β-Pinene Derived Products With Enhanced In Vitro Antimicrobial Activity

Xuezhen Feng1, Zhuanquan Xiao2, Yuling Yang1, Shangxing Chen1, Shengliang Liao1, Hai Luo1, Lu He1, Zongde Wang1, and Guorong Fan1

Abstract

The development of new antimicrobials has always been a research hotspot. In this study, β-pinene-based derivatives were synthesized, and their antimicrobial activity was evaluated. The purpose was to develop some novel, promising new fungicides. Three β-pinene derivatives containing bis-hydronopyl were prepared, and their antifungal and antibacterial activities were evaluated against 6 plant pathogenic fungi and 4 bacterial species; a preliminary structure-activity relationship is discussed. The results indicated that the derivatives containing the blend of alkyl group and bis-hydronopyl had potent inhibitory activities against plant fungal pathogens and bacteria. Among these molecules, bis-hydronopyl dimethyl ammonium bromide showed excellent effects on Colletotrichum acutatum with a half-maximal effective concentration of 0.538 µg/mL, which was lower than that of carbendazim. Scanning electron microscope showed that after administration of bis-hydronopyl dimethyl ammonium bromide (compound 3a), the C. acutatum mycelia were sunken and deformed in comparison with the control group. Furthermore, the inhibitory activities of the methyl derivatives against the plant pathogens were better than those of the ethyl derivatives. These results provide new insights into the inhibition of fungi and bacteria by β-pinene derivatives, which may lead them to be used as precursor molecules for novel pesticides and antimicrobials in further research.

Keywords
β-pinene, hydronopyl, antifungal activity, phytopathogenic fungi, bacteria

Received: October 16th, 2020; Accepted: January 8th, 2021.

Plant pathogenic fungi have become a serious threat to global crop production and food security, which has resulted in huge losses to human beings.1-3 In addition, pathogenic bacteria are the cause of many human diseases.4 In the past few decades, the large-scale use of chemical fungicides and antibiotics has been the principal tool to eliminate these disasters.5 However, the widespread application of chemical fungicides and antibiotics often leads to drug resistance of fungi and bacteria. Drug-resistant bacterial and fungal strains are causing a serious threat to global human health, and so it is very urgent to look for new alternatives.6,7 Therefore, the development of safe and eco-friendly alternatives can not only control the increase of microorganisms but also reduce the environmental risks.8

β-Pinene is a natural compound with antibacterial activity, which can be used to participate in many chemical reactions.9-13 A large number of β-pinene derivatives can be synthesized by chemical modification, and some of these derivatives have been proved to have increased antibacterial activities.14-19 For example, Gavrilov et al20 synthesized 3 series of β-pinene-based derivatives, and the reaction mixes of the sulfoxides had high activity against Penicillium tardum. Recently, cationic antimicrobial agents have been shown to be very effective bacteriostatic agents and are used in many applications, such as pesticides, hospital protective clothing, medical implants, wound dressings, and food packaging materials.21,22 Among these cationic antimicrobial agents, quaternary ammonium salts are probably the most widely developed and used.23-25 Jin et al reported that hydronopyl quaternary ammonium salts from β-pinene have good antifungal activity against various plant pathogenic fungi. N-hydronopyl-γ-dimethylylpyridine ammonium bromide was shown to exert good antifungal activity against Colletotrichum gloeosporioides and Pestalotiopsis actinidia.26

1College of Forestry, Jiangxi Agriculture University, East China Woody Fragrance and Flavor Engineering Research Center of National Forestry and Grassland Administration, Nanchang, China
2College of Chemistry, Jiangxi Normal University, Nanchang, China

Corresponding Authors:
Zongde Wang and Guorong Fan, College of Forestry, Jiangxi Agriculture University, East China Woody Fragrance and Flavor Engineering Research Center of National Forestry and Grassland Administration, Nanchang 330045, China.
Emails: zongdewang@163.com; fgr008@126.com
Herein, a series of quaternary ammonium salts were synthesized from β-pinene in order to obtain derivatives with potent antimicrobial activity. This study can be used as a guide for the development of new and efficient botanical antimicrobial agents.

Materials and Methods

Synthetic Procedures and Structural Characterization of the Title Compounds

Materials and structural characterization techniques. Pure chemicals were purchased from Jiangxi Jingke Scientific Instrument Co., Ltd., China. Fourier transform infrared (FT-IR) spectroscopy was performed using a Nicolet IS10 FT-IR spectrometer (America). Melting points (m.p.) were determined with a WRS-2 melting point apparatus (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China) and are uncorrected. Proton nuclear magnetic resonance (NMR) was performed on a Bruker 400 spectrometer with tetramethylsilane and deuterated chloroform (CDCl3) used as internal control and solvent, respectively. Mass spectrometry (MS) was carried out on a Bruker mass SL spectrometer (Bruker, Germany). The synthesis of quaternary ammonium salts.

(1R,2R,5R)-Hydronopyl bromide (1), (1R,2R,5R)-hydronopyl chloride (2), and (1R,2R,5R)-hydronopyl ammonium halide (2a-c) were synthesized from β-pinene as previously reported. Then, bis-hydronopyl quaternary ammonium salts (3a-c) were obtained by the reaction between (1R,2R,5R)-hydronopyl bromide (1) and (1R,2R,5R)-hydronopyl ammonium halide (2a-c). The synthetic routes and structures of compounds 3a-c are shown in Scheme 1.

(1R,2R,5R)-Hydronopyl dimethyl amine (2a, 0.05 mol) and compound 1 (0.05 mol) were slowly added to 30 mL acetone at 60 °C. After 48 hours of reaction, the acetone was vacuum removed. Following 3 light petroleum washes and recrystallization from ethyl acetate, a white solid bis-hydronopyl dimethyl ammonium bromide (3a) was obtained; m.p., 213.7-215.2 °C; Yield, 82%. FT-IR ν (cm⁻¹): 2997.43-2867.23 (C-H, 1227.71 (C-N); 1H NMR (CDCl3, 400 MHz) δH: 3.41 (4H, t, J = 8.8 Hz, 21−CH2), 3.41 (10 H, s, 2α−CH2), 2.83 (2H, m, 2α−CH), 1.99-1.70 [16H, m, 2 (−CH−10CH2), 1CH, 5−CH, 3−CH, 4−CH2)], 1.40 (2H, m, 2β−CH), 1.15 (6H, s, 2β−CH2), 0.97 (6 H, s, 2−CH3), 0.86 (2 H, d, J = 10 Hz, 2−CH); 13C NMR (CDCl3, 100 Hz) δC: 62.29 (C−11), 51.61 (C−α), 46.41 (C−2), 41.03 (C−8), 38.56 (C−9), 38.34 (C−10), 30.16 (C−γ), 27.90 (C−γ), 26.02 (C−γ), 23.30 (C−C), 22.12 (C−C); LC-MS, C24H44NBr: 346.3 (M+−Br), 504.0, 506.0, 508.0 (M++Br).

(1R,2R,5R)-Hydronopyl dimethyl amine (2b, 0.04 mol) and compound 1 (0.05 mol) were slowly added to 40 mL ethyl acetate at 60 °C. After 72 hours of reaction, the ethyl acetate was vacuum removed. Following 3 light petroleum washes and recrystallization from ethyl acetate, a white solid bis-hydronopyl ethyl ammonium bromide (3b) was obtained; m.p., 198-201.7 °C; Yield, 80%. FT-IR ν (cm⁻¹): 2985.86-2866.27 (C-H, 1121.63 (C-N); 1H NMR (CDCl3, 400 MHz) δH: 3.61 (4H, q, J = 6.8 Hz, 2α−CH2), 3.33 (4H, t, J = 8.4 Hz, 21−CH2), 2.33 (2H, m, 2−CH), 2.06-1.67 [18H, m, 2 (−CH−10CH2), 1CH, 5−CH, 3−CH2, 4−CH2)], 1.38 (6H, m, 2 β−CH3), 1.17 (6H, s, 2 α−CH3), 0.99 (6H, s, 2α−CH3), 0.85 (2H, d, J = 10 Hz, 2−CH); 13C NMR (CDCl3, 100 Hz) δC: 57.02 (C−11), 54.08 (C−α), 46.25 (C−2), 41.01 (C−8), 38.57 (C−9), 38.49 (C−10), 33.34 (C−γ), 29.53 (C−γ), 27.92 (C−γ), 26.04 (C−γ), 23.33 (C−C), 23.31 (C−C), 8.16 (C−γ); LC-MS, C22H44NBr: 374.4 (M+−Br), 532.4, 534.4, 536.4 (M++Br).

(1R,2R,5R)-Hydronopyl piperidine (2c, 0.05 mol) and compound 1 (0.06 mol) were slowly added to 40 mL ethyl acetate at 60 °C. After 72 hours of reaction, the ethyl acetate was vacuum removed. Following 3 light petroleum washes and recrystallization from ethyl acetate, a white solid bis-hydronopyl piperidine ammonium bromide (3c) was obtained; m.p., 226.9-230.3 °C; Yield, 86.5%. FT-IR ν (cm⁻¹): 2975.25-2867.23 (C-H), 1066.65 (C-N); 1H NMR (CDCl3, 400 MHz) δH: 3.74 (4H, s, 2α−CH2), 3.48-3.20 (4H, m, 2α−CH2), 2.32 (2H, m, 2−CH), 1.98-1.63 (2H, m, 2−CH2), 1.83 (6H, d, J = 9 Hz, 2−CH3); 13C NMR (CDCl3, 100 Hz) δC: 57.02 (C−11), 54.08 (C−α), 46.25 (C−2), 41.01 (C−8), 38.57 (C−9), 38.49 (C−10), 33.34 (C−γ), 29.53 (C−γ), 27.92 (C−γ), 26.04 (C−γ), 23.33 (C−C), 23.31 (C−C), 8.16 (C−γ); LC-MS, C24H44NBr: 374.4 (M+−Br), 532.4, 534.4, 536.4 (M++Br).

Scheme 1. Synthetic routes of bis-hydronopyl quaternary ammonium salts (3a-c).
Determination of effective concentration. The antimicrobial activities of 3a-c against phytopathogenic fungi were evaluated using the half-maximal effective concentration (EC₅₀) values. The regression equation was obtained by using the natural logarithm of the compound concentrations as the abscissa, and the biometric probability value calculated by MGI, as the ordinate. The EC₅₀ values were calculated using the regression equation when the MGI values were 50%.

Determination of minimum inhibitory concentration of bacteria. The antimicrobial properties of 3a-c against E. coli, P. putida, S. aureus, and B. subtilis were evaluated by the resazurin coloration method. The concentrations of bacterial suspensions were 1.5 × 10⁸ CFU/mL. One hundred microliters of each bacterial suspension and 100 µL of the compound solutions with different concentrations were transferred to a 96-well plate, after which 20 µL of a 0.1% resazurin solution was added into the 96-well plate, and the contents of each well were mixed by vortex shaking. Instead of the compound solution, 0.9% saline was added as a control plate. Kanamycin sulfate (98%) was used as the positive control. Then, the mixtures were incubated at 37 °C for 24 hours.

Results and Discussion

Chemistry

The FT-IR spectrum of 3a showed an absorption band at 2997-2867 cm⁻¹ due to the amidic V' C-H group and the characteristic stretching frequency of δ C-H was observed at 1450 cm⁻¹. The characteristic absorption band of the C-N group appeared at 1227 cm⁻¹. The absorption peaks at 2985-2866, 1469, and 1121 cm⁻¹ represented the infrared absorption group appeared at 1227 cm⁻¹. The absorption peaks at 2985-2866, 1469, and 1121 cm⁻¹ represented the infrared absorption band of the C-H group and the characteristic stretching frequency of δ C-H, respectively, which indicated that the expected structure of 3b had been formed.

Table 1. Antifungal Activities of 3a Against Plant Pathogenic Fungi.

| Strain    | MGI (%) at a concentration of (µg/mL) | EC₅₀ (µg/mL) | y = a + bx | R² | 95% CI |
|-----------|--------------------------------------|-------------|-----------|----|--------|
| A. eucalperis | 100 100 50 25 12.5 | 0.9086 4.4754-28.5483 | 4.754-28.5483 |
| P. nicoitanae | 100 100 50 25 12.5 | 0.9356 3.3465-9.7678 | 3.3465-9.7678 |
| C. gloeosporioides | 99.16 93.82 76.40 54.78 35.96 | 20.6670 | 17.2874-24.7072 |
| C. versicolor | 100 95.97 83.98 50.40 39.33 | 19.1249 | 9.0370-9.0370 |
| C. acutatum | 98.28 93.70 91.40 89.68 86.82 | 0.5380 | 0.8627-4.6136 |
| F. oxysporum | 100 98.25 96.79 90.40 85.01 | 11.4824 | 4.7350-27.8448 |

Abbreviations: EC₅₀, half-maximal effective concentration; MGI, mycelial growth inhibition.
The FT-IR spectrum of 3c exhibited absorption wavelengths at 2975-2867 cm⁻¹ corresponding to a V C-H group, and at 1434 cm⁻¹ belonging to the δ C-H group. The C-N group absorption was observed at 1066 cm⁻¹. In the ¹H NMR spectrum, the proton signal of the CH₂ group attached to the N group was observed at δ 3.2-3.6 ppm as a doublet of doublets; for example, in the spectrum of compound 2b, this was seen at δ 3.471 and 3.226 ppm. In the ¹³C NMR spectra, the carbon signal of the CH₂ group attached to the N group was observed at δ 51.640-62.354 ppm; for example, in the spectrum of compound 3b, this was seen at δ 57.018 and 54.060 ppm. The mass spectrum of 3a and 3b showed the expected pseudomolecular ion peaks at m/z 346.3 (M⁺ − Br) and 374.4 (M⁺ + Br), and 504.0, 506.0, 508.0 (M⁺ + Br) and 532.4, 534.4, 536.4 (M⁺ + Br), corresponding to the molecular formula. There was a difference in (CH₂)₂ (28) between 3a and 3b.

**Biological Activity**

**Activity against phytopathogenic fungi.** The antifungal activities of the β-pinene-based derivatives against six plant pathogens are shown in Tables 1–4. For all the tested samples, the inhibition rates improved with the increase in sample concentration. The EC₅₀ values of the β-pinene-based derivatives against the 6 typical phytopathogenic fungi were less than 30 µg/mL. Among them, compound 3a showed the best activity with an EC₅₀ value of 11.30 µg/mL. For C. acutatum, compound 3a also exhibited an outstanding antifungal activity, with an EC₅₀ value of 0.54 µg/mL.

Compound 3c showed the best effect on F. oxysporum, with an EC₅₀ value of 10.15 µg/mL. Compared with the positive control, chlorothalonil, the β-pinene-based derivatives exhibited better antifungal activity against P. nicotianae, C. versicolor, and C. acutatum.

Of all the compounds tested, 3a exhibited the most significant antifungal activity against C. acutatum (EC₅₀ = 0.538 µg/mL). The ultrastructural features of C. acutatum mycelium upon administration of 3a were examined by SEM, as shown in Figure 1. After being cultured for 72 hours Figure 1(A and B) show that the mycelia were smooth and full in the control experiment, indicating that the fungus was in good condition. However, the morphology of the mycelia treated with an EC₅₀ concentration of 3a was changed. Serious mycelial deformation was observed, with sunken surfaces and the mycelia were easily fractured when harvested (Figure 1(C and D)).

Although it was hard to give a comprehensive structure-activity relationship to these derivatives, we could find some interesting hints from the antifungal experiment. For T. cucumeris, P. nicotianae, C. versicolor, and C. acutatum, the antifungal activities of the compounds were 3b < 3c < 3a. For C. gloeosporioides and F. oxysporum, the antifungal activities of the compounds were 3b < 3a < 3c. In our previous report, for quaternary ammonium salts containing a hydronopyl group,
the antifungal activities of 2 ethyl groups linked on the hydrophilic group N⁺ was stronger than that of 2 methyl groups. However, when there were 2 hydronopyl groups in the structure of the quaternary ammonium salts, the rule for the antifungal activity was opposite. Several reports suggest that polycations can kill various microbes by adsorption to the cell membranes and disruption of their integrity. Therefore, quaternary ammonium salts could effectively inhibit the growth of fungi because of their polycationic nature, but the molecular weight of the quaternary ammonium salt and its inhibitory effect might not be positively correlated under different conditions. The molecular weight of 3b with an ethyl alkyl chain was higher than that of 3a with a methyl alkyl chain. However, the ethyl in 3b could hinder the combination of the positively charged amino groups of the quaternary ammonium salt with the negatively charged substances on the cell wall to form polyelectrolyte complexes, which cause the integrity of the hard cell membrane to be destroyed. Researchers found that the antifungal activity of amides bearing piperidine moieties against Cladosporium sphaerospermuma was comparable with that observed for the reference compounds miconazole and nystatin. Interestingly, the introduction of piperidine to the hydrophilic group N⁺ of the derivatives (3c) could improve the antifungal activity against C. gloeosporioides and F. oxysporum.

Antibacterial activity. Screening of the activities of bis-hydronopyl quaternary ammonium salts against bacteria was performed using Gram-negative (E. coli, P. putida) and Gram-positive bacteria (S. aureus, B. subtilis). The minimum inhibitory concentration (MIC) values were determined using the resazurin coloration method. Resazurin is a redox indicator used to differentiate between metabolically active and inactive cells. The resazurin reagent is blue; however, it gives off a bright-red color when reduced, indicating the presence of live bacteria. The MIC values for the bis-hydronopyl quaternary ammonium salts and kanamycin sulfate against E. coli, P. putida, S. aureus, and B. subtilis are shown in Figure 2.

Compared with the positive control kanamycin sulfate, 3a had better bacteriostatic activity against P. putida, S. aureus, and

### Table 4. Antifungal Activities of Chlorothalonil Against Plant Pathogenic Fungi.

| Strain         | MGI (%) at a concentration of (μg/mL) | EC₅₀  | y = a + bx | R²         | 95% CI             |
|----------------|--------------------------------------|-------|------------|------------|--------------------|
| T. cucumeris   |                                      |       |            |            |                    |
| 200            | 91.40                                | 3.5676| y = 4.6122 + 0.7020x | 0.9336     | 1.0504-12.1164     |
| 100            | 80.23                                | 3.4152| y = 4.3712 + 0.4756x | 0.9415     | 19.9710-48.2796    |
| 50             | 76.51                                | 2.8628| y = 4.2913 + 0.4287x | 0.9481     | 19.9710-48.2796    |
| 25             | 75.93                                | 2.2951| y = 3.6760 + 0.3629x | 0.9512     | 19.9710-48.2796    |
| 12.5           | 64.88                                | 1.7324| y = 2.9521 + 0.3066x | 0.9554     | 19.9710-48.2796    |
| 6.25           | 64.02                                | 1.3328| y = 2.2205 + 0.2590x | 0.9596     | 19.9710-48.2796    |
| 3.125          | 63.24                                | 0.9636| y = 1.6789 + 0.2106x | 0.9636     | 19.9710-48.2796    |
| C. gloeosporioides |                                 |       |            |            |                    |
| 200            | 59.83                                | 3.0568| y = 4.6428 + 0.2790x | 0.9215     | 9.8998-36.7365     |
| 100            | 57.89                                | 3.0408| y = 4.3712 + 0.4756x | 0.9415     | 19.9710-48.2796    |
| 50             | 56.23                                | 2.6824| y = 3.6760 + 0.4287x | 0.9481     | 19.9710-48.2796    |
| 25             | 54.02                                | 2.2951| y = 3.6760 + 0.4287x | 0.9481     | 19.9710-48.2796    |
| 12.5           | 45.15                                | 1.7324| y = 2.9521 + 0.3629x | 0.9512     | 19.9710-48.2796    |
| 6.25           | 43.95                                | 1.3328| y = 2.2205 + 0.3066x | 0.9554     | 19.9710-48.2796    |
| 3.125          | 42.46                                | 0.9636| y = 1.6789 + 0.2590x | 0.9636     | 19.9710-48.2796    |
| C. versicolor  |                                      |       |            |            |                    |
| 200            | 71.14                                | 4.0526| y = -3.0509 + 0.9517x | 0.9119     | 58.5682-213.0209   |
| 100            | 69.30                                | 3.8979| y = -2.5805 + 1.3637x | 0.9663     | 44.0478-80.2596    |
| 50             | 58.89                                | 3.7433| y = -2.3254 + 1.2076x | 0.9547     | 41.2848-79.2596    |
| 25             | 56.89                                | 3.5889| y = -2.0703 + 1.0517x | 0.9438     | 38.7512-74.1832    |
| 12.5           | 45.29                                | 2.9345| y = -1.4154 + 0.8958x | 0.9325     | 31.5728-69.0064    |
| 6.25           | 43.29                                | 2.5899| y = -0.7604 + 0.7409x | 0.9215     | 25.5542-61.0372    |
| 3.125          | 41.29                                | 2.2454| y = 0.0000 + 0.6864x  | 0.9104     | 13.9252-58.6372    |
| E. oxysporum   |                                      |       |            |            |                    |
| 200            | 79.05                                | 4.6984| y = 4.6984 + 0.4739x | 0.9895     | 2.7994-6.6930      |
| 100            | 72.86                                | 4.6353| y = 4.6353 + 0.4678x | 0.9853     | 2.7994-6.6930      |
| 50             | 70.48                                | 4.5722| y = 4.5722 + 0.4622x | 0.9812     | 2.7994-6.6930      |
| 25             | 62.86                                | 4.4091| y = 4.4091 + 0.4566x | 0.9771     | 2.7994-6.6930      |
| 12.5           | 59.29                                | 4.2460| y = 4.2460 + 0.4510x | 0.9730     | 2.7994-6.6930      |
| 6.25           | 43.86                                | 3.6928| y = 3.6928 + 0.4454x | 0.9690     | 2.7994-6.6930      |
| 3.125          | 41.46                                | 3.3396| y = 3.3396 + 0.4399x | 0.9650     | 2.7994-6.6930      |

Abbreviations: EC₅₀, half-maximal effective concentration; MGI, mycelial growth inhibition.

Figure 1. Effect of bis-hydronopyl dimethyl ammonium bromide (3a) on mycelial morphology in Colletotrichum acutatum (A, C); 2000 times, plates untreated and treated with EC₅₀ value concentration of 3a; (B, B); 6000 times, plates untreated and treated with EC₅₀ value concentration of 3a. EC₅₀, half-maximal effective concentration.

Figure 2. MIC values of bis-hydronopyl quaternary ammonium salts against Escherichia coli, Pseudomonas putida, Staphylococcus aureus, and Bacillus subtilis. MIC, minimum inhibitory concentration.
**Conclusions**

Derivatives of β-pinene (3a-c) were produced, and their inhibitory properties against phytopathogenic fungi and bacteria were assessed. Results showed that compound 3a containing the methyl and bis-hydronopyl group had a higher activity in comparison with the positive control. Specifically, compound 3a against *P. nicotianae, C. versicolor, C. acutatum, P. putida, S. aureus,* and *B. subtilis* exhibited an EC₅₀ or MIC value lower than that of compounds 3b, 3c and the positive control. SEM images showed that compound 3a could alter the morphological structure of the mycelium of *C. acutatum.* The structure-activity relationship analysis showed that the antibacterial activity of ammonium salts containing bis-hydronopyl against plant pathogens and bacteria were less dependent on molecular weight. The introduction of piperidine to the hydrophilic group N⁺ could improve the antibacterial activity against *C. gloeosporioides, F. oxysporum,* and *S. aureus.* Therefore, the β-pinene-based derivatives can be used as precursor molecules for further pesticide and bacteriostatic agent development.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Key Projects of Key R&D Program of Jiangxi Province (2019AB60011).

**ORCID IDs**

Xuezhen Feng [https://orcid.org/0000-0001-7149-6080](https://orcid.org/0000-0001-7149-6080)
Shangxing Chen [https://orcid.org/0000-0002-7512-6316](https://orcid.org/0000-0002-7512-6316)
Guorong Fan [https://orcid.org/0000-0002-6966-277X](https://orcid.org/0000-0002-6966-277X)

**Supplemental Material**

Supplemental material for this article is available online.

**References**

1. Zhang J, Tan W, Zhang Z, et al. Synthesis, characterization, and the antifungal activity of chitosan derivatives containing urea groups. *Int J Biol Macromol.* 2018;109(4):1061-1067. doi:10.1016/j.ijbiomac.2017.11.092
2. Knocke W. Fungal infection of plants. *Plant Cell.* 1996;8(10):1711-1722. doi:10.2307/3870224
3. Mendgen K, Hahn M. Plant infection and the establishment of fungal biotrophy. *Trends Plant Sci.* 2002;7(8):352-356. doi:10.1016/S1360-1385(02)02297-5
4. Asghar MA, Zahir E, Shahid SM, et al. Iron, copper and silver nanoparticles: Green synthesis using green and black tea leaves extracts and evaluation of antibacterial, antifungal and aflatoxin B₁ adsorption activity. *LiWT.* 2018;90(3):98-107. doi:10.1016/j.liwt.2017.12.009
5. Yang G, Jin Q, Xu C, Fan S, Wang C, Xie P. Synthesis, characterization and antifungal activity of coumarin-functionalized chitosan derivatives. *Int J Biol Macromol.* 2018;106(8):179-184. doi:10.1016/j.ijbiomac.2017.08.009
6. Lagrouh F, Dakka N, Bakri Y. The antifungal activity of Moroccan plants and the mechanism of action of secondary metabolites from plants. *J Mycol Med.* 2017;27(3):303-311. doi:10.1016/j.jymymed.2017.04.008
7. Łukowska-Chojnacka E, Mierzejewska J, Milner-Krawczyk M, Bondaryk M, Staniszewska M. Synthesis of novel tetrazole derivatives and evaluation of their antifungal activity. *Bioorg Med Chem.* 2016;24(22):6058-6065. doi:10.1016/j.bmc.2016.09.066
8. Xie Y, Wang Z, Huang Q, Zhang D. Antifungal activity of several essential oils and major components against wood-rot fungi. *Ind Crops Prod.* 2017;108(6):278-285. doi:10.1016/j.indcrop.2017.06.041
9. Rivas da Silva AC, Lopes PM, Barros de Azevedo MM, Costa DCM, Alviano CS, Alviano DS. Biological activities of α-pinene and β-pinene enantiomers. *Molecules.* 2012;17(6):6305-6316. doi:10.3390/molecules17066305
10. de Macêdo Andrade AC, Rosalen PL, Freires Ia, et al. Antifungal activity, mode of action, docking prediction and anti-biofilm effects of (+)-β-pinene enantiomers against *Candida* spp. *Curr Top Med Chem.* 2018;18(29):2481-2490. doi:10.2174/156802661866181115103104
11. Wilson CI, Solar JM, El Ghaouth A, Wisniewski ME, Wisniewski ME. Rapid evaluation of plant extracts and essential oils for antifungal activity against Botrytis cinerea. Plant Dis. 1997;81(2):204-210. doi:10.1094/PDIS.1997.81.2.204

12. Feng XZ, Xiao Z, Zhang L, et al. Antifungal activity of β-pinene-based hydronopoly quaternary ammonium salts against phytopathogenic fungi. Nat Prod Commun. 2020;15(8):1-6. doi:10.1177/1934578X20948365

13. Moreira ACP, Lima EDO, Souza ELD, Castellano LR, Castro RD. Inhibitory effect of Cinnamomum zeylanicum Blume (Laureaceae) essential oil and beta-pinene on the growth of dematiaceous molds. Digestion. 1972;7(38):33-38.

14. Nikitina LE, Startseva VA, Vavilenko IA, et al. Synthesis and antifungal activity of compounds of the pinane series. Pharm Chem J. 2009;43(5):251-254. doi:10.1007/s11094-009-0282-3

15. Liao S, Shang S, Shen M, et al. One-pot synthesis and antimicrobial evaluation of novel 3-cyanopyridine derivatives of (−)-β-pinene. Bioorg Med Chem Lett. 2016;26(6):1512-1515. doi:10.1016/j.bmcl.2016.02.024

16. Li J, Tian X, Gao Y, Shang S, Feng J, Zhang X. A value-added use of volatile turpenite: antifungal activity and QSAR study of β-pinene derivatives against three agricultural fungi. RSC Adv. 2015;5(82):66947-66955. doi:10.1039/C5RA10660E

17. Gao Y, Hao J, Li J, Song Z, Shang S. Structural modification of turpenite with natural chiral preservation and low-risk application prospects in crop protection. ACS Omega. 2019;4(4):6392-6398. doi:10.1021/acsomega.9b00241

18. Gao Y, Wang Y, Li J, Shang S, Song Z. Improved application of natural forest product terpene for discovery of potential botanical fungicide. Ind Crops Prod. 2018;126(210):103-112. doi:10.1016/j.indcrop.2018.10.008

19. Gao Y, Li J, Li J, Song Z, Shang S, Rao X. High add value application of turpentine in crop production through structural modification and QSAR analysis. Molecules. 2018;23(2):356. doi:10.3390/molecules23020356

20. Gavrilo VV, Startseva VA, Nikitina LE, et al. Synthesis and antifungal activity of sulfides, sulfoxides, and sulfones based on (1S)-(−)-β-pinene. Pharm Chem J. 2010;44(3):126-129. doi:10.1007/s11094-010-0413-x

21. Hoque J, Akkapeddi P, Yadav V, et al. Broad spectrum antibacterial and antifungal polymeric paint materials: synthesis, structure-activity relationship, and membrane-active mode of action. ACS Appl Mater Interfaces. 2015;7(3):1804-1815. doi:10.1021/am507482y

22. Zhang J, Tan W, Luan F, et al. Synthesis of quaternary ammonium salts of chitosan bearing halogenated acetate for antifungal and antibacterial activities. Polymers. 2018;10(5):530. doi:10.3390/polym10050530

23. Muñoz-Bonilla A, Fernández-García M. Polymeric materials with antimicrobial activity. Prog Polym Sci. 2012;37(2):281-339. doi:10.1016/j.progpolymsci.2011.08.005

24. Wan X, Zhang Y, Deng Y, et al. Effects of interaction between a polycation and a nonionic polymer on their cross-assembly into mixed micelles. Soft Matter. 2015;11(21):4197-4207. doi:10.1039/C5SM00380F

25. Gilbert P, Moore LE. Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol. 2005;99(4):703-715. doi:10.1111/j.1365-2672.2005.02664.x

26. Jin LI, Xiao ZQ, Fan GR, et al. Synthesis and antifungal activity of a series of N-hydromethylpyridine ammonium halides. Chem Ind Forest Prod. 2017;37(3):122-128.

27. Chen JZ, Xiao ZQ, LF X, Wang ZD, Hang ZJ. The synthesis and structural analysis of hydronopoly tertiary amine compounds. J Jiangxi Normal U: Nat Sci Ed. 2016;40(2):179-182.

28. Zhao LH, Xiao ZQ, Chen JZ, Wang ZD, Fan GR, Chen SX. Synthesis and structural analysis of hydronopol and its halides. Chem Ind Forest Prod. 2012;32(1):39-42.

29. Shirakawa M, Uehara I, Tanaka M, Makoto S, Iwao U, Megumi T. Mycorrhizosphere bacterial communities and their sensitivity to antibacterial activity of ectomycorrhizal fungi. Microbes Environ. 2019;34(2):191-198. doi:10.1264/jmte2.18146

30. Abdel-Aziz AA-M, Asiny YA, Al-Agamy MMH, Design A-AHMH. Design, synthesis and antibacterial activity of fluoroquinolones containing bulky arenesulfonyl fragment: 2D-QSAR and docking study. Eur J Med Chem. 2011;46(11):5487-5497. doi:10.1016/j.ejmech.2011.09.011

31. Lubna H, Fahim U. Establishment and application of the resazurin micro-plate method for rapid diagnosis of bovine mastitis-causing staphylococci. Pak J Bio Sci. 2011;11(1):2017.

32. Guembe M, Alonso B, Cruces R, Sanchez-Carrillo C, Perez A. Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm. Agro Food Ind Hi Tech. 2016;27(6):135-137.

33. Feng XZ, Xiao ZQ, PY L, Fan GR, Wang ZD. Synthesis and antibacterial activity of hydronopol dimethyl alkyl ammonium halide. Chem Ind Forest Prod. 2019;39(1):35-40.

34. Lee SB, Koepsel RR, Morley SW, Matyjaszewski K, Sun Y, Russell AJ. Permanent, nonleaching antibacterial surfaces. 1. synthesis by atom transfer radical polymerization. Biomacromolecules. 2004;5(3):877-882. doi:10.1021/bm034352k

35. Lin J, Qui S, Lewis K, Kilbanov AM. Mechanism of bactericidal and fungicidal activities of textiles covalently modified with alkylated polyethylenimine. Bioelectrochem Bioeng. 2003;83(2):168-172. doi:10.1002/bit.10651

36. Kögler R, Bouloussa O, Rondelez F. Evidence of a charge-density threshold for optimum efficiency of bioidal cationic surfaces. Microbiology. 2005;151(Pt 5):1341-1348. doi:10.1099/mic.0.27526-0

37. Guo Z, Xing R, Liu S, et al. The influence of molecular weight of quaternized chitosan on antifungal activity. Carbohydr Polym. 2008;71(4):694-697. doi:10.1016/j.carbpol.2007.06.027

38. Badawy MEI. Structure and antimicrobial activity relationship of quaternary N-alkyl chitosan derivatives against some plant pathogens. J Appl Polym Sci. 2010;117(2):960-969. doi:10.1002/ app.31492

39. Navickiene HM, Alécio AC, Kato MJ, et al. Antifungal amides containing bulky arenesulfonyl fragment: 2D-QSAR and docking study. Eur J Med Chem. 2011;46(11):5487-5497. doi:10.1016/j.ejmech.2011.09.011

40. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian...
cell cytotoxicity. *Eur J Biochem*. 2000;267(17):5421-5426. doi:10.1046/j.1432-1327.2000.01606.x

41. Zhong W, Dong C, Liuyang R, et al. Controllable synthesis and antimicrobial activities of acrylate polymers containing quaternary ammonium salts. *Reactive and Functional Polymers*. 2017;121(12):110-118. doi:10.1016/j.reactfunctpolym.2017.10.010

42. Chen CZ, Beck-Tan NC, Dhurjati P, van Dyk TK, LaRossa RA, Cooper SL. Quaternary ammonium functionalized poly(propylene imine) dendrimers as effective antimicrobials: structure-activity studies. *Biomacromolecules*. 2000;1(3):473-480. doi:10.1021/bm0055495

43. Kim JH, Shyam PK, Kim MJ, Lee H-J, Lee JT, Jang H-Y. Enantioselective synthesis and antioxidant activity of 3,4,5-substituted piperidine derivatives. *Bioorg Med Chem Lett*. 2016;26(13):3119-3121. doi:10.1016/j.bmcl.2016.04.092

44. Murata Y, Miyamoto E, Ueda M. Antimicrobial and anti-plaque activity of N’-alkyl-N-(2-aminoethyl)piperidine against dental plaque bacteria. *J Pharm Sci*. 1991;80(1):26-28. doi:10.1002/jps.2600800107

45. Rokhyatou S, Abdoulaye G, Sandrine C, Christian C. Synthesis and antimalarial activity of 1,4-disubstituted piperidine derivatives. *Molecules*. 2020;25(2):299-315.

46. Subba P, Parameshwar Naik G, Krishnamurthy G, Jithendra Kumara KS, Sunil Kumar N, Sathish N. Anti-inflammatory, antibacterial and molecular docking studies of novel spiro-piperidine quinazolinone derivatives. *J Taibah Univ Sci*. 2017;11(3):497-511.