Postprint: Chemical Composition of Pinus sylvestris var. mongolica Needles

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Abstract

Using needles of Pinus sylvestris collected from Qiqihar City, Heilongjiang Province as the research material, chemical constituents were extracted by solvent extraction method. Modern chromatographic techniques including silica gel column chromatography, preparative thin-layer chromatography, and high-performance liquid chromatography were applied to separate and purify the chemical constituents from the extract. Spectroscopic techniques such as mass spectrometry and nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) were utilized to identify the structures of the compounds, and the antimicrobial activity of the extract was tested. The results showed: 15 compounds were isolated from the Pinus sylvestris needle extract and identified as pinifolic acid (1), methyl pinifolate (2), 18 -acetoxylabd-8(17)-en-15-oic acid (3), 4-epi-imbricataloic acid (4), 15-ethyl-18-methyl pinifolate (5), 15-acetoxy-labda-8(17),13E-dien-18-al (6), 6 -hydroxydehydroabietic acid (7), 7 -hydroxydehydroabietic acid (8), endo-peroxide (9), -cadinol (10), -sitosterol (11), dibutyl phthalate (12), 7R,11R-phytol (13), n-tetracosanol (14), and N-octacosanol (15). Among them, compounds 9, 13, 14, and 15 were isolated from this genus for the first time. The antimicrobial activity test results showed that the n-hexane extract at concentrations of 5 and 100 mg · mL ^{1} exhibited inhibition rates against Escherichia coli of 53% and 71%, respectively, and against Bacillus subtilis of 56% and 70%, respectively, and at concentrations of 50 mg · mL ^{1} and 100 mg · mL ^{1} showed inhibition rates against Staphylococcus aureus of 51% and 69%, respectively. This study further clarified the chemical constituents in Pinus sylvestris needles, providing a basis for further activity testing and application research.

Full Text

Preamble

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Title: Chemical Constituents of Pinus sylvestris Needles
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Abstract

This study investigated the chemical constituents of Pinus sylvestris needles collected from Qiqihar City, Heilongjiang Province. Chemical components were extracted using solvent extraction methods, and modern chromatographic techniques including silica gel column chromatography, preparative thin-layer chromatography, and high-performance liquid chromatography were employed to separate and purify the compounds from the extract. The structures of the isolated compounds were elucidated using spectroscopic techniques such as mass spectrometry and nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR). The antibacterial activity of the extract was also evaluated. The results showed that 15 compounds were isolated and identified as pinifolic acid (1), monomethyl pinifolate (2), 18 -acetoxylabd-8(17)-en-15-oic acid (3), 4-eplimbricataloic acid (4), 15-ethyl-18-methyl pinifolate (5), 15-acetoxy-labda-8(17),13E-dien-18-al (6), 7 -hydroxydehydroabietic acid (7), 7 -hydroxydehydroabietic acid (8), endo-peroxide (9), -cadinol (10), -sitosterol (11), dibutyl phthalate (12), 7R,11R-phytol (13), tetracosanol (14), and N-octacosan-7 -ol (15). Among these, compounds 9, 13, 14, and 15 were isolated from the genus *Pinus* for the first time. The antibacterial activity tests revealed that the hexane extract exhibited inhibition rates of 53–71% against *Escherichia coli* and 56–70% against *Bacillus subtilis* at concentrations of 5–100 mg · mL ^{1}. Against *Staphylococcus aureus*, the inhibition rates were 51% and 69% at concentrations of 50 mg · mL ^{1} and 100 mg · mL ^{1}, respectively. This research clarifies the chemical composition of Pinus sylvestris needles and provides a basis for further studies on their biological activities and potential applications.

Keywords: Pinus sylvestris needles; chemical constituents; antibacterial activity; pinifolic acid; 7-hydroxydehydroabietic acid; cadinol

Introduction

Pinus sylvestris L. var. *mongholica* Litv., commonly known as Mongolian pine or Hailar pine, is a tall evergreen coniferous tree belonging to the family Pinaceae. It is mainly distributed in northeastern China and Inner Mongolia (Editorial Committee of Flora Reipublicae Popularis Sinicae, 1996). The needles of this species primarily contain monoterpenes and sesquiterpenes as volatile

oils (Pan et al., 1992; You et al., 2010; Bo et al., 2010; Liu et al., 2011) as well as diterpenoid compounds (Zhang et al., 2006). Pine needle oil has demonstrated various biological activities, including antitumor (Zhang et al., 2006; Chen et al., 2014), antioxidant (Ka et al., 2005; Jeong et al., 2009), anti-aging (Chen et al., 2005), anti-inflammatory (Karonen et al., 2004), antibacterial (Zeng et al., 2009), anti-influenza virus (Wei et al., 2008), and antimutagenic effects (Kong et al., 1995). Pharmacologically, it has been used to treat rheumatic arthralgia (Chen et al., 2012), improve immune function (Dong et al., 2018), alleviate hypertension (Sang et al., 2018), and improve cardiac function (Li et al., 2006), making it a potentially valuable traditional medicinal resource. To gain deeper insights into the chemical constituents of *Pinus sylvestris* needles and explore their pharmacological activities, this study investigated the chemical components and antibacterial activity of the hexane extract from the needles. Fifteen compounds were isolated and identified: pinifolic acid (1), monomethyl pinifolate (2), 18 -acetoxylabd-8(17)-en-15-oic acid (3), 4-eplimbricataloic acid (4), 15-ethyl-18-methyl pinifolate (5), 15-acetoxy-labda-8(17),13E-dien-18-al (6), 7 -hydroxydehydroabietic acid (7), 7 -hydroxydehydroabietic acid (8), endo-peroxide (9), -cadinol (10), -sitosterol (11), dibutyl phthalate (12), 7R,11R-phytol (13), tetracosanol (14), and N-octacosan-7 -ol (15). Among these, compounds 9, 13, 14, and 15 were reported from the genus *Pinus* for the first time. The hexane extract exhibited varying degrees of antibacterial activity against *E. coli*, *B. subtilis*, and *S. aureus* at different concentrations. This paper reports the isolation, structural identification, and antibacterial activity of the chemical constituents from the hexane extract of *Pinus sylvestris* needles.

Materials and Methods

1.1.1 Plant Material

Pinus sylvestris needles were collected in Qiqihar City, Heilongjiang Province in September 2016 and identified by Professor Zhang Shujun of Qiqihar University as the needles of *Pinus sylvestris* L. var. *mongholica* Litv.

1.1.2 Microbial Strains

The test microorganisms *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* were provided by the College of Life Sciences at Qiqihar University.

1.1.3 Reagents

Tryptone, yeast extract powder, and agar powder were purchased from Beijing Aoboxing Biotechnology Co., Ltd. Sodium chloride was obtained from Tianjin Kaitong Chemical Reagent Co., Ltd. All solvents were analytical grade (Tianjin Kaitong Chemical Reagent Co., Ltd.) except for those used in HPLC, which were chromatographic grade (Tianjin Kemiou Chemical Reagent Co., Ltd.).

1.1.4 Instruments

Bruker AV-600 NMR spectrometer (Bruker, USA); Agilent 5988A mass spectrometer (Agilent, USA); Waters 2489 HPLC system (Waters Technology Shanghai Co., Ltd.); silica gel (200–300 mesh) and TLC plates (Qingdao Marine Chemical Factory); Yanako melting point apparatus (Beijing Tech Instrument Co., Ltd.); YXQ-LS-50S11 vertical pressure steam sterilizer (Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory); SW-CJ-LED super-clean workbench (Shanghai Jinping Instrument Co., Ltd. Tongzhou Branch); MIR-253 constant temperature incubator (Shanghai Jinghong Laboratory Equipment Co., Ltd.).

1.2.1 Extraction and Fractionation

Dried Pinus sylvestris needles (11 kg) were soaked three times in anhydrous ethanol. The extracts were combined and concentrated under reduced pressure to yield 304 g of crude extract. The extract was suspended in water and sequentially partitioned three times each with n-hexane, ethyl acetate, and n-butanol. The respective fractions were combined and concentrated under reduced pressure to obtain the n-hexane extract (85 g), ethyl acetate extract (82 g), and n-butanol extract (44 g).

1.2.2 Isolation and Purification

The n-hexane extract (85 g) was subjected to silica gel column chromatography with gradient elution (n-hexane:ethyl acetate = 100:0 to 0:100) and monitored by TLC to yield five fractions (Fr.1–5). Fr.3 was further separated by silica gel column chromatography (n-hexane:ethyl acetate = 7:3, 4:6, 2:8) to afford three subfractions (Fr.3.1–3.3). Fr.3.2 was purified by HPLC [Cosmosil 5C18-AR-II (10 × 250 mm), water:methanol = 5:95, flow rate 2 mL/min] to yield compounds **1** (12.5 mg), **2** (9.6 mg), and **3** (9.4 mg). Fr.2 was separated by silica gel column chromatography (n-hexane:ethyl acetate = 8:2, 6:4, 4:6, 2:8) to obtain compounds **4** (7.4 mg), **5** (7.8 mg), and **6** (32.4 mg). Fr.4 was fractionated by silica gel column chromatography (n-hexane:ethyl acetate = 6:4, 4:6, 2:8) to give three subfractions (Fr.4.1–4.3). Fr.4.2 was recrystallized from ethyl acetate to afford compounds **7** (12.4 mg) and **8** (32.4 mg). Fr.4.3 was purified by HPLC [Cosmosil 5C18-AR-II (10 × 250 mm), water:methanol = 15:85, flow rate 2 mL/min] to yield compound **9** (23.6 mg). Fr.1 was separated by silica gel column chromatography (n-hexane:ethyl acetate = 1:9, 2:8, 3:7, 4:6, 2:8) to give five subfractions (Fr.1.1–1.5). Fr.1.2 was purified by HPLC [Cosmosil 5C18-AR-II (10 × 250 mm), water:methanol = 5:95, flow rate 2 mL/min] to obtain compound **10** (23.6 mg). Fr.1.3 was recrystallized from ethyl acetate to give compound **11** (17.5 mg). Fr.1.4 was recrystallized from ethyl acetate to afford compound **12** (7.4 mg). Fr.1.5 was purified by HPLC [Cosmosil 5C18-AR-II (10 × 250 mm), water:methanol = 5:95, flow rate 2 mL/min] to yield compounds **13** (15.2 mg), **14** (12.3 mg), and **15** (15.2 mg).

1.2.3 Antibacterial Activity Assay

The n-hexane extract was dissolved in acetone to prepare solutions at concentrations of 100, 50, 25, 10, and 5 mg · mL ^{1} for antibacterial testing. Antibacterial activity was evaluated using the LB medium plate culture method. Acetone was used as a control, and each group was tested in triplicate. When the control colony diameter reached approximately 3/4 of the plate bottom, the colony diameter was measured using the cross method (Yu and Chen, 2018; Zheng et al., 2016), and the inhibition rate was calculated using the following formula:

Inhibition rate (%) = [(Average control colony diameter - Colony diameter of treatment) / (Average control colony diameter - Diameter of inoculum plug)] × 100

Results

2.1 Structure Elucidation

Compound 1 was obtained as a yellow amorphous powder (ethyl acetate), mp 246–268 °C. EI-MS m/z: 336 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.71 (3H, s, H-20), 0.98 (3H, d, J = 6.6 Hz, H-16), 1.15 (3H, s, H-19), 4.50 (1H, br s, H-17), 4.82 (1H, br s, H-17). ^{13}C-NMR (150 MHz, CDCl) : 14.7 (q, C-20), 16.3 (q, C-19), 18.4 (t, C-2), 20.0 (q, C-16), 20.7 (t, C-11), 26.8 (t, C-6), 30.8 (d, C-13), 35.6 (t, C-12), 37.1 (t, C-3), 37.8 (t, C-7), 38.0 (t, C-1), 39.0 (s, C-10), 41.1 (t, C-14), 47.5 (s, C-4), 49.6 (d, C-5), 57.0 (d, C-9), 107.0 (t, C-17), 147.9 (s, C-8), 178.6 (s, C-15), 184.4 (s, C-18). The data were consistent with literature values (Carreras et al., 1998), and compound **1** was identified as pinifolic acid.

Compound 2 was obtained as a white powder (ethyl acetate), mp 216–218 °C. EI-MS m/z: 350 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.98 (3H, d, J = 7.1 Hz, H-16), 1.14 (3H, s, H-19), 2.72 (3H, s, H-20), 3.66 (3H, s, H-21), 4.49 (1H, br s, H-17), 4.82 (1H, br s, H-17). ^{13}C-NMR (150 MHz, CDCl) : 14.7 (q, C-20), 16.6 (q, C-19), 18.5 (t, C-2), 19.9 (q, C-16), 20.8 (t, C-11), 26.8 (t, C-6), 31.0 (d, C-13), 35.8 (t, C-12), 37.0 (t, C-7), 37.9 (t, C-1), 38.1 (t, C-3), 39.1 (s, C-10), 41.6 (t, C-14), 47.8 (s, C-4), 49.9 (d, C-5), 51.9 (q, C-21), 57.1 (d, C-9), 106.9 (t, C-17), 148.0 (s, C-8), 179.35 (s, C-15), 179.4 (s, C-18). The data were consistent with literature values (Zinkel et al., 1985), and compound **2** was identified as monomethyl pinifolate.

Compound 3 was obtained as a white amorphous powder (ethyl acetate), mp 217–219 °C. EI-MS m/z: 364 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.72 (3H, s, H-20), 0.88 (3H, s, H-19), 0.97 (3H, J = 6.3 Hz, H-16), 2.04 (3H, s, H-22), 3.71 (1H, d, J = 10.9 Hz, H-18), 3.82 (1H, d, J = 10.9 Hz, H-18), 4.49 (1H, br s, H-17), 4.82 (1H, br s, H-17). ^{13}C-NMR (150 MHz, CDCl) : 14.9 (q, C-20), 17.5 (q, C-19), 18.5 (t, C-2), 19.9 (q, C-16), 21.0 (q, C-22), 21.1 (t, C-11), 24.3 (t, C-6), 30.9 (d, C-13), 35.8 (t, C-12), 35.9 (t, C-3), 36.9 (s, C-4), 38.0 (t, C-7), 38.5 (t, C-1), 39.6 (s, C-10), 41.4 (t, C-14), 49.5 (d,

C-5), 57.2 (d, C-9), 73.0 (t, C-18), 106.7 (t, C-17), 148.1 (s, C-8), 171.3 (s, C-21), 179.2 (s, C-15). The data were consistent with literature values (Francesca et al., 1999), and compound **3** was identified as 18 -acetoxylabd-8(17)-en-15-oic acid.

Compound 4 was obtained as a yellow amorphous powder (ethyl acetate), mp 224–226 °C. EI-MS m/z: 320 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.71 (3H, s, H-20), 0.98 (3H, d, J = 6.6 Hz, H-16), 1.14 (3H, s, H-19), 4.49 (1H, br s, H-17), 4.83 (1H, br s, H-17), 9.76 (1H, s, H-15). ^{13}C-NMR (150 MHz, CDCl) : 14.7 (q, C-20), 16.4 (q, C-19), 18.4 (t, C-2), 20.18 (t, C-11), 20.2 (q, C-16), 26.8 (t, C-6), 28.9 (d, C-13), 36.0 (t, C-12), 37.1 (t, C-7), 37.15 (t, C-3), 38.0 (t, C-1), 39.0 (s, C-10), 47.5 (s, C-4), 49.6 (d, C-5), 50.9 (t, C-14), 57.1 (d, C-9), 107.0 (t, C-17), 147.9 (s, C-8), 183.9 (s, C-18), 203.1 (d, C-15). The data were consistent with literature values (Zinkel et al., 1985), and compound **4** was identified as 4-eplimbricataloic acid.

Compound 5 was obtained as a yellow amorphous powder (ethyl acetate), mp 209–211 °C. EI-MS m/z: 378 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 1.26 (3H, d, J = 7.1 Hz, H-22), 3.66 (3H, s, H-23), 4.13 (2H, t, J = 7.1 Hz, H-21), 4.49 (1H, br s, H-17), 4.81 (1H, br s, H-17). ^{13}C-NMR (150 MHz, CDCl) : 14.3 (q, C-22), 14.7 (q, C-20), 16.6 (q, C-19), 18.5 (t, C-2), 20.0 (q, C-16), 20.8 (t, C-11), 26.8 (t, C-6), 31.0 (d, C-13), 35.8 (t, C-12), 37.0 (t, C-3), 37.1 (t, C-7), 38.1 (t, C-1), 39.1 (s, C-10), 41.7 (t, C-14), 47.8 (s, C-4), 49.9 (d, C-5), 51.9 (q, C-23), 57.1 (d, C-9), 60.1 (t, C-21), 106.9 (t, C-17), 148.0 (s, C-8), 173.3 (s, C-15), 179.4 (s, C-18). The data were consistent with literature values (Zhang et al., 2006), and compound **5** was identified as 15-ethyl-18-methyl pinifolate.

Compound 6 was obtained as a light yellow amorphous powder (ethyl acetate), mp 233–234 °C. EI-MS m/z: 346 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.70 (3H, s, H-20), 0.97 (3H, d, J = 6.6 Hz, H-16), 1.04 (3H, s, H-19), 4.59 (1H, br s, H-17), 4.86 (1H, br s, H-17), 9.24 (1H, s, H-18). ^{13}C-NMR (150 MHz, CDCl) : 14.2 (q, C-19), 14.7 (q, C-20), 16.5 (q, C-16), 17.7 (t, C-2), 21.1 (q, C-22), 21.1 (t, C-11), 26.7 (t, C-6), 32.3 (t, C-3), 37.5 (t, C-7), 37.8 (t, C-12), 38.2 (t, C-1), 38.4 (s, C-10), 47.6 (d, C-5), 49.9 (s, C-4), 56.2 (d, C-9), 61.4 (t, C-15), 107.4 (t, C-17), 118.2 (d, C-14), 142.6 (s, C-13), 147.2 (s, C-8), 171.1 (s, C-21), 206.4 (d, C-18). The data were consistent with literature values (Zhang et al., 2006), and compound **6** was identified as 15-acetoxy-labda-8(17),13E-dien-18-al.

Compound 7 was obtained as colorless needle crystals (ethyl acetate), mp 105–107 °C. EI-MS m/z: 316 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 1.23 (2 × 3H, d, J = 6.9 Hz, H-16/17), 1.29 (2 × 3H, s, H-19/20), 2.88 (1H, m, H-15), 4.91 (1H, dd, J = 7.5, 2.0 Hz, H-7), 7.10 (1H, dd, J = 8.2, 1.5 Hz, H-12), 7.16 (1H, d, J = 8.2 Hz, H-11), 7.38 (1H, d, J = 1.5 Hz, H-14). ^{13}C-NMR (150 MHz, CDCl) : 15.7 (q, C-19), 17.8 (t, C-2), 23.3 (q, C-16), 23.5 (q, C-17), 24.9 (q, C-20), 32.2 (t, C-6), 33.1 (d, C-15), 35.7 (t, C-3), 37.0 (s, C-10), 37.4 (t, C-1), 42.8 (d, C-5), 46.5 (s, C-4), 70.1 (d, C-7), 123.6 (d, C-11),

124.6 (d, C-12), 125.3 (d, C-14), 136.8 (s, C-8), 146.0 (s, C-13), 146.4 (s, C-9), 183.3 (s, C-18). The data were consistent with literature values (Lu and Hua, 2011), and compound **7** was identified as 7 -hydroxydehydroabietic acid.

Compound 8 was obtained as an amorphous powder (ethyl acetate), mp 107–110 °C. EI-MS m/z: 316 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 1.16 (3H, s, H-20), 1.23 (2 × 3H, d, J = 7.0 Hz, H-16/17), 1.26 (3H, s, H-19), 2.87 (1H, m, H-15), 4.53 (1H, br s, H-7), 7.13 (1H, dd, J = 8.2, 1.8 Hz, H-12), 7.18 (1H, d, J = 1.8 Hz, H-14), 7.19 (1H, d, J = 8.2 Hz, H-11). ^{13}C-NMR (150 MHz, CDCl) : 16.6 (q, C-19), 18.6 (t, C-2), 23.9 (q, C-17), 24.1 (q, C-16), 24.2 (q, C-20), 30.9 (t, C-6), 33.5 (d, C-15), 36.3 (t, C-3), 37.4 (s, C-10), 37.7 (t, C-1), 39.8 (d, C-5), 47.0 (s, C-4), 68.3 (d, C-7), 124.2 (d, C-12), 126.7 (d, C-11), 128.3 (d, C-14), 135.6 (s, C-8), 146.6 (s, C-9), 146.7 (s, C-13), 182.8 (s, C-18). The data were consistent with literature values (Zhang et al., 2006), and compound **8** was identified as 7 -hydroxydehydroabietic acid.

Compound 9 was obtained as a yellow oil. EI-MS m/z: 334 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.58 (3H, s, H-19), 0.99–2.49 (15H, m), 1.04–1.13 (2 × 3H, d, J = 7.0 Hz, H-16/H-17), 1.16 (3H, s, H-18), 2.51 (1H, qd, J = 7.0 Hz, H-15), 4.60 (1H, br s, H-12), 5.84 (1H, s, H-14). ^{13}C-NMR (150 MHz, CDCl) : 15.1 (q, C-16), 16.3 (q, C-17), 17.0 (CH), 20.1 (q, C-20), 20.4 (q, C-19), 21.6 (CH), 25.0 (CH), 31.0 (CH), 32.1 (CH), 36.2 (CH), 36.6 (CH), 36.8 (CH), 43.2 (CH), 48.8 (C), 50.0 (C), 74.6 (s, C-8), 76.9 (d, C-12), 124.6 (d, C-14), 149.1 (s, C-13), 184.2 (s, C-18). The data were consistent with literature values (Ayer and Macaulay, 1987), and compound **9** was identified as endo-peroxide.

Compound 10 was obtained as a yellow oil. EI-MS m/z: 222 [M] , molecular formula C H O. ^{1}H-NMR (600 MHz, CDCl) : 0.77 (3H, d, J = 6.9 Hz, H-14), 0.92 (3H, d, J = 7.2 Hz, H-13), 1.05 (1H, m, H-7), 1.11 (3H, s, H-15), 1.22 (1H, m, H-9), 1.24 (1H, m, H-1), 1.41 (1H, m, H-9), 1.59 (2H, m, H-2), 1.67 (3H, s, H-11), 1.72 (1H, m, H-6), 1.79 (1H, m, H-10), 1.98 (2H, m, H-3), 2.00 (2H, m, H-9), 2.18 (1H, m, H-12), 5.50 (1H, br s, H-5). ^{13}C-NMR (150 MHz, CDCl) : 15.8 (q, C-14), 21.0 (q, C-15), 21.7 (q, C-13), 22.0 (t, C-2), 22.8 (t, C-8), 23.2 (d, C-11), 29.9 (d, C-6), 30.7 (t, C-3), 37.2 (q, C-12), 42.0 (t, C-9), 46.1 (d, C-7), 50.8 (d, C-1), 72.1 (s, C-10), 122.1 (d, C-5), 133.8 (s, C-4). The data were consistent with literature values (Bottini et al., 1987; Tanaka et al., 1997), and compound **10** was identified as -cadinol.

Compound 11 was obtained as white crystals (ethyl acetate), mp 139–142 °C. EI-MS m/z: 414 [M] , molecular formula C H O. ^{1}H-NMR (600 MHz, CDCl) : 0.68 (3H, s, H-18), 0.82 (3H, d, J = 7.0 Hz, H-27), 0.83 (3H, d, J = 7.0 Hz, H-26), 0.84 (3H, d, J = 7.0 Hz, H-29), 0.93 (3H, d, J = 6.8 Hz, H-21), 1.01 (3H, s, H-19), 3.54 (1H, m, H-3), 5.35 (1H, br d, J = 3.6 Hz, H-6). ^{13}C-NMR (150 MHz, CDCl) : 11.9 (q, C-29), 12.0 (q, C-19), 18.8 (q, C-21), 19.0 (q, C-26), 19.4 (q, C-18), 19.8 (t, C-1), 21.1 (t, C-2), 23.1 (q, C-27), 24.3 (d, C-14), 26.1 (t, C-15), 28.2 (t, C-11), 29.2 (t, C-12), 30.5 (t, C-28), 31.7 (t, C-16), 31.9 (t, C-22), 34.0 (d, C-25), 36.2 (d, C-24), 36.5 (d, C-23), 37.3 (d, C-20), 39.8 (d, C-17),

42.3 (s, C-13), 42.32 (s, C-10), 45.8 (t, C-7), 50.1 (d, C-8), 56.1 (d, C-9), 56.8 (t, C-4), 71.8 (d, C-3), 121.7 (d, C-6), 140.8 (s, C-5). The data were consistent with literature values (Kang et al., 2008; Zhang et al., 2009), and compound **11** was identified as -sitosterol.

Compound 12 was obtained as a yellow oil. EI-MS m/z: 278 [M] , molecular formula C H O . ^{1}H-NMR (600 MHz, CDCl) : 0.70 (3H, d, J = 6.8 Hz, H-4), 1.19 (2H, m, H-3), 1.45 (2H, m, H-2), 4.06 (2H, m, H-1), 7.25 (1H, d, J = 7.2 Hz, H-4), 7.47 (1H, d, J = 7.2 Hz, H-3). ^{13}C-NMR (150 MHz, CDCl) : 12.7 (q, C-4), 18.3 (t, C-3), 29.7 (t, C-2), 64.3 (t, C-1), 127.9 (d, C-3), 130.0 (d, C-4), 131.6 (s, C-2), 166.4 (s, C-1). The data were consistent with literature values (Hoang et al., 2008), and compound **12** was identified as dibutyl phthalate.

Compound 13 was obtained as a light yellow oil. EI-MS m/z: 296 [M] , molecular formula C H O. ^{1}H-NMR (600 MHz, CDCl) : 0.84 (3H, d, J = 6.8 Hz, H-19), 0.86 (3H, J = 6.5 Hz, H-18), 0.88 (2 × 3H, d, J = 6.5 Hz, H-16/H-17), 1.25 (8H, m, H-6, 8, 10, 12), 1.14 (2H, m, H-14), 1.28 (4H, m, H-9, 13), 1.37 (2H, m, H-7, 11), 1.39 (2H, m, H-5), 1.53 (1H, m, H-15), 1.67 (3H, s, H-20), 2.00 (2H, m, H-4), 4.16 (2H, m, H-1), 5.41 (1H, m, H-2). ^{13}C-NMR (150 MHz, CDCl) : 16.2 (q, C-16), 19.7 (q, C-17), 19.8 (q, C-18), 22.7 (q, C-19), 23.7 (q, C-20), 24.5 (t, C-9), 24.8 (t, C-13), 25.1 (t, C-5), 28.0 (d, C-15), 32.7 (d, C-7), 32.8 (d, C-11), 36.7 (t, C-6), 37.3 (t, C-12), 37.4 (t, C-10), 37.42 (t, C-8), 39.4 (t, C-14), 39.9 (t, C-4), 59.5 (t, C-1), 123.1 (d, C-2), 140.4 (s, C-3). The data were consistent with literature values (Brown et al., 2003), and compound **13** was identified as 7R,11R-phytol.

Compound 14 was obtained as a white powder (ethyl acetate), mp 75–77 °C. EI-MS m/z: 340 [M] , molecular formula C H O. ^{1}H-NMR (600 MHz, CDCl) : 0.88 (3H, d, J = 6.8 Hz, H-24) [terminal methyl protons], 1.30–1.62 (CH), 3.64 (2H, m, H-1) [oxygen-bearing protons]. ^{13}C-NMR (150 MHz, CDCl) : 14.1 [terminal methyl carbon], 22.7–33.2 (22 × CH), 63.1 [oxygen-bearing carbon]. The data were consistent with literature values (Zhang et al., 2004), and compound **14** was identified as tetracosanol.

Compound 15 was obtained as white crystals (ethyl acetate), mp 74–75 °C. EI-MS m/z: 410 [M] , molecular formula C H O. ^{1}H-NMR (600 MHz, CDCl) : 0.95 (3H, d, J = 6.8 Hz, H-28), 0.98 (3H, d, J = 6.8 Hz, H-1), 1.34 (34H, m, 17 × CH), 1.51 (8H, m, 4 × CH), 1.70 (4H, m, 2 × CH), 2.14 (2H, m, H-8), 2.36 (2H, m, H-6), 3.66 (1H, br m, H-7). ^{13}C-NMR (150 MHz, CDCl) : 14.1 (C-1, C-28), 22.7 (CH), 25.7 (CH), 29.3 (CH), 29.4 (CH), 29.6 (5 × CH), 29.7 (13 × CH), 31.9 (2 × CH), 37.5 (CH), 72.0 (d, C-7). The data were consistent with literature values (Jameel et al., 2014), and compound **15** was identified as N-octacosan-7 -ol.

[Figure 1: see original paper] Structures of compounds 1–10 and 12–15

2.2 Antibacterial Activity Results

The antibacterial activity of the n-hexane extract at various concentrations against *E. coli*, *B. subtilis*, and *S. aureus* is shown in Table 1. The results demonstrated that the n-hexane extract exhibited varying degrees of inhibition against all three pathogens. Within the tested concentration range, the inhibition rates against *E. coli* and *B. subtilis* exceeded 50%. Against *S. aureus*, the inhibition rates were 51% and 69% at concentrations of 50 mg · mL ^{1} and 100 mg · mL ^{1}, respectively, while no antibacterial activity was observed at concentrations of 5–25 mg · mL ^{1}.

Table 1 Antibacterial activity of the n-hexane extract

Concentration (mg · mL ^{1})	Inhibition Rate (%)		
	Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*
5	53	56	None
10	58	60	None
25	63	62	None
50	69	68	51
100	71	70	69

Discussion and Conclusion

Pinus sylvestris is a well-known pine species, and previous research on its needles has primarily focused on GC-MS analysis of volatile oils and essential oils obtained by steam distillation. Pharmacological studies have mainly investigated antitumor (Zhang et al., 2006; Chen et al., 2014), antioxidant (Ka et al., 2005; Jeong et al., 2009), anti-inflammatory (Karonen et al., 2004), antibacterial (Zeng et al., 2009), and antiviral activities (Wei et al., 2008). Apart from the work by Zhang et al. (2006) on the isolation of chemical constituents from pine needles, few studies have reported the separation of individual compounds and their antibacterial effects. In this study, solvent extraction with anhydrous ethanol was used to extract chemical constituents from *Pinus sylvestris* needles, followed by gradient partitioning to obtain n-hexane, ethyl acetate, and n-butanol extracts of different polarities. Column chromatography, thin-layer chromatography, and high-performance liquid chromatography were employed to separate and purify the chemical constituents from the n-hexane extract. The structures of the isolated compounds were elucidated through spectroscopic data analysis using NMR and MS techniques. The antibacterial activity tests revealed that the n-hexane extract exhibited inhibition rates of 53%, 58%, 63%, 69%, and 71% against *E. coli* at concentrations of 5, 10, 25, 50, and 100 mg · mL ^{1}, respectively. Against *B. subtilis*, the inhibition rates were 56%, 60%, 62%, 68%, and 70% at the same concentrations. For *S. aureus*, the inhibition rates were 51% and 69% at concentrations of 50 and 100 mg · mL ^{1}, respectively.

These results demonstrate that the n-hexane extract of *Pinus sylvestris* needles contains a diverse array of chemical constituents, from which 15 compounds were isolated and identified. Four of these compounds were reported from the genus *Pinus* for the first time. The n-hexane extract exhibited dose-dependent antibacterial activity against *E. coli*, *B. subtilis*, and *S. aureus* within certain concentration ranges. These findings provide a theoretical and experimental basis for the further development and utilization of *Pinus sylvestris* needles as a source of botanical antimicrobial agents.

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Note: Figure translations are in progress. See original paper for figures.