisomerase Inhibitors. Topoisomerases are essential enzymes which have the special ability to change the topological structures of DNA by cleaving the DNA backbone and then rejoining it in the cells [50–52]. Those ubiquitous proteins are reported to be involved in many cellular processes such as replication, transcription, recombination, and critical for cell growth and proliferation [53–55]. For the importance of their cellular role, some topoisomerases are reported to be selected as the targets of anticancer drugs and antibiotics [56–58]. Bacterial-type IIA topoisomerases, topoisomerase IV, and DNA gyrase, for example, are well utilized clinical targets for antibacterial chemotherapy [59–61]. However, resistance of bacterial pathogens to current antibiotics has grown to be an urgent crisis. Compounds that can inhibit the activities of bacterial-type IA topoisomerases have been extensively studied in recent years [62–65].

Yang and coworkers reported that linear oligonucleotides containing mismatch or bulge can act as the irreversible inhibitors of bacterial topoisomerase I [66, 67], but no direct evidence such as the formation of covalent complex between DNA and proteins was provided in their studies. In our recent studies, particularly designed small DNA circles with high bending stress were synthesized [68]. It is demonstrated that small DNA circles showed high inhibitory effect on the activity of bacterial topoisomerases I and the single-stranded regions associated with bending deformation in DNA circles are believed to be the crucial factor for trapping the enzymes and decreasing the effective concentration of the topoisomerase in the reaction solution, as shown in Figure 2 [68]. Although DNA-based biopharmaceuticals have the advantages of low cytotoxicity and can be prepared easily [69, 70], the risk of host genome integration should be taken into consideration during the further design of the new type of DNA-based antibacterial agents. In addition, it should be pointed out that the small DNA circles alone can hardly penetrate into bacteria and exhibit bacteria growth inhibition effect in the current stage. However, it is possible to facilitate the delivery of oligonucleotides into bacteria with the development of the drug-delivery carriers based on nanotechnology and pharmaceutics.

3. Antimicrobial Peptide

Antimicrobial peptides (AMPs) are usually short biomolecules (15–50 amino acids) encoded by genes [71]. Most of these short peptides contain basic amino acids and hydrophobic residues, which are arranged in three dimensions on the surface of the peptide. The earliest research on AMPs can be traced back to the time when Fleming discovered lysozyme [72]. This discovery has developed a new field of research. In the 1960s, Zeya discovered that the basic peptides in polymorphonuclear (PMN) have antimicrobial properties [73, 74]. Over time, cecal peptide was discovered in 1980s [75]. The discovery and identification of defensive peptides from mammalian granules of neutrophils have taken a major step in the study of AMPs. In 1987, it was found that AMPs were rich in Xenopus laevis, named "magainins" [76]. Magainins were obtained by the secretory gland in the body. It also showed for the first time that AMPs are not only molecules of lower invertebrates but also part of higher vertebrates. Ever since, researchers around the world have made outstanding contributions to the continued rapid development of AMPs.

3.1. AMP Classification. Thousands of AMPs have been extracted from single-celled microorganisms of plants, insects, and bacteria. The antimicrobial peptide database (APD3)

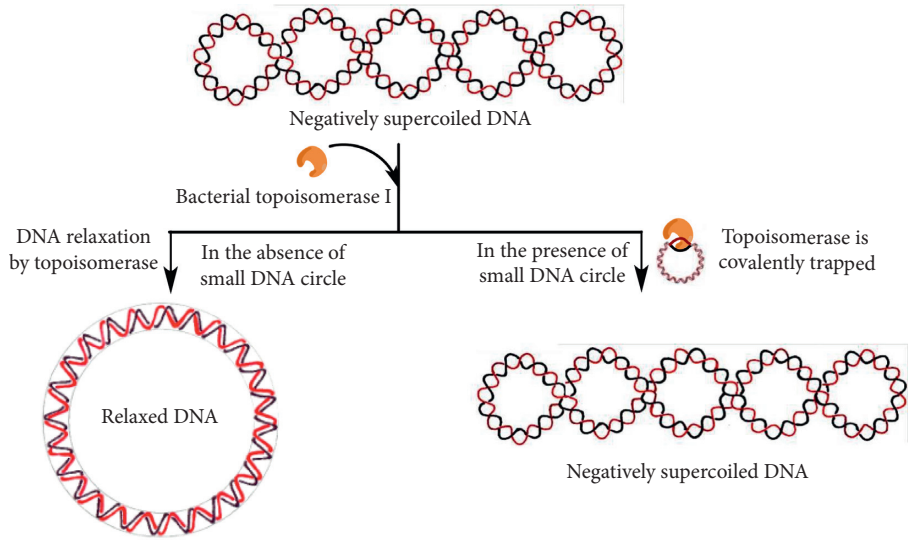

FIGURE 2: Illustration of the supercoiled plasmid relaxation catalyzed by bacterial topoisomerase I in the presence or absence of small DNA circle.

contains more than 3000 AMPs [77, 78]. It is worth noting that bacteria also produce a variety of AMPs in order to limit the growth of other microorganisms. AMPs can be ranked according to their physical properties, sequence properties, and conformation of antimicrobial peptides interacting with cell membranes. The APD3 database divides them into animal AMPs, plant AMPs, and bacterial AMPs, as shown in Table 1. Animal AMPs are further divided into human AMP, bovine AMPs, porcine AMPs, and insect AMPs [77].

3.2. Animal AMPs

3.2.1. Human AMP. It has been known that many patients with chronic obstructive pulmonary disease (COPD) sustain bacterial adhesion infections. Peptide deaminase (PADI) levels are used to elevate when suffering from COPD [97]. It is found that LL-37 was citrated due to an excess of PADI, which made the peptide more susceptible to be degraded by proteases. Citrated LL-37 reduced the antimicrobial activity against *Haemophilus* and *S. aureus*. Lima evaluated several HDPs including LL-37 and found that LL-37 had good potential in antimicrobial and immunomodulatory activities [98]. The antimicrobial effect of exogenous LL-37 was studied on the preterm infants *in vitro*, and the results indicated that LL-37 can enhance host immunity and exhibit significant anti-SA, SE, and CA activities [99] (SA, SE, and CA represent Staphylococcus aureus, Staphylococcus epidermidis, and Candida Albicans, respectively). L-37 and its homologues may be promising drugs for the prevention or treatment of neonatal sepsis.

3.2.2. Bovine and Porcine AMPs. Baumann et al. reported that bovine cathelicidins had the ability to inhibit bacterial growth and enhance the host immune system [100]. The results showed that BMAP-27 had the best killing effect on *S. aureus*, *E. coli*, and *Streptococcus uberis* (three main mastitis pathogens causing bovine mastitis). Pore-forming ability and antimicrobial mechanism of BMAP-27 are similar to LL-37 which has the amphiphilic alpha helix structure leading to membrane rupture. Surprisingly, although BMAP-27 completely lost its amphiphilic character, it still showed antimicrobial function, indicating the net charge contributing partially to the bactericidal effect.

PMAP-36 was isolated from *porcine* and has a variety of immunomodulatory effects *in vitro* [101]. It was found that PMAP-36 can disrupt the inner membrane of bacteria and is still active with a significant antibacterial effect at the concentration as low as 2.5 μM [101]. Modified PMAP-36 analogs were also designed in another work. It showed that the original antibacterial activity was retained when shortening the length of peptide chains within a certain range, and the hemolysis rate was also reduced at the same time [102]. Appropriate modification of existing AMPs may provide a promising strategy for developing new agents to overcome drug resistance.

3.2.3. Insect AMPs. Insect AMPs also show stronger antimicrobial effects at low concentrations. Abaecin showed no detectable activity against *E. coli* when tested at a concentration of 200 μM alone, and hymenopteran can affect the growth of bacterial cells only at concentrations greater than 2 μM [103]. However, the effective concentration of hymenopteran is 1.25 μM and was observed when it was used in combination with abaecin. The result indicated that abaecin can enhance the interactions between *E. coli* and hymenopteran. The naturally occurring enhanced interactions suggested that the combination of AMPs can be used to treat against Gram-negative pathogens with acquired resistance [104].

3.3. Plant AMPs

3.3.1. Brassica AMPs. Plant AMPs mainly interact with phospholipids in the microbial cell wall and lead to

Table 1: Physicochemical properties of AMPs mentioned in this review.

Tree	Source	Peptide	Length	Net charge	Hydrophobic residue (%)	3D structure	Reference
Animal	Human	LL-37	37	6	37	Helix	[79]
	Bovine	Bactenecin	12	4	66	Unknown	[80]
		BMAP-27	27	10	40	Helix	[81]
	Chicken	CATH-2	27	9	37	Helix	[82]
	Porcine	PMAP-36	37	13	37	Helix	[83]
		Protegrin	18	7	44	Beta	[84]
	Honeybee	Abaecin	34	4	23	Rich	[85]
	Tsetse	Cecropin	17	8	47	Helix	[86]
Plant	Brassica	BhDef1-2	—	—	—	—	[87]
	Aizoaceae	Novel peptides	—	—	—	—	[88]
	Alfalfa	alfAFP	45	3	33	Bridge	[89]
	Vigna radiata	Vrd2	47	3	29	Combine helix and beta structure	[90]
	Potato	Snakin-1	63	8	31	Helix	[91]
Bacteria	Paenibacillus	Nisin	43	1	34	Nonhelixbeta	[92]
		TriA1	13	2	46	Nonhelixbeta	[93]
	Pediococcus	Pediocin	44	3	34	Combine helix and beta structure	[94]
		LMW	16	1	50	Unknown	[95]
	Pediococcus	Garvicin KS	60	5	60	Unknown	[96]

membrane penetration [105]. Two new defensin genes, BhDef1 and BhDef2, were isolated from the *Brassica hybrid cv Pule* by Kaewklom et al. [87]. Six of the eight synthetic BhDef polypeptides have antimicrobial activity against Gram-positive and Gram-negative bacteria. Among them, BhDef14 had the highest activity against the two test pathogens *MRSA* and *Salmonella typhi* with an MIC of 1.88 mg/ml and 0.66 mg/ml, respectively.

3.3.2. Portulaca AMPs. Samriti et al. isolated new AMPs from the leaves of *Portulaca oleracea* [88], which had antimicrobial activity against *S. aureus* and *Bacillus subtilis*, but no antimicrobial activity against *E. coli* and *Candida albicans* [106]. The existence of omptins may be the cause of this phenomenon, a class of proteases present in the outer membrane of Gram-negative bacteria. Bacterial proteases have the ability to convert active AMPs into inactive fragments, making host bacteria resistant to these active AMPs. Another reason may be the low concentration of protein/peptide, resulting in insufficient supply of protein. In addition, the maximum inhibition zone against *S. aureus* and *Bacillus subtilis* was 8 mm and 4 mm, respectively, using the agarose diffusion method [106].

3.3.3. Alfalfa AMPs. It has demonstrated that chitin-binding domain (CBD) may increase the antimicrobial activity of alfAFP, one peptide from *Alfalfa* [107]. The results suggested that the introduction of this recombinant gene into other crops such as potatoes may result in GM crops (genetically modified crops) having higher resistance to fungal pathogens. Badrhadad et al. put the restructuring gene into tobacco by agrobacterium-mediated transformation, forming the recombinant protein (CBD-alfAFP) [108]. The experimental results showed that the total protein extract obtained from the transgenic plants ($P < 0.05$) significantly inhibited the growth of various bacteria and fungi compared with the nontransgenic plants, indicating fusion of CBD and alfAFP is an effective way to control pathogen damage.

3.4. Bacterial AMPs. The nisin synthesized by lactic acid bacteria (LAB) can suppress bacteria proliferation and was used as a natural preservative [109]. The study indicates that adding bacteriocin to packaging materials is an effective way to improve meat fresh keeping, rather than adding it directly to meat. In addition, nisin is nontoxic and can be digested by proteases with little or no effect on intestinal flora [110]. Finding new strategies based on synergistic combinations is key to fight resistant bacteria. Combinations of different bacteriocins can synergistically or additively eliminate bacteria [111]. The synergistic effects of the four bacteriocins nisin, pediocin, and enterocin MT104b and MT162b were studied based on food spoilage bacteria using the checkerboard method. The results showed that nisin combined with MT104b had a synergistic effect on killing *S. aureus*, while nisin combined with pediocin, nisin combined with MT162b, pediocin combined with MT104b, and pediocin combined with MT162b had a synergistic effect on *Sakei*. When Nisin is combined with pediocin, it has an additive effect on *Listeria monocytogenes* [111].

Recent work also conducted in-depth research on the synergistic bacteriocins. The activity of garvicin KS, a novel bacteriocin produced by *Lactococcus garvieae*, was studied [112, 113]. Unlike other bacteriocins, garvicin KS inhibits *Acinetobacter* but does not inhibit other Gram-negative bacteria. The combination action of polymyxin B and garvicin KS has a synergistic effect on *Acinetobacter* and *E. coli*, but not on *P. aeruginosa*. The mixture of nisin and polymyxin B has a similar effect. The synergistic mixture of polymyxin B, garvicin KS, and nisin resulted in rapid and complete eradication of *Acinetobacter* and *E. coli*. Low concentrations of garvicin KS or nisin can also rapidly and completely eliminate *S. aureus* when bacteriocins work synergistically with farnesol. In addition, garvicin KS is also

used as a promising healing AMP due to broad-spectrum inhibition and synergy with other antibiotics [114].

3.5. Mechanism of Action of AMPs. Most bioactive peptides are initially present as inactive precursors and are released in different ways *in vivo* [115, 116]. Generally, AMPs are released from their precursor proteins and participate in immune response. Several models, for example, "barrel-rod model," "carpet model," and "ring hole model," have been proposed to explain peptide insertion and membrane permeability, as shown in Figure 3. It is believed that the positively charged AMPs first interact with the negatively charged lipid groups on the outer surface of the cell membrane [117]. The amphiphilic feature facilitates the alternative insertion of the hydrophobic residue in AMPs. When the concentration of AMPs is relatively low, the peptide binds in parallel with the lipid bilayer. Once a certain threshold concentration is reached, the peptide molecule is perpendicular to the membrane and inserted into the lipid bilayer to form a transmembrane pore [118]. During the course of this action, spontaneous conformation changes in AMPs cause their disturbance in the cell membrane. Changes in the membrane structure, such as thinning, formation of pores, and localized disturbances, may result in the reorientation of peptide molecules in the membrane, and AMPs may also migrate through the membrane and diffuse into the cytoplasm to reach the intracellular target region [119]. The binding of AMPs on the surface of the cytomembrane changes the potential structural properties difference inside and outside the bacterial cell membrane and leads to dysregulation of ion channels and disorder of intracellular ion balance, which ultimately causes death of the bacteria [120]. Mammals, however, can produce proteases that has an ability to digest most of AMPs efficiently [121]. In addition, mammalian cell membranes are neutral, and the cholesterol can also stabilize the cell membrane structure, which is another reason that the activity of AMPs is alleviated in mammalian cell membranes [121]. The facts mentioned above indicate that AMPs can be used as excellent bacteriostatic agents for low toxicity to the human body.

4. Chitosan

Chitosan is a natural nontoxic biopolymer derived by deacetylation of chitin, a major component of the shells crab, shrimp, and crawfish. Many attentions have been attracted because of its unique biological activities and pervasive application in biopharmaceutical and chemical industries. The molecular weight of chitosan is between 3,800 and 20,000 Da, and the ratio of D-glucosamine to N-acetyl-D-glucosamine in polymer chain is known to be the degree of deacetylation (DD) that determines most of properties of chitosan [122]. As a polycationic polymer, chitosan has been investigated as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts, and fungi [123–125].

The use of chitosan is limited because of its insolubility in water, high viscosity, and tendency to coagulate with proteins at high pH. Many efforts to prepare functional derivatives by chemical modifications to increase the solubility in water have been reported [126]. In order to address the problem, scientists expand the application range by chemical modification of the chain without changing the original backbone, as shown in Figure 4 [127]. In addition, with the development of nanometer materials, researchers have turned their studies to the formation of chitosan nanoparticle complex that can improve their performance in antimicrobial behaviors [128].

4.1. Chitosan Derivatives

4.1.1. Quaternized Derivatives. In order to overcome the fact that chitosan is only suitable for acidic conditions, scientists make quaternized nitrogen atoms to produce derivatives with long-term charge and good water solubility (a wide range of pH values). For example, N,N,N-trimethyl chitosan is the first quaternized chitosan derivative with good antimicrobial activity [135], and improved antibacterial activity can be achieved by extensive quaternization of chitosan, as shown in Table 2. It has been demonstrated that low concentration of trimethyl chitosan (TMC) appears to be more effective against *E. coli*. The maximum activity is attained when 1 mg/ml of TMC was used [136]. In another studies, researchers synthesized three different ammonium salts with carboxylic acid end groups by the quaternary reaction of bromohexanoic acid with tertiary amines. The complexes were then coupled to the chitosan skeleton. The results showed no significant change in the antimicrobial activity against *S. aureus*. However, improved antimicrobial efficiency against *P. aeruginosa* was observed under the same condition [137]. Moreover, the quaternized derivatives exhibited good thermal stability, which is suitably applied to later development and utilization [138].

4.1.2. Sulfonated Derivatives. Sulfonated chitosan (SCS) was prepared by attaching 1,3-propane sulfonate to the chitosan backbone. Dragostin et al. measured the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of sulfonated chitosan derivatives using broth microdilution methods, and antimicrobial activity of the designed derivatives is higher than pure chitosan [134]. It is shown that the most active derivative is chitosan-sulfadiazine, and its MIC value is 0.03 and 1.25 mg/mL against *E. coli* and *S. aureus,* respectively, which is much lower than the values of unmodified chitosan. The promising antimicrobial activity is believed to be closely connected with the sulfonamides that replace chitosan glucosamine [134]. In addition, the *in vivo* models studies of rat burn wound showed that chitosan-sulfonamide derivatives have better healing effects than unmodified chitosan and can enhance epithelial formation. The MIC values against *E. coli* and *S. aureus* were also messaged in the studies, and the antibacterial activity increased by four times than those of water-soluble chitosan [130].

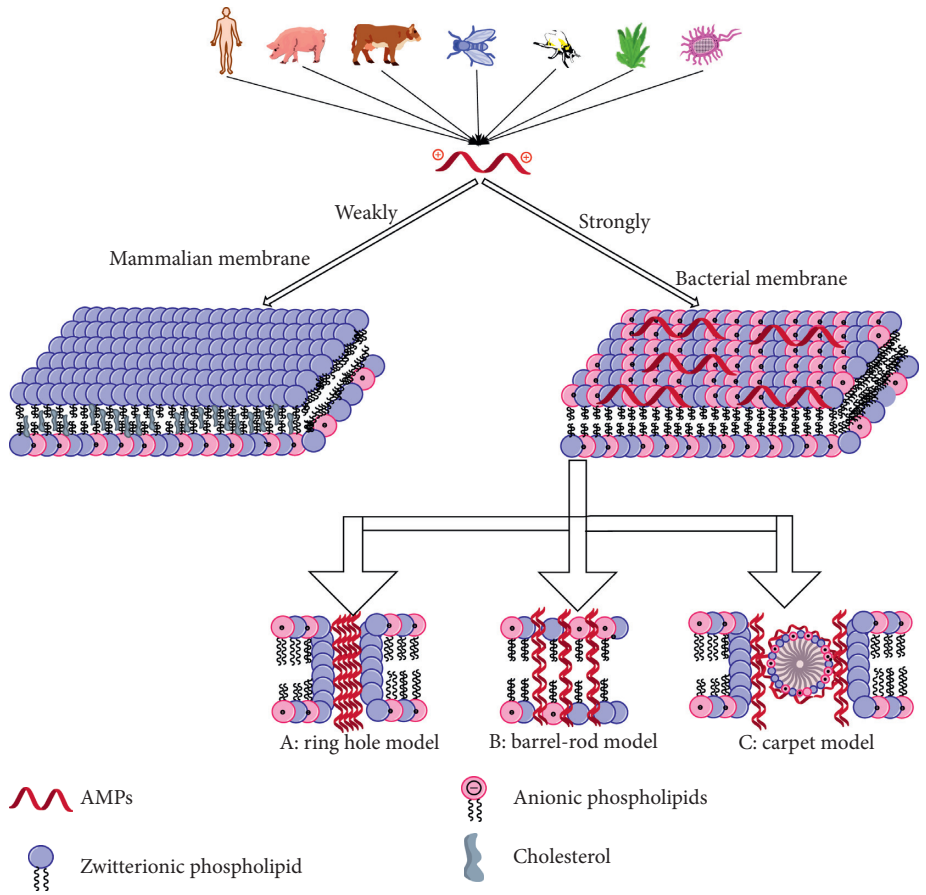

Figure 3: Mechanism of antibacterial peptides (AMPs): (a) in the ring hole model, the peptides insert into the membrane and aggregate into unstructured micelles by inducing continuous bending of the lipid monolayers; (b) in the barrel-rod model, the peptides vertically insert into the hydrophobic core region of the lipid membrane to form pores, like the rod inside the barrel; (c) in the carpet model, the peptides cover the membrane like a carpet, destroying the cell membrane and eventually leading to the formation of micelles. Due to the structural difference between the cell membrane of mammalian and bacterial, AMPs weakly react to mammalian cell membranes, but strongly to bacterial cell membranes.

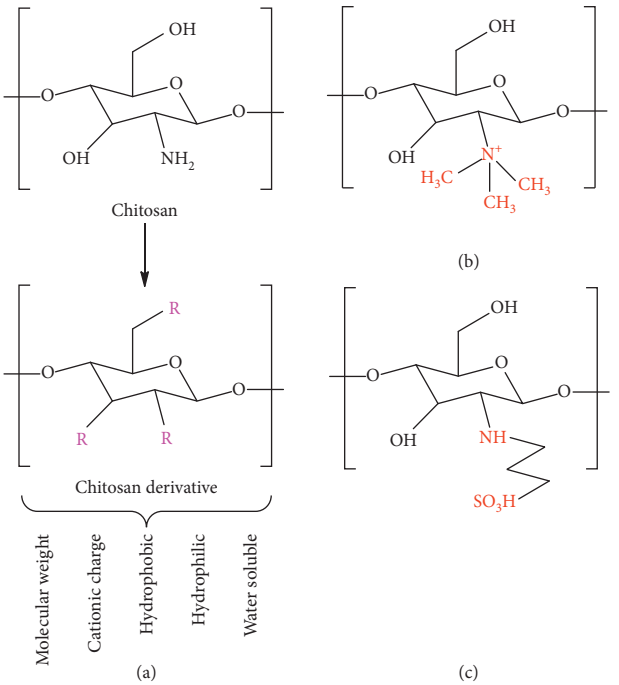

Figure 4: Chemical structures of (a) chitosan, (b) trimethyl chitosan (TMC), and (c) sulfonated chitosan (SCS). Chitosan derivatives with different properties can be prepared by modification at *R* (*R* quaternary ammonium, sulfonation, hydroxyalkyl, alkyl, and aminoalkyl).

Table 2: MIC (μg/mL) of chitosan and its derivatives against *E. coli* and *S. aureus*.

Compounds	MIC (μg/mL)		Reference
	E. coli	*S. aureus*	
Chitosan	>256	>256	[129–134]
N-Hydroxypropyl chitosan	31.3	31.3	[129]
N-Aminoethyl chitosan	62.5	125	[131]
N-Ethyl chitosan	64	32	[132]
N-Dodecyl chitosan	128	64	[132]
2-Hydroxy-3-trimethylammonium chitosan	31.25	7.8	[133]
Sulfadiazine chitosan	—	30	[134]

4.2. Chitosan Nanoparticle Complexes

4.2.1. Chitosan-Ag Complex. Chitosan has high chelating ability with various metal ions under acidic conditions. It is well known that metal ions can bind to the cell wall of microbial molecules, and it may be fatal to bacteria. Chitosan-mediated chelation of metal ions is often considered as a possible way to perform its antimicrobial action [139]. Silver ions can attach to proteins on the surface of cell membranes, which can cause changes in the structure and permeability of the cell membrane, ultimately leading to bacterial death [140]. In addition, silver ions can interact with microbial nucleic acids to inhibit microbial replication [140]. Chitosan metal complexes have strong antimicrobial activity. Kalaivani et al. used chitosan-mediated silver nanoparticles to detect the zone of *Bacillus sp.* and *S. aureus* by the agar disc diffusion method [141]. The inhibition diameters were 15 mm and 13 mm, respectively. At the same time, ampicillin and amphotericin-β were used as comparison to determine their antimicrobial activity against bacteria and fungi. The zone of inhibition diameters was smaller than chitosan-mediated silver nanoparticles, indicating the complexes had high antimicrobial activity.

4.2.2. Chitosan-ZnO Complex. It has been reported that ZnO nanoparticles release reactive oxygen species (ROS) [142]. ROS and Zn^{2+} can attack the negatively charged cell wall and cause bacterial leakage, eventually leading to bacterial death. In another studies, a porous chitosan film was prepared by casting *chitosan*-ZnO complex on silicon microsphere. By testing the efficiency against Gram-negative bacteria *Klebsiella* and Gram-positive bacteria *Bacillus*, it showed that the chitosan-ZnO composite film has the better antibacterial and antifouling activities when compared with the chitosan synthesized in acid solutions [143]. In addition, the complex showed more inhibition to *Klebsiella* than *Bacillus*. This difference may be caused by the chemical composition and structure of the cell membranes of the two bacteria. Recently, an unfocused chitosan/ZnO composite film was first prepared by a simple one-pot procedure [144]. The antimicrobial activities of chitosan against *S. aureus* and *E. coli* were studied by the colony counting method. Antimicrobial analysis showed that the effectiveness of all composite film can catch up to 2–4 times compared with pure chitosan. In another study, scientist successfully synthesized zinc oxide nanoparticles by microwave heating using chitosan as a stabilizer [145]. The amount of zinc oxide particles show linear relationship with the antibacterial activity. The antimicrobial activity of the complex against *S. aureus* and *E. coli* was determined to be 16 mm and 13 mm by measuring the inhibition zone. On the contrary, the optical density of the complex as an inhibitor is more than 1.5 times than pure chitosan when cultured at 37°C for 12 hours.

4.3. Mechanism of Action of Chitosan. Chitosans showed higher antibacterial activities than chitosan oligomers and markedly inhibited growth of most bacteria tested although inhibitory effects differed with Mws of chitosan. Li et al. studied the effects of different degree of deacetylation (DD) and pH on the antimicrobial activity of ultrahigh molecular weight chitosan [146]. The results showed that the antimicrobial activity against *E. coli* and *S. aureus* enhanced with the increase in chitosan DD when pH was 6.0. The minimum bactericidal concentration of chitosan amphiphilic bacteria was 0.0156%, when DD gets to 100%. The ultralong molecular chain of high-molecular-weight chitosan facilitates the combination of bacteria and then significantly enhances its antimicrobial activity. The nature of the antimicrobial activity is also relevant to the protonation of the amino groups and the formation of cations [147]. It is characterized by inhibiting bacterial growth first, and then the cells gradually break down in the bacteriostatic process.

The antimicrobial activity mainly depends on the presence of $-NH_3$. According Gomes' reports, it is proved that the surface of the nanomembrane with the highest antimicrobial activity had more NH^{3+} groups [148]. Numerous studies have shown that the NH^{3+} group of chitosan can interact with the negatively charged components carried by bacteria, causing a dramatic change on the membrane surface. This process increases membrane permeability, leads to cell membrane instability, induces leakage of intracellular components, and ultimately leads to cell death. It is also suggested that the chelation ability of chitosan has an important influence in antimicrobial ability [139]. Chitosan binds to trace metals on the surface of the cell wall, competing with Mg^{2+} and Ca^{2+} for electronegative sites. Owing to the substitution, the cell wall may lose its integrity or affect the activity of the degrading enzyme, making the cells unable to grow normally. The other mode of antimicrobial action proposed relies on the ability of chitosan to interact with cellular RNA through the cell membrane, then inhibiting DNA transcription and protein synthesis [149]. Besides chitosan, natural antimicrobials, including plant extracts and their essential oils, enzymes, peptides, bacteriocins, bacteriophages, and fermented ingredients, have all been shown to have the potential for use as alternatives to chemical antimicrobials [150].

5. Conclusion and Future Prospects

Functional nucleic acids, chitosan, and antimicrobial peptides exhibit huge antibacterial potential and greatly reduce

bacterial resistance. Compared with nonmodified molecules, the derivatives show stronger antibacterial activity after proper chemical and nanoparticle modification. As an alternative strategy, the development of antibacterial agents composed of biomolecules may provide a new way to avoid the current antibiotic resistance. Great progress and achievement have been made in understanding the antimicrobial mechanism with functional biomolecules *in vitro*. Some reports have shown significant efficacy in animal models of infection using doses in a clinically relevant range. We think that the future is bright for new antibacterial agents composed of functional biological molecules that has great potential in effect and are safe and nonresistant.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Natural Science Foundation of Jiangsu Province (no. BK20181028), the Startup Foundation (GXL2014038), the Jiangsu Innovative Research Program for Talent from the World's Famous Universities, and the Priority Academic Program Development (PAPD) Program of Jiangsu Province at Nanjing Forestry University. This work was also funded by the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (17KJB150011) and the Startup Foundation (JSNU2016YB02) at Jiangsu Second Normal University.

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Hindawi
Journal of Chemistry
Volume 2019, Article ID 2467970, 10 pages
https://doi.org/10.1155/2019/2467970

Research Article

Enaminone-Derived Pyrazoles with Antimicrobial Activity

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Received 15 April 2019; Revised 3 September 2019; Accepted 24 September 2019; Published 7 November 2019

Guest Editor: Maria Grazia Bonomo

A series of pyrazoles derived from the substituted enaminones were synthesized and were evaluated for antimicrobial activity. All the compounds were characterized by the spectral data and elemental analysis. The synthesized compounds were initially screened for their antimicrobial activity against *ATCC 6538*, *NCTC 10400*, NCTC 10418, and *ATCC 27853*. During initial screening, compounds (**P1**, **P6**, and **P11**) presented significant antimicrobial activity through disc diffusion assay. These compounds were further evaluated for antimicrobial activity at different time points against Gram-positive and Gram-negative bacteria and presented significant activity for 6 hours. The activity was found to be greater against Gram-positive bacteria. In contrast at 24 hours, the activity was found only against Gram-positive bacteria except compound **(P11)**, showing activity against both types of bacteria. Compound (**P11**) was found to have highest activity against both Gram-positive and Gram-negative bacteria.

1. Introduction

Enaminones are chemical compounds consisting of an amino group linked through a C=C to a carbonyl group. They are versatile synthetic intermediates that combine the ambident nucleophilicity of enamines with the ambident electrophilicity of enones. They are typical push-pull ethylenes in which the amine group pushes and the carbonyl pulls electron density. The carbonyl group, conjugated to the enamine moiety, gives this system enough stability to be easily prepared, isolated, and stored under atmospheric conditions at room temperature [1, 2]. Enaminones are the attractive intermediates for the synthesis of therapeutically active heterocycles such as quinolines, dibenzodiazepines, pyridinones, pyrazoles, oxazoles, and tetrahydrobenzoxazines [3–7]. They have been used as a starting material for the synthesis of antibacterial [8], anti-inflammatory [9], anticonvulsant [10], and antitumor agents [11]. Enaminones are versatile synthones because of their promptness to both electrophilic and nucleophilic attack [12]. They have been used in the synthesis of various heterocyclic compounds. Besides reducing the reaction time and increasing the yield and efficiency of the process, most of the research focused on a cleaner way to obtain enaminones.

Pyrazoles display a broad spectrum of potential pharmacological activities and are present in a number of pharmacologically active molecules such as phenazone, amidopyrene, methampyrone, azolid, tandearil, indoxacarb, and anturane. Pyrazoles are the most important scaffold having their broad spectrum of applications in the pharmaceutical field [13]. Pyrazole derivatives are reported to exhibit good anti-inflammatory [14], analgesic [15], anticonvulsant [16], and antidepressant activity [17]. Recently some arylpyrazoles were reported to have nonnucleoside HIV-1 reverse transcriptase inhibitor activity [18]. *N*-substituted pyrazoline derivatives also exhibit biological activities like antimicrobial [19], antitumor [20], antidepressant [21], and antitubercular activity [22].

In continuation of our work on pyrazoles [23], herein, we have synthesized a series of *N*-arylpyrazoles, which were derived from substituted enaminones and were found to possess an interesting profile as antimicrobial agents.

2. Materials and Methods

2.1. Experimental. Solvents were procured from Merck. Thin layer chromatography (TLC) was performed on Silica gel 60 F_{254} coated plates (Merck) to check the purity of compounds. Melting points were determined by using the Gallenkamp melting point apparatus. ^{1}H and ^{13}C NMR were recorded in the Bruker NMR 500/700 MHz and 125/176 MHz spectrophotometers. The samples were run in DMSO-d_6 with tetramethylsilane (TMS) as an internal standard. The molecular masses of compounds were determined by Agilent triple quadrupole 6410 TQ GC/MS equipped with ESI (electrospray ionization) source. The CHN Elementar (Analysensysteme GmbH, Germany) was used for elemental analysis of the compounds.

*2.2. Synthesis of Substituted Enaminones **(1–11)**.* A mixture of substituted acetophenone (0.02 mol) and dimethyl formamide-dimethylacetal (DMF-DMA) **(II)** (0.023 mol) was refluxed for 12 h under solvent-free condition on a heating mantle, and then, the mixture was left to cool slowly at room temperature. The precipitate was obtained. Diethyl ether was added to the precipitate, and filtration was performed under vacuum. The obtained product was recrystallized from absolute ethanol [24].

*2.3. Synthesis of Pyrazoles (**P1–P11**).* A mixture of enaminone (0.01 mol) and hydrazine hydrate 99% (0.01 mol) was refluxed in absolute alcohol for 3 hours. The mixture was poured into cold water. The precipitate was obtained. The product was obtained by filtration under vacuum. The product was recrystallized from ethanol [25].

3-(2,4,6-Trimethoxyphenyl)-1*H*-pyrazole **(P1)**: yield: 70%; m. p.: 148–150°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = (3.75 (3H, s, -OCH_3), 3.85 (3H, s, -OCH_3), 3.92 (3H, s, -OCH_3), 6.15–6.17 (2H, m, Ar-H), 6.80 (1H, d, J = 7 Hz, pyrazole), 7.79 (1H, d, J = 7 Hz, pyrazole), 11.81 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 55.5 (C10), 55.9 (C11, C12), 94.3 (C6), 98.9 (C8), 106.0 (C4), 106.1 (C2), 131.7 (C3), 148.0 (C1), 158.5 (C5), 158.9 (C9), 160.4 (C7); MS: m/z = 234.25 $[M]^+$; analysis: for $C_{12}H_{14}N_2O_3$, calcd. C 61.53, H 6.02, N 11.96%; found C 61.77, H 6.03, N 11.99% [26].

3-(2,4,5-Trimethoxyphenyl)-1*H*-pyrazole **(P2)**: yield: 65%; m. p.: 145–147°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 3.78 (3H, s, -OCH_3), 3.85 (3H, s, -OCH_3), 3.86 (3H, s, -OCH_3), 6.65 (1H, d, J = 7 Hz, pyrazole), 6.78 (1H, d, J = 7 Hz, pyrazole), 7.31–7.73 (2H, m, Ar-H), 12.80 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 56.1 (C10, C11, C12), 98.7 (C6), 99.1 (C8), 104.2 (C4), 105.4 (C2), 129.1 (C3), 139.5 (C1), 143.2 (C5), 149.8 (C7), 150.7 (C9); MS: m/z = 234.25$[M]^+$; analysis: for $C_{12}H_{14}N_2O_3$ calcd. C 61.53, H 6.02, N 11.96%; found C 61.76, H 6.04, N 11.98% [27].

3-(3,4-Dimethoxyphenyl)-1*H*-pyrazole **(P3)**: yield: 75%; m. p.: 123–125°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 3.78 (3H, s, -OCH_3), 3.62 (3H, s, -OCH_3), 6.68 (1H, d, J = 7 Hz, pyrazole), 6.98 (1H, d, J = 7 Hz, pyrazole), 7.33–7.70 (3H, m, Ar-H), 12.78 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 55.9 (C10, C11), 101.8 (C6), 102.0 (C8), 109.2 (C4), 118.0 (C2), 130.1 (C3), 140.4 (C1), 148.6 (C5), 149.2 (C9), 150.5 (C7); MS: m/z = 204.22 $[M]^+$; analysis: for $C_{11}H_{12}N_2O_2$ calcd. C 64.69, H 5.92, N 13.72%; found C 64.84, H 5.90, N 13.70% [28].

4-[4-(1*H*-Pyrazol-3-yl)phenyl]morpholine **(P4)**: yield: 70%; m. p.: 210–212°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 1.9 (4H, s, 2 × CH_2, morpholine), 3.73 (4H, s, 2 × CH_2, morpholine), 6.10 (4H, m, Ar-H), 6.88 (1H, d, J = 7 Hz, pyrazole), 7.56 (1H, d, J = 7 Hz, pyrazole), 11.80 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 48.6 (C10, C11), 66.5 (C12, C13), 114.9 (C2, C6, C8), 126.0 (C4, C9), 131.3 (C3), 143.3 (C1), 150.6 (C7); MS: m/z = 229.27 $[M]^+$; analysis: for $C_{13}H_{15}N_3O$ calcd. C 68.10, H 6.59, N 18.33%; found C 68.36, H 6.60, N 18.40% [29].

1-[4-(1*H*-Pyrazol-3-yl)phenyl]piperidine **(P5)**: yield: 60%; m. p.: 170–172°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 1.23 (6H, s, piperidine), 3.16 (4H, s, piperidine), 6.0–6.98 (4H, m, Ar-H), 7.47 (1H, d, J = 7 Hz, pyrazole), 7.63 (1H, d, J = 7 Hz, pyrazole), 12.8 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 24.3 (C12), 25.6 (C13), 49.7 (C10, C11), 101.2 (C2), 116.0 (C6, C8), 126.3 (C4, C9), 151.3 (C3, C1, C7); MS: m/z = 227.30 $[M]^+$; analysis: for $C_{14}H_{17}N_3$ calcd. C 73.98, H 7.54, N 18.49%; found C 73.69, H 7.56, N 18.55%.

3-(2,4-Dimethoxyphenyl)-1*H*-pyrazole **(P6)**: yield: 80%; m. p.: 125–127°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 3.81 (3H, s, -OCH_3), 3.83 (3H, s, -OCH_3), 6.61 (1H, d, J = 7 Hz, pyrazole), 6.66 (1H, d, J = 7 Hz, pyrazole), 7.50–7.62 (3H, m, Ar-H), 12.8 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 55.7 (C10), 55.9 (C11), 99.1 (C6), 103.9 (C8), 105.4 (C2, C4), 105.9 (C3), 128.9 (C1), 139.4 (C5), 157.3 (9), 160.6 (C7); MS: m/z = 204.22 $[M]^+$; analysis: for $C_{11}H_{12}N_2O_2$ calcd. C 64.69, H 5.92, N 13.72%; found C 64.74, H 5.91, N 13.76% [30].

3-(4-Methoxyphenyl)-1*H*-pyrazole **(P7)**: yield: 90%; m. p.: 126–128°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 3.78 (3H, s, -OCH_3), 6.63 (1H, d, J = 7 Hz, pyrazole), 6.99 (1H, d, J = 7 Hz, pyrazole), 7.55–7.78 (4H, m, Ar-H), 12.8 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 55.5 (C10), 101.7 (C6, C8), 114.4 (C4), 126.9 (C2), 130.1 (C3), 140.4 (C1), 142.2 (C5), 160.4 (C9), 159.1 (C7); MS: m/z = 174.19 $[M]^+$; analysis: for $C_{10}H_{10}N_2O$ calcd. C 68.95, H 5.79, N 16.08%; found C 68.77, H 5.77, N 16.14% [31].

3-(2,5-Dimethoxyphenyl)-1*H*-pyrazole **(P8)**: yield: 80%; m. p.: 108–110°C; ^{1}H NMR (500 MHz, DMSO–d_6): δ = 3.81 (3H, s, -OCH_3), 3.83 (3H, s, -OCH_3), 6.61 (1H, d, J = 7 Hz, pyrazole), 6.66 (1H, d, J = 7 Hz, pyrazole), 7.50–7.62 (3H, m, Ar-H), 12.8 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): δ = 55.7 (C10), 55.9 (C11), 99.1 (C6), 103.9 (C8), 105.4 (C4), 105.9 (C2), 128.9 (C3), 139.4 (C1), 157.3 (C5, C9), 160.6 (C7); MS: m/z = 204.22 [M]+; analysis: for $C_{11}H_{12}N_2O_2$ calcd. C 64.69, H 5.92, N 13.72%; found C 64.87, H 5.94, N 13.77% [32].

3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1*H*-pyrazole **(P9)**: yield: 55%; m. p.: 68–70°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 4.26$ (4H, s, $2 \times OCH_2$), 6.62 (1H, d, $J = 7$ Hz, pyrazole), 6.88 (1H, d, $J = 7$ Hz, pyrazole), 7.30–7.73 (3H, m, Ar-H), 12.8 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 64.5$ (C10, C11), 101.8 (C6), 114.1 (C8), 117.6 (C4), 118.7 (C2), 127.8 (C3), 130.1 (C1), 143.2 (C5), 143.8 (C9), 150.1 (C7); MS: m/z = 202.20 $[M]^+$; analysis: for $C_{11}H_{10}N_2O_2$ calcd. C 65.34, H 4.98, N 13.85%; found C 65.40, H 4.96, N 13.80%.

3-[4-(1*H*-Imidazol-1-yl)phenyl]-1*H*-pyrazole **(P10)**: yield: 68%; m. p.: 178–180°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 6.80$ (1H, d, $J = 7$ Hz, pyrazole), 7.14 (1H, d, $J = 7$ Hz, pyrazole), 7.70–8.32 (7H, m, Ar-H), 12.99 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 102.4$ (C6), 118.3 (C8), 120.9 (C10), 126.8 (C11, C12), 130.3 (C2, C4), 130.6 (C3), 135.9 (C1), 136.3 (C5, C9), 149.6 (C7); MS: m/z = 210.23 $[M]^+$; analysis: for $C_{12}H_{10}N_4$ calcd. C 68.56 H 4.79, N 26.65%; found C 68.70, H 4.80, N 26.70%.

3-(4-Bromophenyl)-1H-pyrazole **(P11)**: yield: 70%; m. p.: 148–150°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 6.75$ (1H, d, $J = 7$ Hz, pyrazole), 7.58 (1H, d, $J = 7$ Hz, pyrazole), 7.79–7.81 (4H, m, Ar-H), 13.00 (1H, s, NH, D_2O exchg.); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 102.4$ (C6), 120.8 (C8), 127.6 (C2), 130.5 (C4), 131.9 (C3), 132.8 (C1), 133.5 (C5, C9), 149.4 (C7); MS: m/z = 190.19 $[M]^+$; analysis: for $C_{10}H_{10}N_2O_2$ calcd. C 63.15, H 5.30, N 14.73%; found C 63.35, H 5.31, N 14.78% [33].

*2.4. Synthesis of Pyrazole Derivatives **(P12–P16)**.* A mixture of pyrazole (0.01 mol), phenacyl bromide (0.01 mol), and triethylamine (0.01 mol) was refluxed in DMF for 10 hours. The precipitates were obtained by pouring the reaction mixture into cold water (50 mL). The products were obtained by filtration under vacuum. The products were washed several times with cold water. The obtained products were recrystallized from glacial acetic acid (5 mL) and ethanol (100 mL) mixture. Analytical and spectral data for the compounds were in good agreement with the expected structures of the compounds.

1-Phenyl-2-[3-(2,4,6-trimethoxyphenyl)-1*H*-pyrazol-1-yl]ethan-1-one **(P12)**: yield: 70%; m. p.: 128–130°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 3.69$ (6H, s, $2 \times$ -OCH_3), 3.81 (3H, s, -OCH_3), 5.80 (2H, s, CH_2O), 6.19 (1H, d, $J = 7$ Hz, pyrazole), 6.27 (1H, d, $J = 7$ Hz, pyrazole), 7.58–8.0 (7H, m, Ar-H); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 55.7$ (C10), 56.1 (C11), 58.1 (C12), 91.4 (C6, C8), 105.3 (C4), 108.4 (C2), 128.5 (C16, C17), 129.3 (C20), 131.5 (C19), 134.3 (C18), 135.1 (C15), 144.3 (C1), 159.5 (C5), 161.1 (C9), 194.3 (C14); MS: m/z = 352.38$[M]^+$; analysis: for $C_{20}H_{20}N_2O_4$ calcd. C 68.17, H 5.72, N 7.95%; found C 68.34, H 5.70, N 7.98%.

1-Phenyl-2-[3-(2,4,5-trimethoxyphenyl)-1*H*-pyrazol-1-yl]ethan-1-one **(P13)**: yield: 70%; m. p.: 140–142°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 3.70$ (3H, s, -OCH_3), 3.83 (3H, s, -OCH_3), 3.86 (3H, s, -OCH_3) 5.92 (2H, s, CH_2O), 6.74 (1H, d, $J = 7$ Hz, pyrazole), 7.40 (1H, d, $J = 7$ Hz, pyrazole), 7.59–8.0 (6H, m, Ar-H); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 56.2$ (C10), 56.5 (C11), 58.3 (C12), 99.2 (C6), 106.6 (C8), 111.9 (C4), 113.8 (C2), 129.4 (C16), 132.8 (C17), 134.4 (C20), 134.9 (C19), 142.1 (C18), 147.6 (C15), 147.6 (C1), 149.5 (C5), 151.4 (C9), 194.3 (C14); MS: m/z = 352.38 $[M]^+$; analysis: for $C_{20}H_{20}N_2O_4$ calcd. C 68.17, H 5.72, N 7.95; found C 68.30, H 5.71, N 7.97%.

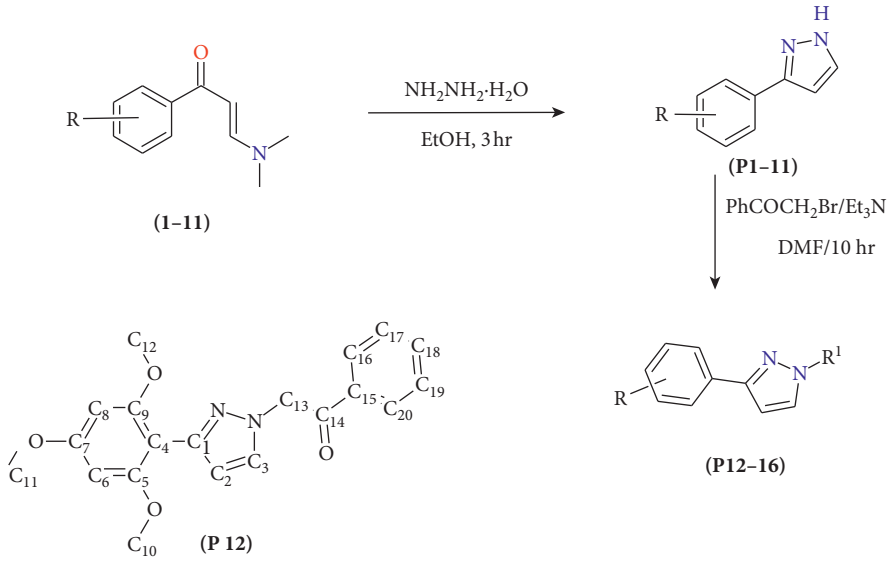
2-[3-(3,4-Dimethoxyphenyl)-1*H*-pyrazol-1-yl]-1-phenylethan-1-one **(P14)**: yield: 70%; m. p.: 118–120°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 3.78$ (3H, s, -OCH_3), 3.80 (3H, s, -OCH_3), 5.90 (2H, s, CH_2O), 6.75 (1H, d, $J = 7$ Hz, pyrazole), 6.99 (1H, d, $J = 7$ Hz, pyrazole), 7.32–8.0 (8H, m, Ar-H); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 55.8$ (C10), 58.3 (C11), 103.0 (C6), 109.0 (C8), 112.3 (C4), 118.0 (C2), 126.7 (C16), 128.5 (C17), 129.4 (C20), 138.7 (C19), 134.4 (C18), 135.0 (C15), 148.8 (C1), 149.2 (C5), 150.8 (C9), 194.2 (C14); MS: m/z = 322.35 $[M]^+$; analysis: for $C_{19}H_{18}N_2O_3$ calcd. C 70.79, H 5.63, N 8.69%; found C 70.99, H 5.65, N 8.72%.

2-[3-(2,4-Dimethoxyphenyl)-1H-pyrazol-1-yl]-1-phenylethan-1-one **(P15)**: yield: 70%; m. p.: 108–110°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 3.80$ (3H, s, -OCH_3), 3.87 (3H, s, -OCH_3), 5.87 (2H, s, CH_2O), 6.68 (1H, d, $J = 7$ Hz, pyrazole), 6.78 (1H, d, $J = 7$ Hz, pyrazole), 7.55–8.0 (7H, m, Ar-H); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 55.6$ (C10), 58.2 (C11), 99.1 (C8), 105.7 (C6), 106.6 (C4), 115.2 (C2), 128.5 (C16), 128.9 (C17), 129.4 (C19), 132.5 (C20), 134.3 (C18), 135.0 (C15), 147.7 (C1), 157.8 (C5), 160.3 (C9), 194.3 (C14); MS: m/z = [M]+; analysis: for $C_{19}H_{18}N_2O_3$ calcd. C 70.79, H 5.63, N 8.69%; found C 70.98, H 5.64, N 8.71%.

2-[3-(4-Methoxyphenyl)-1*H*-pyrazol-1-yl]-1-phenylethan-1-one **(P16)**: yield: 70%; m. p.: 75–77°C; ^{1}H NMR (500 MHz, DMSO–d_6): $\delta = 3.78$ (3H, s, -OCH_3), 5.88 (2H, s, CH_2O), 6.79 (1H, d, $J = 7$ Hz, pyrazole), 6.98 (1H, d, $J = 7$ Hz, pyrazole), 7.59–8.0 (9H, m, Ar-H); ^{13}C NMR (125.76 MHz, DMSO–d_6): $\delta = 55.5$ (C10), 102.8 (C6), 114.4 (C8), 126.5 (C4, C2), 126.8 (C16), 128.5 (C17), 129.4 (C19), 133.7 (C20), 134.4 (C18), 135.0 (C15), 150.6 (C1), 159.1 (C5, C9), 194.2 (C14); MS: m/z = 292.33 $[M]^+$; analysis: for $C_{18}H_{16}N_2O_2$ calcd. C 73.95, H 5.52, N 9.58%; found C 73.77, H 5.50, N 9.53%.

2.5. Bacterial Culture. Bacterial growth inhibitory activity of synthesized compounds was checked against Gram-positive and Gram-negative bacteria. In order to test antibacterial activity of synthesized compounds, *ATCC 6538*, *NCTC 10400* (Gram-positive); NCTC 10418, and *ATCC 27853* (Gram-negative) were obtained from the microbiology unit at Department of Pharmaceutics, College of Pharmacy, King Saud University. Initially, the bacteria were grown at 37°C for overnight in Mueller-Hinton agar. The actively growing bacteria were transferred to Mueller-Hinton broth and further incubated overnight at 37°C. The resultant active bacteria were used for antibacterial activity.

2.6. Screening of Synthesized Compounds for Antibacterial Activity. The synthesized compounds were initially screened for their antibacterial activity against *ATCC 6538* and *NCTC* 10418 with the use of agar well diffusion assay [34]. The bacterial culture turbidity was established to 0.5



Compound	R	R^1
P1	2,4,6-$(OCH_3)_3$	-
P2	2,4,5-$(OCH_3)_3$	-
P3	3,4-$(OCH_3)_2$	-
P4	4-Morpholino	-
P5	4-Piperidino	-
P6	2,4-$(OCH_3)_2$	-
P7	4-(OCH_3)	-
P8	2,5-$(OCH_3)_2$	-
P9	4-Imidazole	-
P10	1,4-Benzodioxane	-
P11	4-Br	-
P12	2,4,6-$(OCH_3)_3$	$-COCH_2C_6H_5$
P13	2,4,5-$(OCH_3)_3$	$-COCH_2C_6H_5$
P14	3,4-$(OCH_3)_2$	$-COCH_2C_6H_5$
P15	2,4-$(OCH_3)_2$	$-COCH_2C_6H_5$
P16	4-(OCH_3)	$-COCH_2C_6H_5$

Scheme 1: Synthetic route of compounds (**P1–P16**).

McFarland turbidity standards. The activity was performed on Mueller-Hinton agar plates. The bacteria were spread over agar plates, and the 5 mm wells were created aseptically in the agar plate. The 20 μL of each tested compound (250 μg/mL) was added to wells, and plates were incubated overnight at 37°C. The zone of inhibition was measured, and comparative activity of synthesized compounds was determined.

2.7. Antibacterial Activity at Different Time Points. The synthesized compounds were solubilized in DMSO with initial concentration of 5 μg/μL. This initial stock was used to adjust different concentrations of tested compounds. The concentration was adjusted in Mueller-Hinton broth as 500, 300, 200, 100, 50, and 0 μg/mL.

2.8. Antimicrobial Activity of Compounds through Optical Density. Antibacterial activity of tested compounds against *ATCC 6538*, *NCTC 10400*, NCTC 10418, and *ATCC 27853* was determined by analysis of bacterial growth at different time points under the influence of different concentrations of tested compounds. The bacterial growth was determined in terms of optical density at 600 nm. The OD_{600} was measured through a microplate reader (BioTek, USA). The 10^{-3} diluted active bacterial culture was prepared from overnight growth of bacteria in Mueller-Hinton agar, and 5 mL of this diluted bacterial culture was added to a 96-well plate containing different concentrations of tested compounds. This plate was incubated at 37°C, and OD_{600} was measured at different time points ranging from 0 to 6 hours. The result was prepared by plotting graph between time vs. OD_{600}. The resultant curve was used to analyze effects of tested compounds on the growth of bacteria. All OD_{600} readings were calculated in terms of OD_{600}(t)–OD_{600} (0 h), where "t" represent for time points (h). Gentamicin (Gibco) was used as positive control for bacterial growth inhibition; in contrast, the bacterial growth in the absence of synthesized compounds was used as negative control.

Table 1: Zone of inhibition of synthesized compounds (250 μg/mL) and standard drug gentamicin (250 μg/mL) against Gram-positive and Gram-negative bacteria after 24 hrs.

Compound	*E. coli*	*S. aureus*
P1	0	1.48
P6	0	1.21
P11	1.90	2.63
Gentamicin	2.50	2.90

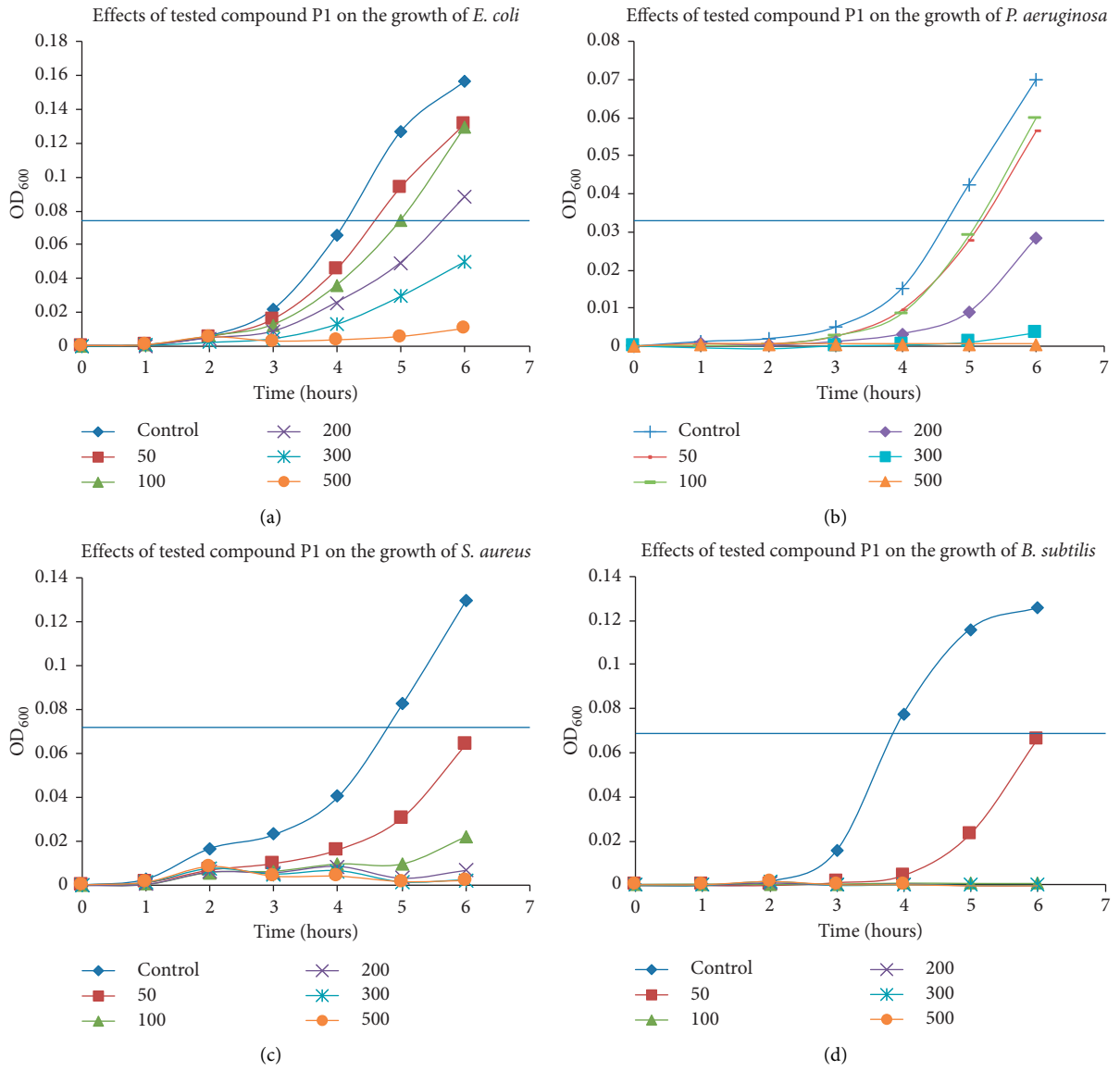


Figure 1: Effects of different concentrations of compound **P1** against different time points. All compound concentrations are in μg/mL.

3. Results and Discussion

Enaminone derivatives **(1–11)** were used as a starting material for the synthesis of various pyrazole derivatives (**P1–P16**). Various enaminones were obtained by the reaction of substituted acetophenones with DMF-DMA in a solvent-free environment [35, 36]. Pyrazole derivatives **(P1–P11)** were obtained, when substituted enaminones were reacted with hydrazine hydrate 99% in presence of absolute alcohol. *N*-substituted pyrazole derivatives (**P12–P16**) were obtained by reacting pyrazole derivatives with phenacyl bromide in dimethylformamide (DMF) in presence of triethylamine. The synthesis of these compounds was performed through an efficient synthetic route (Scheme1). The structures of all the synthesized compounds were confirmed by proton and carbon NMR spectroscopy. ^{1}H

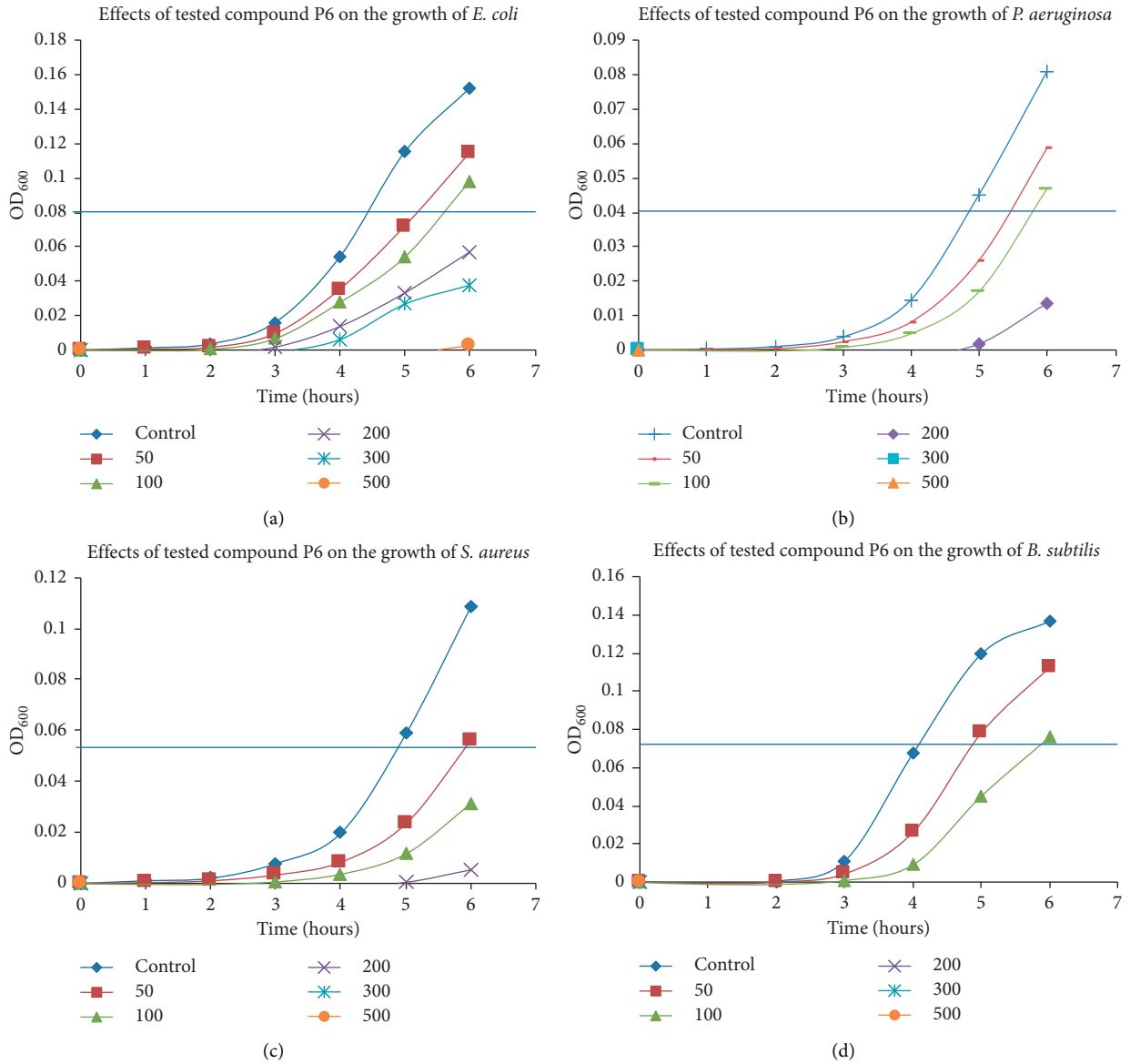


Figure 2: Effects of different concentrations of compound **P6** against different time points. All compound concentrations are in μg/mL.

NMR spectrum of **(1)** displayed two doublets at δ H 5.80–5.82 and 7.63–7.65 ppm (d, $J = 14$ Hz) due to the ethylenic protons. The value of coupling constant ($J = 14$ Hz) for the ethylenic protons indicates that the enaminones existed in the *E*-configuration which was also confirmed by single-crystal X-ray crystallography [37]. All the pyrazoles presented the D_2O exchangeable broad singlet at δ H 11.8–13.00 ppm corresponding to the NH proton. The aromatic protons appeared at δ H 6.0–8.32 ppm. The two pyrazole protons appeared as doublets with $J = 7$ Hz at δ H 6.62–7.47 ppm and δ H 6.66–7.79 ppm. In case of *N*-substituted pyrazoles, (OCH_3) protons were observed at δ H 3.69–3.86 ppm. The two pyrazole protons appeared as doublets with $J = 7$ Hz at δ H 6.19–6.79 ppm and δ H 6.27–7.40 ppm. The aromatic protons appeared at δ H 7.32–8.00 ppm. The CH_2 protons were observed at δ H 5.80–5.90. The presence of all carbon atoms for all the compounds was confirmed by ^{13}C NMR spectra. Molecular weights of compounds were confirmed by GC/MS spectroscopy. The detailed spectral results of ^{1}H NMR spectra, ^{13}C NMR spectra, and mass spectra are given in the experimental part. The spectral and analytical data confirmed the composition of the synthesized compounds **(P1–P16)**.

3.1. Disc Diffusion Assay. The antimicrobial activity of all the synthesized compounds was determined by disc diffusion assay, and their data are presented in Table 1. Only three compounds (**P1**, **P6**, and **P11**) showed good antimicrobial activity.

3.2. Bacterial Growth Inhibition at Different Time Points. The compounds (**P1**, **P6**, and **P11**), selected in initial screening steps, were further evaluated for their

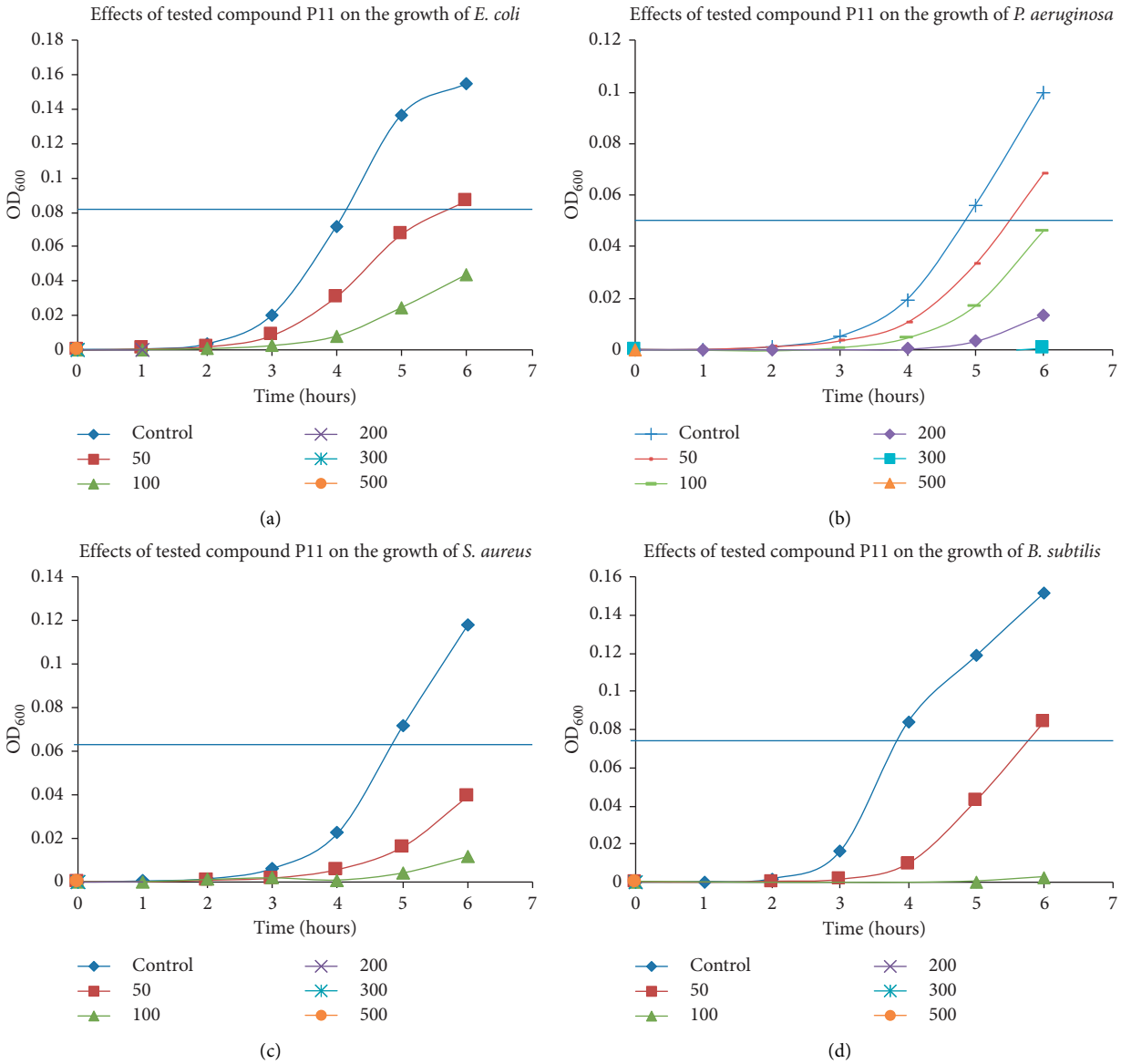


Figure 3: Effects of different concentrations of compound **P11** against different time points. All compound concentrations are in μg/mL.

antimicrobial activity at different time points. The data of these compounds against tested bacteria are presented in Figures 1–4. The concentration of the compound inhibiting 50% growth in comparison with control after 6 hours was also calculated, and data are presented in Table 2. All the synthesized compounds were screened, and only three compounds (**P1**, **P6**, and **P11**) showed antimicrobial activity during the screening step through disc diffusion assay. Among these three active compounds, only one compound **(P11)** showed antimicrobial activity against both Gram-positive and Gram-negative bacteria after 24 hours. All the three compounds were showing good antimicrobial activity against Gram-positive bacteria. Compound **P11** presented maximum activity among all active compounds against both Gram-positive and Gram-negative bacteria. The compounds were tested for antimicrobial activity at different time points against Gram-positive and Gram-negative bacteria; it was found that all of these compounds were showing good activity against Gram-positive and Gram-negative bacteria for 6 hours, but the activity was higher against Gram-positive bacteria. In contrast, at 24 hours, the activity was found only against Gram-positive bacteria except one compound (**P11**), showing activity against both bacteria. The reason for this discrepancy may be due to many reasons. Limited efficacy of compounds may be present for certain time only and can give result at 6 hours but not at 24 hours. In addition, degradation of compounds in DMSO solvent was checked for 24 hours. It was found that all the compounds were stable. Moreover, mechanism for resistance in Gram-negative bacteria is different than Gram-positive bacteria that may also cause these results. However, the water solubility of these compounds was very poor, and therefore, we used DMSO as cosolvent and performed this study as per the given protocol [38]. Gentamicin showed very good

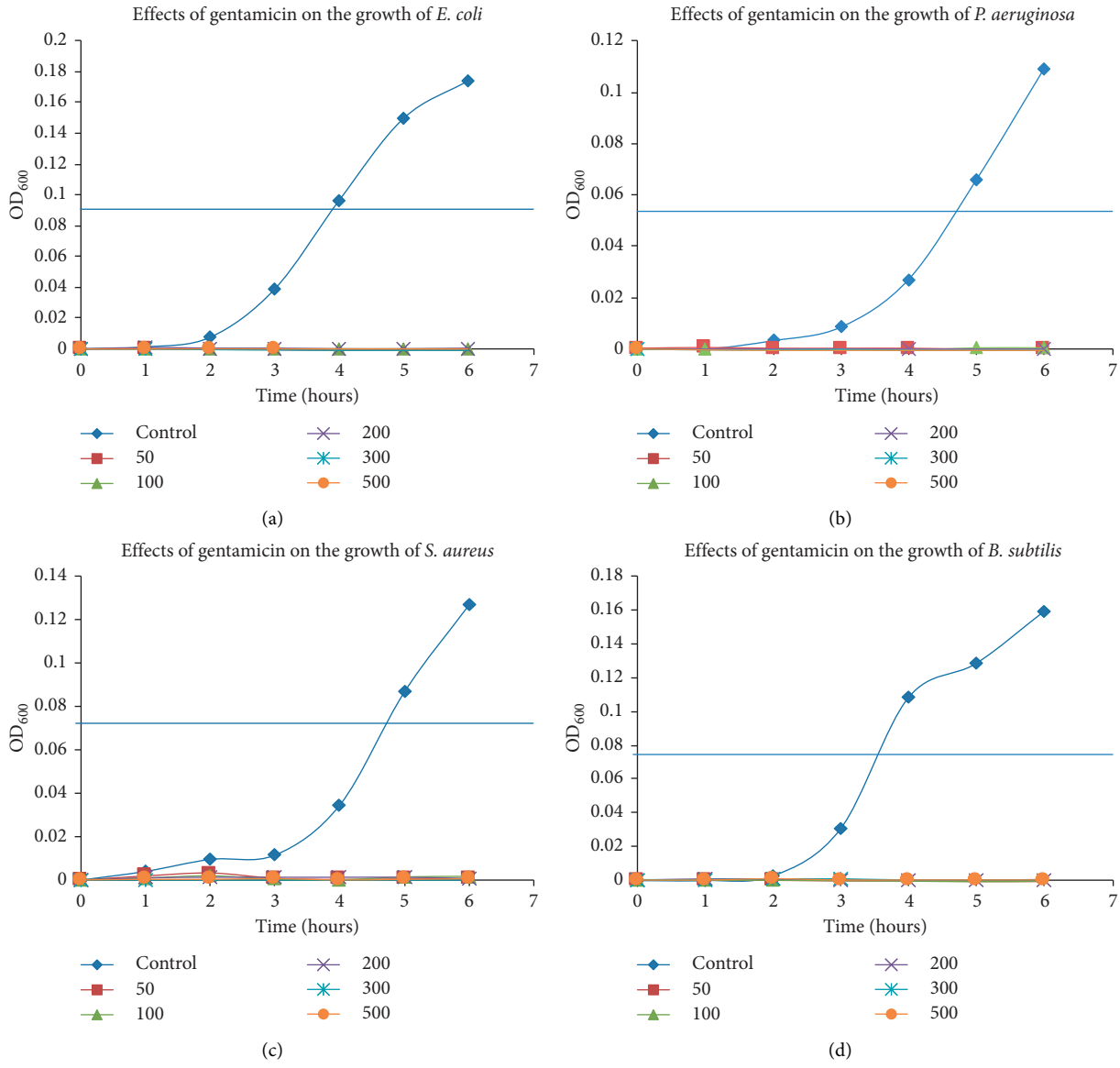


Figure 4: Effects of different concentrations of gentamicin against different time points. All compound concentrations are in μg/mL.

Table 2: Concentration of compounds required to reduce bacterial growth by 50% in comparison with control gentamicin.

Compound*	*E. coli*	*P. aeruginosa*	*S. aureus*	*B. subtilis*
P1	<100	<100	<50	<50
P6	<100	<100	<50	<100
P11	<50	<50	<50	<50
Gentamicin	<50	<50	<50	<50

*All concentrations are in μg/mL.

antimicrobial activity against tested compounds, and it was not allowing any growth of microbes even at least concentration.

4. Conclusion

In conclusion, a series of pyrazoles and *N*-substituted pyrazoles were synthesized in good yield from enaminones by an efficient synthetic route. All the synthetic compounds were characterized by spectral data and elemental analysis. Antimicrobial activity of the compounds was determined by the disc diffusion assay. In the initial screening, only compounds (**P1**, **P6**, and **P11**) showed significant antimicrobial activity. These three compounds were further evaluated for their antimicrobial activity at different time points. Compound number **P11** presented maximum activity

among all active compounds against both Gram-positive and Gram-negative bacteria. Active compounds were screened for antimicrobial activity at different time points against Gram-positive and Gram-negative bacteria, and it was found that all of these compounds were showing good activity against Gram-positive and Gram-negative bacteria for 6 hours, but the activity was higher against Gram-positive bacteria. In contrast, at 24 hours, the activity was found only against Gram-positive bacteria except one compound (**P11**) showing activity against both types of bacteria.

Data Availability

Samples of the compounds (**P1–P16**) in pure form are available from the authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this research group (no. RG 1435–006).

Supplementary Materials

The spectral data of compounds (**P1–P16**) used to support the findings of this study are included within the supplementary information file. (*Supplementary Materials*)

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Hindawi
Journal of Chemistry
Volume 2019, Article ID 5264943, 7 pages
https://doi.org/10.1155/2019/5264943

Research Article

Antibacterial and Antifungal Activities of the Medicinal Plant *Veronica biloba*

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Received 19 July 2019; Revised 27 September 2019; Accepted 3 October 2019; Published 7 November 2019

Guest Editor: Maria Grazia Bonomo

Plants are naturally God gifted for the synthesis of medicinal compound and provide a great help in a new discovery in the area of chemical diversity because of the unknown availability either as a standardized extract or as a pure compound. The medicinal plant *Veronica biloba* extracts obtained through Soxhlet and maceration methods were subjected to preliminary antimicrobial screening against pathogenic microorganisms. Fractionation was performed using liquid-liquid extracts such as ethyl acetate, water, dichloromethane, and hexane extract of plant, and the fractions were tested for antifungal activity and antibacterial activity using well-diffusion method at sample concentration of 10–30 μL. The result indicated that all extracts exhibited antimicrobial activity against all test pathogens. The ethyl acetate extract showed greater activity than other corresponding extracts. Among various extracts, only the ethyl acetate extract show potential against bacterial (gram negative and gram positive) and fungus test strain greater than standard Nystatin test control. Thus, the extract of *Veronica biloba* could be used to treat microbial (fungus and bacterial strain) infection.

1. Introduction

Plants are naturally God gifted for the synthesis of medicinal compound. Their isolation from medicinal plants and the characterization of the active compound they contain provide a great help in the preparation of new drugs to treat many diseases and have a high therapeutic value [1]. The plant extract, which is also called natural product, provided a great help in a new discovery in the area of chemical diversity because of the unknown availability either as standardized extract or as pure compound [2]. According to pharmaceutical studies, approximately 10 to 20% of plants are used in a positive way in health care to treat harmful diseases such as cancer [3]. The classical example is reported on the *bark of yew tree*, which mainly contains *taxol* and is used in ovarian cancer and breast cancer [2]. Isolation or extraction of medicinal plants mainly produced one or several substances that are responsible for any activity and are closely related to each other [4]. Plants are the main source of drugs in modern medicinal system, folk medicinal system, traditional medicinal system, food supplement, and for synthetic drug [5]. A recent research study shows that medicinal plants show mainly antioxidant activity. The phenolic compounds such as flavonoids, lignins, and vitamins A, C, and E, and tannins all are antioxidants and are present mainly in plants [6]. The people interested in conventional medicine called drugs from plant because of some reason they are efficient, effective therapy, and have no side effects, while ecological awareness show that natural products are harmless. Whereas wrong use or abusive use of synthetic drugs cause many problems and have many side effects [7]. The *Veronica* (Plantaginaceae) genus consisting of about 450 known species exists in both hemisphere and temperate region [8]. They have 79 popular species of which 26 are endemic [9]. Due to the great importance of *Veronica* species throughout the world, they are selected for our investigation. They

possess phytochemical and chemotaxonomic values, as well as biological activities. They are traditionally used in Turkish and Chinese medicine for curing respiratory diseases, influenza, in wound healing, and as diuretics, tonics, restoratives, and expectorants [10]. Some studies reported on the potential anticancer activity of a veronica species [11]. In the present study, antibacterial and antifungal activity potential of the medicinal plant *Veronica biloba* is reported.

2. History

According to the report of the World Health Organization, about 80% people used traditional medicine for primary health care treatment. In Asia, plants as medicine show long history with human involvement in the environment. Herbal medicines contain different types of novel and unique substances to treat infectious and chronic diseases [12]. The tradition of using plant products to treat a number of diseases starts with the beginning of human civilization. The earliest document shedding light on the use of medicinal plants is Hindu Culture, written between 4500 and 1600 BC [13]. The use of traditional medicine or natural products is oldest as with the human civilization medicine from plants has therapeutic properties and history write that from long time the main source of drug was plants, minerals, and animal products [14]. The synthetic chemical drugs show bad health-related side effects, microbial resistance man tend to ethnopharmacognosy obtained thousands of phytochemical from plant with less or, no side effect, safe and mainly effective with many biological activities such as analgesic, antimicrobial, wound-healing, antioxidant, anticancer, antidiarrheal activities. Some people claim that natural products are beneficial for health. So, clinical trials tend to verify that claim of bioactive part, their formulation, safeguard, and side effects before the drug is provided to the patients. According to the report of the World Health Organization, 12 mega biodiversity countries nearly have 20,000 medicinal plants [15]. The isolation of penicillin from microorganisms is clearly important with the development of anti-infective therapy. Approximately 25% of drugs used throughout the world are obtained from plants. The World Health Organization reported 252 essential active compounds and about 121 are in current use. More than 11% of synthetic drugs are obtained from natural plant source. Some valuable drugs isolated from plants are quinidine and quinine isolated from *Cinchona*, atropine from *Atropa belladonna*, and codeine and morphine from *Papaver somniferum*. The clinical trial obtained drug from natural source at least 60% of anti-infective, antitumor, drug available in market [16]. Naturally isolated active part of the plant is important and is used to cure physiological, pharmacological and biochemical study such as phorbol ester, cannabinoids, forkolin, muccarine, colchicines, and yohimbine [17]. Many of these cannot be yet economically synthesized and are mainly isolated from cultivated or wild plants [18].

3. Field of Knowledge

The research on a plant origin for a therapeutic medicinal material discovery or development is expensive and is a hard task [19]. Developing a new drug requires about 100–360 million US$ and at least ten years' work on it. Up to 1992, 10,000 compounds were tested, with only 1 of 4 being approved active for drugs. The National Cancer Institute found three biologically active compounds to treat human immunodeficiency virus in 50,000 tested plant extracts and three active compounds for antitumor activity in 33,000 tested plant extracts [17]. They involved the basic knowledge of science, pharmacology, botany, toxicology, and chemistry. These particular disciplines should not be considered as secondary for one another. To account for a medicine from plants, other fields of knowledge, which include organic chemistry, anthropology, biotechnology, agronomy, and a fundamental pharmaceutical, have important roles in the designing any new drug from plants [20]. When a medicinal plant is found, the methods applying for therapeutic treatment (as home-made) are herbal teas or preparation of pharmaceutical powder pills, tinctures, capsules, fluid extract, standard enrich, or crude extract Finally, a plant that contains active natural compound that is itself responsible for drug can be isolated and purified by extraction process, such as ergotamine (as a precursor, for example, diogenin), digoxin, and quinine [21].

4. Selection of Plant

The approach for a suitable plant selection is hard and very important. In pharmacology, discussions depend on the requirement to isolate a natural active compound or make a herbal medicine, which involves several roots of traditional usage, toxicity, chemical content, and randomized several requirements [22, 23]. The common cultural medicine is called ethnopharmacology or ethnobotany. It shows how usage of natural folk medicine is highly important and how ethnic groups utilized it, and their procedure of preparation provides information on pharmacological activity and extraction process. A different culture has its own health care system and health illness [24]. Selection of active compounds against insects and bacteria depend on environment of the plant [18]. However, a specific potent therapeutic drug in biological research has been found in a toxic plant [17]. For a pharmacological activity, certain plant families and genera are selected based on chemotaxonomic or phylogenetic information [25, 26]. For selection of plants, researchers decide a randomized search for active pharmacological species; for example, discovery of an antitumor drug follows this strategy and for choosing a selective plant, study scientific literature or, if identified, find a new way [23, 27, 28]. A cultivated plant usually selected can provide genetically guarantying homogeneous material with extinction threatened species [29]. In recent few years in the research area, a number of publications studied biologically active plant-derived compounds that are anti-inflammatic, antibiotic, antitumor, contraceptive, and kidney medication and for psychiatric treatment. However, a priority is shown towards a viral, cardiovascular, and tumor diseases [18]. A taxol naturally active compound diterpenes show anti-tumor activity obtained from *Taxus*. About 2500 mg taxol isolation required more than 12,000 trees to be cut down and 27,000

tons of *T. bacata* and *T. brevifolia* bark was obtained. Due to high requirement of *Taxus*, it is necessary to find alternative sources in other plants or should be synthesized in a considerable amount [30].

5. Experimental Methodology

5.1. Identification of Plant. The medicinal plant species *Veronica biloba*, *biolobed*, *two-lobed speedwell*, of genus *Veronica* was identified and confirmed with the help of botanical expert Prof. Muhammad Israr of Botany Department, Govt. Post Graduate College Mardan, and also through various literature survey comparisons.

5.2. Collection of the Plant. The medicinal plant used in the project/experiment was the whole plant selected. Fresh whole plants in their flowering stage were collected from Sang-e-mar mar, Near Par Hoti District Mardan, and also from Surkh Dheri, Rustam, Mardan. The plant collection was done during the month of February–March. Healthy plants are collected/selected from a fertile land.

5.3. Drying and Grinding of the Plant. After cleaning of the collected plants, they were cut into small pieces by using knives and scissor. They were stored for drying under shade, and to avoid/protect from surrounding contamination and dust present in the environment. The drying was done in a room for about two weeks (2-week), without any exposure to light. After completely drying the plants, obtain uniform-sized powder and ensure to enhance the surface area for better extraction process.

5.4. Extraction

5.4.1. Soxhlet Extraction. 30 g of finely grinded uniform-size powder of the plant sample is kept in a thimble, a porous bag made from cellulose strong filter (paper prepared manually), and then thimble is inserted into thimble chamber of Soxhlet. Extraction was carried out in 300 ml ethanol kept in the bottom flask of Soxhlet. The upper part was fitted with a condenser by introducing water inflow and outflow. The solvent was heated at moderate temperature around 40°C over mantox heater, and the solvent vaporizes and goes to sample thimble chamber, condenses, and falls back when the liquid extract reaches the siphon arm and emptied into down a bottom flask again and again. The process was continued for 48 hrs until solvent drop cannot leave residue when evaporated. Furthermore, fractionation is carried out on water, dichloromethane, *n*-hexane, and ethyl acetate. The four fractions were then concentrated to get dried extract for further analysis of biological activities.

5.4.2. Maceration. In this method, 20 g grinded powdered plant sample is kept in a closed jar (made from Pyrex glass), and 200 ml absolute ethanol is added. The jar is allowed for up to 3 weeks at room temperature, and proper shaking is performed on a daily basis to release plant-soluble phytochemicals. The extract obtained via soaking is filtered through a normal filter paper (Whatman filter paper) to get concentrated ethanolic extract with evaporation of the solvent. Both the extracts were analyzed by thin-layer chromatography (TLC) to confirm their similarity pattern if any. Furthermore, same fractionation was done as for above Soxhlet fraction obtained in water, dichloromethane, *n*-hexane, and ethyl acetate. A fraction was then concentrated to obtain the desired dry extract for further analysis of biological activities.

5.5. Antibacterial Activity

5.5.1. Preparation of Fraction Extracts Solution. The dried four fractions obtained were dissolved to make a solution of concentration in dimethylsulfoxide (DMSO) of 20 mg/ml. For proper mixing, the solution was kept in centrifuge for 25 minutes at 13000 rpm. The standard antibiotics gentamicin (10 mg/discs), ampicillin (10 mg/discs), and ofloxacin (1 mg/ml) were used for comparing the activity with each active fraction.

5.5.2. Microbes Used in the Test. The microorganisms used in the study were obtained from the Microbiology Department of Abdul Wali Khan University Mardan. Gram-negative bacteria used was *Escherichia coli*, and the gram-positive bacteria was *Staphylococcus aureus.*

5.5.3. Culture: Media Preparation. Microorganism suspension was prepared as McFarland standard. For the antibacterial sensitivity test analysis, the MHA (Müller–Hinton Agar) was used for bacterial media preparation. The culture media were prepared in 250 ml distilled water by dissolving 9.5 g of MHA. The obtained amber color solution is mixed thoroughly and boiled with frequent agitation to dissolve agar powder completely and a clear to slightly opalescent gel is obtained. Then, autoclave the media for sterilization at 15 lbs pressure, at 121°C temperature, for 15 minutes. Allow sterilized media to cool at room temperature in laminar flow hood, and then pour 25 ml of the media into each Petri plate and leave for few minutes to allow the media to solidify. After solidification, spread the culture microbes on media by using cotton swab and cover the whole media with turn 90° degree rotation without leaving any gap. Make 6 bores in each Petri plate separated from each other by 2.5 cm distance. 30 μl of each fraction is poured in the first 4 bores, antibiotics in the second last bore, and solvent in the last bore. For positive control, two plates are placed for both microbes with no antibiotics and extract fraction added, whereas for sterility of media negative control, one Petri plate is placed without any microbes. Store all Petri plates for incubation in biochemical oxygen demand (BOD) incubator at 37°C for 24 hrs. Table 1 shows the result of antibacterial activity. The inhibited zone for each fraction and active drug measured are calculated as mean ± standard deviation (SD).

Table 1: Result of antibacterial activity of *Veronica biloba*.

Extract	Concentration (μl)	*Staphylococcus aureus*	*Escherichia* coli
Dichloromethane	30 μl	4.3 ± 0.2 mm	6.3 ± 0.5 mm
Water	30 μl	5.1 ± 0.2 mm	4.5 ± 0.5 mm
n-Hexane	30 μl	4.5 ± 0.5 mm	6.8 ± 0.2 mm
Ethyl acetate	30 μl	10.5 ± 1 mm	7.3 ± 0.2 mm
Ampicillin (+control)	10 mg	15.5 ± 0.3 mm	—
Gentamicin (+control)	10 mg	—	11.9 ± 0.4 mm
Ofloxacin (+control)	1 mg	20 ± 0.5 mm	11.5 ± 0.15 mm
DMSO (–control)	30 μl	1 ± 0 mm	1 ± 0 mm

The data are expressed as mean ± SD (standard deviation) in mm of zone of inhibition shown by each fraction. Compared using ANOVA, with significance level set at alpha of 0.05.

5.6. Antifungal Activity. The antifungal activity was performed on nutrient agar with the fungus *Aspergillus fumigatus* obtained from the Biochemistry department of Malakand University, Chakdara. The culture was done as McFarland standard sterilized media prepared at 121°C for 14 minutes in autoclave. The well-diffusion method was applied as per requirement after streaking cultured for 12–14 hrs. 10 μl of extract fraction was used for activity analysis, 10 μl of Nystatin was used as the standard sample, and 10 μl of oxytetracycline was used as the test control. Store all Petri plates for an incubation period of 72 hrs at temperature 20°C. Table 2 shows the result of antifungal activity. Inhibited zone was calculated for each fraction as mean ± SD (standard deviation).

Table 2: Result of antifungal activity of *Veronica biloba*.

Extract	Concentration (μl)	*Aspergillus fumigatus*
Dichloromethane	10 μl	8.3 ± 0.5 mm
n-Hexane	10 μl	12.1 ± 0.2 mm
Water	10 μl	10.6 ± 0.5 mm
Ethyl acetate	10 μl	12.3 ± 0.5 mm
Oxytetracycline (test control)	10 μl	26 ± 0 mm
Nystatin (standard)	10 μl	6.7 ± 0.5 mm
DMSO (negative control)	10 μl	1 ± 0 mm

The data are expressed as mean ± SD (standard deviation) in mm of zone of inhibition shown by each fraction. Compared using ANOVA, with significance level set at alpha of 0.05.

6. Results and Discussion

The current attempt was made due to resistance development in bacteria and fungi (microbes) to available drugs. Agar well-diffusion method was used for antimicrobial screening using a standard protocol of clinical laboratory prescribed by national committee [31]. Extracted plant antibiotics are safe, effective, and have no or little side effects [32]. The active phytochemicals are responsible for biological activity such as antimicrobial against pathogens provide help in discovery of new antibiotic drugs [33–35]. The present study investigated the antimicrobial and antifungal potential of a medicinal plant *Veronica biloba* for the first time.

The antibacterial report of the medicinal plant *Veronica biloba* fractionation extracts is summarized in Table 1. The *Veronica biloba* extracts show dose-dependent potential activity and affect the tested pathogens. The crude ethyl acetate extract is more potent (shown in Figure 1 and Table 1) against both bacterial strains *Staphylococcus aureus* and *Escherichia coli*. The *Veronica biloba* ethyl acetate extracted fraction showed 10.5 ± 1 mm maximum zone of inhibition at 30 μL concentration with *S. aureus* and 7.3 ± 0.2 mm at 30 μL with *E. coli* (shown in Table 1). However, the aqueous extracted fraction showed 5.1 ± 0.2 mm and 4.5 ± 0.5 mm inhibited zone with *S. aureus* and *E. coli*, respectively, at 30 μL. The hexane extracted fraction showed 6.3 ± 0.5 mm and 4.3 ± 0.2 mm zone of inhibition with *S. aureus* and *E. coli*, respectively, which is comparatively less than that using the ethyl acetate fraction (shown in Figure 1). Dichloromethane fraction showed less activity with *S. aureus* 4.3 ± 0.2 mm and 6.3 ± 0.5 mm with

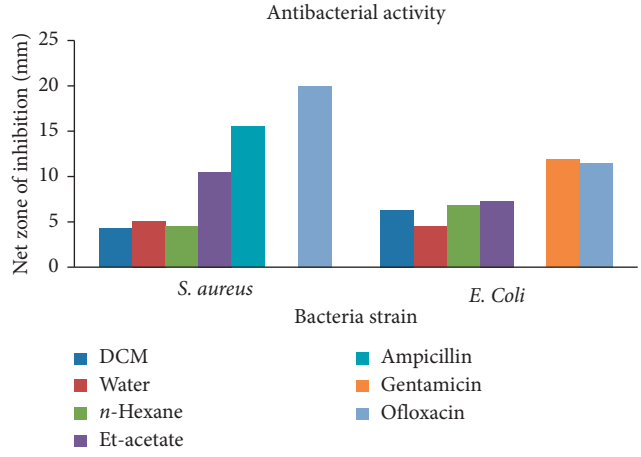

Figure 1: Inhibited zone shown by each fraction of *Veronica biloba* against bacterial strain.

E. coli zone of inhibition as compared with the standard antibiotics ofloxacin 11.5 ± 0.15 mm, ampicillin 11.9 ± 0.4 mm less inhibition causes, while gentamicin 15.5 ± 0.3 mm, showed more zone of inhibition (shown in Figure 1 and Table 1).

The antifungal assay result of *Veronica biloba* is summarized in Table 2. The crude extracted fractions of *Veronica biloba* strongly inhibited the fungus *Aspergillus fumigatus* in the same concentration. The maximum inhibition was shown by ethyl acetate extract 12.3 ± 0.5 mm zone of inhibition at 10 μL concentration (shown in Figure 2). However, hexane extract cause 12.1 ± 0.2 mm, water

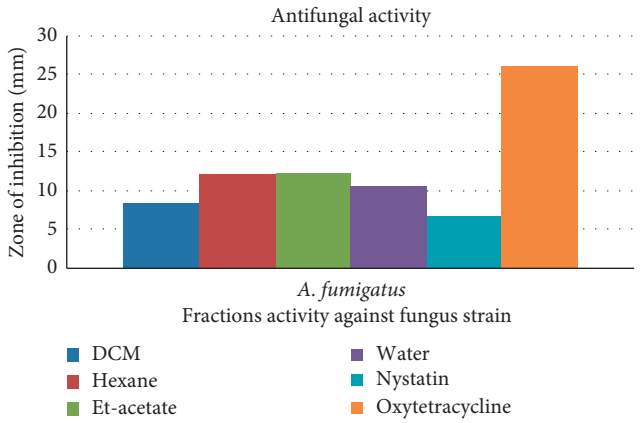

FIGURE 2: Inhibited zone shown by each fraction of *Veronica biloba* against fungus strain.

10.6 ± 0.5 mm, and dichloromethane 8.3 ± 0.5 mm inhibition at 10 μL concentration. The standard nystatin (test control) show at 10 μL concentration 6.7 ± 0.5 mm less zone of inhibition as compared to a medicinal plant *Veronica biloba* extracted fractions (shown in Figure 2 and Table 2).

Muanza et al. [36] reported that the antifungal and antibacterial activities of fractionation crude extract of *zairen* found potent activity in ethyl acetate and aqueous fraction only. Duraipandiyan [37] reported antibacterial and antifungal activities on *Toddalia asiatica* in hexane, ethyl acetate, methanol, and water extracted fraction. The most potential activity was observed in ethyl acetate. Sanches et al. [38] found that aqueous extract of *guava* was effective against *S. aureus*. This resembles with our work. The more potential activity of plant extracts with gram-negative and gram-positive bacteria strains depends on the nature of both microbes cell wall. The efflux system, mainly in gram-negative strains, is responsible for activity [39–41].

The sensitivity difference between two strain gram-negative and gram-positive bacteria is because of membrane composition and structural dissimilarities [42]. Several studies reported high potential activity on gram-positive strain compared to gram-negative bacteria strain from other plants species [43–48]. The *S. aureus* potential sensitivity is due to the cell membrane and cell wall structure [49]. Negative strain bacteria possess external impermeable membrane, which can basically reduce the antibiotics in cell and show resistance to it [50].

The medicinally bioactive compound present in plants mainly show antimicrobial activity because it contains terpenoids, steroids, saponins, tannins, and flavonoids [51]. The *Veronica* genus contain tannins, phenol, saponins, carboxylic acid, steroids, and flavonoid contents due to which it shows strong antimicrobial activity [52]. The highest noticeable antimicrobial biological activity reported on Veronica species is in [52]. Mocan et al. reported on the presence of antimicrobial active compounds in three *Veronica* species, i.e., *Veronica orchidea*, *teucrium*, and *officinalis* [53]. Javad Sharifi-Rad et al. [54] reported antifungal and antibacterial agents in *Veronica persica poir* species. Stojkvoic et al. [55] reported potent antibacterial agent in the extract of *Veronica montana* species. Exarchou et al. [56] confirmed the antibacterial activity of *Veronica* extracts through the presence of a hispidulin compound. Dunkic et al. [57] found antimicrobial compounds in *Veronica spicata* species of this genus.

Phytochemicals, for example, flavonoids, polyphenols, saponins, steroids, tannins, terpenoids, and alkaloids, are natural active compounds present in plants, which are significantly used to treat diseases and also used as nutrient and dietary supplement [58–62]. Flavonoids are mainly polyphenol, and their presence can increase the antibiotics potential against microbes [63, 64]. The flavonoids form complexes with cell wall of bacteria protein and extracellular components and are very important and effective antimicrobial compounds [65]. Terpenoids are involved in weakening microorganism cell wall and membranous tissue dissolution [66]. The interaction of saponins with microbes causes enzyme protein leakage from the cell [67]. Steroid in antimicrobials is responsible for liposome leakage from lipid bilayer membrane [68]. This is the first antimicrobial report on a medicinal plant *Veronica biloba* against *E. coli*, *S. aureus*, and *Aspergillus fumigatus* pathogens that possesses an extensive useful activity.

7. Conclusion

We have concluded that the extract of *Veronica Biloba* could be used to treat microbial (fungus and bacterial strain) infection. It can be used either in combination with traditional medication or used alone as an antibiotic.

The fraction extracts of the medicinal plant *Veronica biloba* (i.e., water, dichloromethane, *n*-hexane, and ethyl acetate) show both antibacterial and antifungal biological activities in a wide range. The ethyl acetate and *n*-hexane extracts fraction show more potential in both activities. The change in concentration, purification, and isolation of these extracts can provide us a more sustainable result. Thus, *Veronica biloba* is a useful medicinal plant and its further assessment is important, which can provide help in the discovery of new antibiotic drug development in the market.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The first author thanks his supervisor Prof. Himayat Ullah of Organic Chemistry Department, Govt. Post Graduate College Mardan, for his great support and work.

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