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Isolation of antibacterial diterpenoids from *Cryptomeria japonica* bark

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The aims of the present study were to determine the antibacterial activity of bark extract of *Cryptomeria japonica* D. Don and to isolate potential antibacterial constituents. The results showed that the ethanolic extract of *C. japonica* bark possessed a good antibacterial activity. Nine compounds including seven diterpenoids (ferruginol (I), isopimaric acid (II), iguestol (III), isopimarol (IV), phyllocladan-16α-ol (V), sandaracopimarol (VI) and sugiol (VII)) and two steroids (β-sitosterol (VIII) and β-sitostenone (IX)) were isolated from active subfractions; β-sitostenone was isolated for the first time from this plant. Among these compounds, ferruginol possessed the strongest antibacterial activity and had MIC values ranging from 6.3 to 12.5 μg mL\(^{-1}\) against all bacteria tested. Isopimaric acid was also an antibacterial natural product. *Cryptomeria japonica* bark extract and its diterpenoids, ferruginol and isopimaric acid, have the ability to inhibit the bacterial growth and can be used as the source for natural bactericides.

**Keywords:** antibacterial activity; *Cryptomeria japonica*; ferruginol; isopimaric acid; taxodiaceae

1. Introduction

Bacterium is one of the most familiar microbes in our life. Intestinal and stomach diseases caused by bacteria are the most common illnesses, for example cholera and dysentery. However, bacteria resistant to drugs have become significantly more alarming to human beings (Appelbaum, 2006; Rice, 2006). Although vancomycin is regarded as a last-ditch antibiotic, emergences of vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) are other problems beyond methicillin-resistant *S. aureus* (MRSA). Generally, the minimum inhibitory concentration (MIC) of vancomycin against *S. aureus* is less than 4 μg mL\(^{-1}\). MIC values of vancomycin against VISA and VRSA are 4–8 and more than 16 μg mL\(^{-1}\), respectively (Todd, 2006). To resolve the problem of drug resistance, the development of new antibacterial agents from natural products is one of the possible alternatives (Drewes, Mudau, Vuuren, & Viljoen, 2006; Erasto, Moleta, & Majinda, 2004; Jospat, Kiplimo, Karubi, & Hailstorks, 2006; Narayanan, Rao, Shannugam, Gopalakrishnan, & Devi, 2007; Ndi, Semple, Griesser, Pyke, & Barton, 2007; Turker, 2008).

*Corresponding author. Email: chtchang@ntu.edu.tw*
Table 1. Minimum inhibitory concentration values (µg·mL⁻¹) of ethanolic crude extract and four fractions from Cryptomeria japonica bark against bacteria.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>E. faecalis</th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Hex. Fract.</td>
<td>250</td>
<td>250</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>EA Fract.</td>
<td></td>
<td>500</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>BuOH Fract.</td>
<td>1000</td>
<td>-</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>H₂O Fract.</td>
<td>1000</td>
<td>-</td>
<td>500</td>
<td>250</td>
</tr>
</tbody>
</table>

Notes: Hex. Fract.: n-hexane soluble fraction; EA Fract.: ethyl acetate soluble fraction; BuOH Fract.: n-butanol soluble fraction; H₂O Fract.: water soluble fraction.

*aNot active at 1000 µg·mL⁻¹.

Cryptomeria japonica (Linn. f.) D. Don (Taxodiaceae), Japanese cedar, is a conifer tree native to Japan and distributed in East Asia. It is traditional and popular for wood construction and furniture materials. Recently, researchers have reported that it owns various bioactivities such as mosquito larvicidal, antimitic, anti-termitic, antimicrobial, anti-silverfish, cytotoxic activities, and as angiotensin converting enzyme inhibitor to relax blood vessels (Arihara et al., 2004; Cha et al., 2007; Cheng, H.T. Chang, S.T. Chang, Tsai, & Chen, 2005; Cheng, Lin, & Chang, 2005; Cheng, H.T. Chang, Wu, & S.T. Chang, 2007; Kofujita, Ota, Takahashi, & Kawai, 2002; Matsushita, Hwang, Sugamoto, & Matsu, 2006; Tsutsumi, Shimada, Miyano, Nishida, & Mitsunaga, 1998; Wang et al., 2006; Yoshikawa, Tanaka, Umeyama, & Arihara, 2006). The objectives of this study are to evaluate antibacterial activities of the ethanolic extract from C. japonica bark against four Gram-positive bacteria and to isolate antibacterial compounds.

2. Results and discussion

2.1. Antibacterial activities of extract and its fractions

Bactericidal effects of the crude extract and the four fractions against the bacteria examined are shown in Table 1. MIC values of crude extract against Enterococcus faecalis, S. aureus, S. epidermidis and MRSA were all 250 µg·mL⁻¹. Among the four fractions, n-hexane soluble fraction was the most active and MIC values of n-hexane soluble fraction ranged from 250 to 500 µg·mL⁻¹. The weaker activities were obtained with the n-butanol and water soluble fractions.

Ajali (2000) evaluated antibacterial activity of several solvent extracts from the bark of Enantia polycarpa against S. aureus: only methanolic extract possessed activity at an MIC value of 3200 µg·mL⁻¹. The antibacterial activity of Mexican traditional medicinal plants has been studied by Rojas, Levaro, Tortoriello, and Navarro (2001): the extracts from Gnaphalium oxyphyllum, Gnaphalium americanum and Crescentia alata exhibited antimicrobial activity against E. faecalis at a concentration of 5000 µg·mL⁻¹. Djipa, Delmee, and Quetin-Leclercq (2000) demonstrated the aqueous extract and acetone extract from the bark of Syzygium jambos against several species of S. aureus, and their MIC values ranged from 500 to 750 µg·mL⁻¹. Compared with such results, the ethanolic extract from C. japonica bark showed better growth

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inhibitory effectiveness against tested bacteria strains. It could be strongly conjectured that the components of the plant play an important role in affecting antibacterial activity.

n-Hexane soluble fraction was further separated by column chromatography into 16 subfractions (B1–B16). Antibacterial activities of the 16 subfractions were investigated against tested bacteria. MIC values of subfractions are summarised in Table 2. Three subfractions, B4, B7 and B15, possessed strong antibacterial activities. The other subfractions showed no or very weak antibacterial activities and this data is not presented in Table 2. MIC values of subfraction B4 were all 250 μg mL⁻¹ against the tested Gram-positive bacteria. Activities of two subfractions B7 and B15 were weaker than that of B4 and showed no activities against S. epidermidis. Subfraction B4 exhibited the highest antibacterial activity.

2.2. Antibacterial activities of compounds

Normal phase high performance liquid chromatography (HPLC) was utilised to obtain antibacterial compounds from bioactive subfractions (B4, B7 and B15). Nine compounds including seven diterpenoids (ferruginol (I), isopimaric acid (II), iguestol (III), isopimarol (IV), phyllocladan-16α-ol (V), sandaracopimarinol (VI) and sugiol (VII)) and two steroids (β-sitosterol (VIII) and β-sitostenone (IX)) were isolated from active subfractions of n-hexane soluble fraction and identified by spectral analyses. Iguestol (III), phyllocladan-16α-ol (V) and β-sitostenone (IX) are identified for the first time from the bark of C. japonica and reported here; and furthermore β-sitostenone (IX) is isolated from this plant for the first time.

The antibacterial activities of the isolated compounds are shown in Table 3. Six compounds exhibited excellent antibacterial activities, their abilities in decreasing order were as follows: ferruginol (I) > isopimaric acid (II) > sugiol (VII) > sandaracopimarinol (VI) > iguestol (III) > isopimarol (IV). Ferruginol (I) exhibited the best ability against four tested bacteria strains and MIC values were ranging from 6.3 to 12.5 μg mL⁻¹. MIC values of isopimaric acid (II) were less than 50 μg mL⁻¹ against bacteria tested, except for MRSA. Sandaracopimarinol (VI) and sugiol (VII) also possessed antibacterial activities and MIC values were less than 32 μg mL⁻¹ against E. faecalis; iguestol (III) and isopimarol (IV) are weaker against the tested bacteria. Matsushita, Hwang, Sugamoto, and Matsu (2006) reported that ferruginol possessed strong inhibitory activity against phytopathogenic bacterium, Ralstonia solanacearum. Based on these results, ferruginol presented a broad antimicrobial spectrum.
Table 3. MIC values (μg ml⁻¹) of compounds against bacteria.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>E. faecalis</th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferruginol</td>
<td>6.3</td>
<td>6.3</td>
<td>12.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Iguestol</td>
<td>250</td>
<td>1000</td>
<td>250</td>
<td>–</td>
</tr>
<tr>
<td>Isopimaric acid</td>
<td>15.6</td>
<td>15.6</td>
<td>31.3</td>
<td>500</td>
</tr>
<tr>
<td>Isopimarol</td>
<td>– a</td>
<td>250</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sandaracopimarinol</td>
<td>31.3</td>
<td>31.3</td>
<td>1000</td>
<td>62.5</td>
</tr>
<tr>
<td>Sugiol</td>
<td>31.3</td>
<td>125</td>
<td>31.3</td>
<td>31.3</td>
</tr>
<tr>
<td>Cefotaxime b</td>
<td>64</td>
<td>2</td>
<td>No test</td>
<td>No test</td>
</tr>
<tr>
<td>Gentamicin b</td>
<td>16</td>
<td>1</td>
<td>No test</td>
<td>No test</td>
</tr>
</tbody>
</table>

Note: a Not active at 1000 μg mL⁻¹; b Positive control.
As for the effects of the structure of the compound on its antibacterial activity, Evans, Furneaux, Gravestock, Lynch, and Scott (1999) have reported that totarol derivatives with phenolic and isopropyl groups possess excellent activities against Gram-positive bacteria. Kobayashi, Nishino, Fukushima, Shiobara, and Kodama (1988) examined antibacterial activity of pisiferic acid and its derivatives including ferruginol, the hydroxyl group at C12 has been estimated to play an important role against Gram-positive bacteria. Those were the reasons that ferruginol and sugiol with both phenolic and isopropyl groups possessed the antibacterial activity. Ferruginol also exhibited the free radical scavenging activity (Wang, Wu, Shyur, Kuo, & Chang, 2002). The relationship between the mechanism and the structures of isolated compounds should be further investigated in the future.

The antibacterial properties of the ethanolic extract and its constituents from *C. japonica* bark were assessed by the present study. The *n*-hexane soluble fraction and its active constituents, ferruginol and isopimaric acid, from *C. japonica* bark are of great potential to be used as a source for natural bactericides and are worth further investigations and utilisation.

3. Experimental

3.1. Plant materials

The bark of *C. japonica* D. Don was collected from the Experimental Forest of National Taiwan University (located in Nan-Tou, Taiwan), aged 43 years. The species was identified, and a voucher specimen was deposited in the School of Forestry and Resource Conservation, National Taiwan University.

3.2. Extraction and isolation

Barks were extracted twice with ethanol at ambient temperature, then the crude extract was further separated into *n*-hexane, ethyl acetate, *n*-butanol and water soluble fractions by liquid–liquid partition. Antibacterial activities of the four fractions were evaluated to screen the active one for further separation. The active fraction, *n*-hexane soluble fraction, was coated onto silica gel (Silica-60, Merck 7734); the eluted solvents were *n*-hexane, ethyl acetate, acetone and ethanol with different proportions. *n*-Hexane soluble fraction was separated into 16 subfractions by open column chromatography (CC) and thin layer chromatography (TLC). To obtain pure compounds, preparative HPLC was used to isolate the compounds from the active subfractions based on an antibacterial activity-guided fractionation procedure.

3.3. Spectral analyses

Structures of compounds were identified by spectroscopic methods including NMR (Nuclear magnetic resonance spectroscopy, 1D NMR and 2D NMR, Bruker Avance 500 MHz), FTIR (Fourier transform infrared spectroscopy, Bio-rad FTS-40), UV/VIS (Ultraviolet and visible absorption spectroscopy, Jasco V-550) and MS (Mass spectroscopy, Finnigan MAT-958).
3.4. Identification of compounds

Compound I (ferruginol). Yellowish oil, C_{20}H_{30}O; EIMS, m/z 286 (20), 272 (60), 257 (76), 229 (66), 105 (74), 59 (100); UV_{max}: 215 and 287 nm; IR (KBr) \nu_{max} cm^{-1}: 3429 (OH), 2926 (C–H), 2867 (C–H), 1623 (C=C) and 1460 (CH_2). \textsuperscript{1}H NMR (\delta_H, CDCl_3, 500 MHz): 6.82 (1H, s, H-14), 6.62 (1H, s, H-10), 4.56 (1H, s), 3.11 (1H, sept, J = 7.0 Hz, H-15), 2.84 (1H, ddd, J = 16.7, 6.5, 2 Hz, H-7a), 2.77 (1H, ddd, J = 16.8, 11.4, 7.5 Hz, H-7b), 1.23 (3H, d, J = 7.0 Hz, H-17), 1.20 (3H, d, J = 7.0 Hz, H-16), 1.16 (3H, s, H-20), 0.93 (3H, s, H-19), 0.93 (3H, s, H-18). \textsuperscript{13}C NMR (\delta_C, CDCl_3, 125 MHz): 19.21 (C-6), 21.60 (C-19), 22.55 (C-16), 22.73 (C-17), 24.77 (C-20), 26.78 (C-15), 29.74 (C-7), 33.29 (C-18), 33.42 (C-4), 37.49 (C-10), 38.84 (C-1), 41.67 (C-3), 50.33 (C-5), 110.95 (C-11), 126.59 (C-14), 127.60 (C-8), 131.38 (C-13), 148.64 (C-9), 150.66 (C-12).

Compound II (isopimaric acid). Colourless solid, C_{20}H_{30}O_2; EIMS, m/z 302 (100), 287 (80), 241 (75), 119 (76), 105 (84); UV_{max}: 217 nm; IR (KBr) \nu_{max} cm^{-1}: 3434 (OH), 2925 (C–H), 1640 (C=C) and 1383 (CH_3). \textsuperscript{1}H NMR (\delta_H, CDCl_3, 500 MHz): 5.78 (1H, dd, J = 17.5, 10.8 Hz, H-15), 5.30 (1H, br d, J = 5.2 Hz, H-7a), 4.90 (1H, dd, J = 17.6, 1.2 Hz, H-16b), 4.84 (1H, dd, J = 10.7, 1.2 Hz, H-16a), 1.25 (3H, s, H-19), 0.89 (3H, s, H-17), 0.84 (3H, s, H-20). \textsuperscript{13}C NMR (\delta_C, CDCl_3, 125 MHz): 15.28 (C-20), 17.13 (C-19), 17.93 (C-2), 20.01 (C-11), 21.48 (C-17), 25.17 (C-6), 35.00 (C-10), 36.06 (C-12), 36.81 (C-13), 36.99 (C-3), 38.79 (C-1), 45.01 (C-5), 46.06 (C-14), 46.27 (C-4), 51.99 (C-9), 109.25 (C-16), 120.95 (C-7), 135.66 (C-8), 150.31 (C-15), 184.69 (C-18).

Compound III (iguestol). Colourless crystal, C_{21}H_{32}O_2; EIMS, m/z 332 (100), 317 (27), 299 (91), 267 (62), 229 (52); UV_{max}: 222 and 284 nm; IR (KBr) \nu_{max} cm^{-1}: 3394 (OH), 2960 (C–H), 1644 (C=C) and 1373 (CH_3). \textsuperscript{1}H NMR (\delta_H, CDCl_3, 500 MHz): 6.45 (1H, s, H-14), 6.00 (1H, s, H-11), 4.22 (1H, ddd, J = 9, 7.3, 5.7 Hz, H-6), 3.15 (1H, sept, J = 7.0 Hz, H-15), 1.33 (3H, s, H-20), 1.20 (3H, d, J = 6.4 Hz, H-17), 1.18 (3H, d, J = 6.4 Hz, H-16), 1.16 (3H, s, H-18), 1.13 (3H, s, H-19). \textsuperscript{13}C NMR (\delta_C, CDCl_3, 125 MHz): 19.15 (C-2), 21.31 (C-19), 23.01 (C-20), 23.65 (C-16), 26.38 (C-17), 28.6 (C-4), 35.76 (C-18), 37.77 (C-1), 41.63 (C-10), 42.22 (C-7), 43.6 (C-3), 58.24 (C-5), 68.58 (C-6), 117.40 (C-14), 131.17 (C-9), 131.94 (C-8), 138.12 (C-13), 143.08 (C-12), 146.10 (C-11).

Compound IV (isopimarol). Colourless solid, C_{20}H_{32}O; EIMS, m/z 288 (49), 273 (27), 257 (100), 187 (24), 109 (43), 105 (40); UV_{max}: 218 nm; IR (KBr) \nu_{max} cm^{-1}: 3430 (OH), 2924 (C–H), 2853 (C–H), 1645 (C=C) and 1458 (CH_2). \textsuperscript{1}H NMR (\delta_H, CDCl_3, 500 MHz): 5.80 (1H, dd, J = 17.5, 10.8 Hz, H-15), 5.33 (2H, br s, H-7), 4.83 (1H, dd, J = 10.8, 1.3 Hz, H-16a), 4.85 (1H, dd, J = 17.6, 1.3 Hz, H-16b), 3.35 (1H, d, J = 10.9 Hz, H-18a), 3.11 (1H, d, J = 10.9 Hz, H-18b), 0.89 (3H, s, H-17), 0.88 (3H, s, H-20), 0.85 (3H, s, H-19). \textsuperscript{13}C NMR (\delta_C, CDCl_3, 125 MHz): 15.8 (C-20), 18.11 (C-19), 18.13 (C-2), 20.20 (C-11), 21.48 (C-17), 23.31 (C-6), 35.19 (C-10), 35.57 (C-3), 36.17 (C-12), 36.85 (C-13), 37.38 (C-4), 39.40 (C-1), 43.70 (C-5), 46.14 (C-14), 51.81 (C-9), 72.27 (C-18), 109.17 (C-16), 121.21 (C-7), 135.66 (C-8), 150.42 (C-15).

Compound V (phyllocladan-16\alpha-ol). White solid, C_{20}H_{34}O; EIMS, m/z 290 (17), 272 (29), 257 (24), 232 (100), 123 (48); UV_{max}: 214 nm; IR (KBr) \nu_{max} cm^{-1}: 3423 (OH) 2927 (C–H), 2864 (C–H), 1456 (CH_2) and 1369 (CH_3). \textsuperscript{1}H NMR (\delta_H, CDCl_3, 500 MHz): 1.31 (3H, s, H-17), 0.85 (3H, s, H-20), 0.82 (3H, s, H-18), 0.76 (3H, s, H-19). \textsuperscript{13}C NMR (\delta_C, CDCl_3, 125 MHz): 14.81 (C-20), 18.33 (C-2), 18.95 (C-11), 20.28 (C-6), 21.94 (C-19), 23.91 (C-17),
Compound VI (sandaracopimarinol). Colourless solid, C_{20}H_{32}O; EIMS, m/z 288 (15%), 273 (10), 257 (100), 135 (20), 121 (24), 93 (23); UV_{max}: 218 nm; IR (KBr) ν_{max} cm^{-1}: 3423 (OH), 2925 (C–H), 2866 (C–H) and 1663 (C=O). \(^1H\) NMR (δ_H, CDCl₃, 500 MHz): 5.75 (1H, dd, J = 17.5, 10.6 Hz, H-15), 5.19 (2H, s, H-14), 4.88 (1H, dd, J = 17.5, 1.3 Hz, H-16a), 4.85 (1H, dd, J = 12.0, 1.3 Hz, H-16b), 3.37 (1H, d, J = 10.9 Hz, H-18a), 3.10 (1H, d, J = 10.9 Hz, H-18b), 1.02 (3H, s, H-17), 0.82 (3H, s, H-20), 0.78 (3H, s, H-19). \(^13\)C NMR (δ_c, CDCl₃, 125 MHz): 15.58 (C-20), 17.92 (C-19), 18.32 (C-2), 18.78 (C-11), 22.38 (C-6), 25.94 (C-17), 34.54 (C-12), 35.44 (C-7), 35.72 (C-3), 37.37 (C-4), 37.76 (C-13), 38.12 (C-10), 38.88 (C-1), 47.87 (C-5), 50.53 (C-9), 72.23 (C-18), 109.66 (C-16), 128.68 (C-14), 136.98 (C-8), 149.09 (C-15).

Compound VII (sugiol). Colourless solid, C_{20}H_{28}O₂; EIMS, m/z 300 (71), 285 (100), 243 (26), 217 (27), 203 (23); UV_{max}: 216, 244 and 295 nm; IR (KBr) ν_{max} cm^{-1}: 3362 (OH), 2921 (C–H), 2850 (C–H), 1644 (C=O) and 1377 (CH₃). \(^1H\) NMR (δ_H, CDCl₃, 500 MHz): 10.25 (1H, s), 7.64 (1H, s, H-14), 6.77 (1H, s, H-11), 3.12 (1H, sept, J = 7 Hz, H-15), 1.16 (3H, d, J = 6.9 Hz, H-16), 1.12 (3H, s, H-20), 1.11 (3H, d, J = 6.9 Hz, H-17), 0.93 (3H, s, H-19), 0.87 (3H, s, H-18). \(^13\)C NMR (δ_c, CDCl₃, 125 MHz): 18.45 (C-2), 21.11 (C-19), 22.18 (C-16), 22.35 (C-17), 23.01 (C-20), 26.02 (C-15), 32.27 (C-4), 35.49 (C-6), 37.43 (C-1), 40.09 (C-10), 40.81 (C-3), 49.56 (C-5), 109.29 (C-11), 122.54 (C-8), 124.97 (C-14), 132.48 (C-13), 155.81 (C-9), 160.07 (C-12), 196.50 (C-7).

Compound VIII (β-sitosterol). White solid, C_{29}H_{50}O; EIMS, m/z 432 (%), 414 (100), 396 (67), 381 (49), 329 (46), 213 (36); UV_{max}: 217 nm; IR (KBr) ν_{max} cm^{-1}: 3362 (OH) 2921 (C–H), 2850 (C–H), 1644 (C=O) and 1377 (CH₃). \(^1H\) NMR (δ_H, CDCl₃, 500 MHz): 5.33 (1H, br d, J = 5.2 Hz, H-6), 3.50 (1H, m, H-3), 2.27 (2H, m), 0.98 (3H, s, H-19), 0.90 (3H, d, J = 6.6 Hz, H-21), 0.84 (3H, t, J = 7.5 Hz, H-29), 0.80 (3H, d, J = 6.9 Hz, H-27), 0.78 (3H, d, J = 6.9 Hz, H-26), 0.65 (3H, s, H-18). \(^13\)C NMR (δ_c, CDCl₃, 125 MHz): 11.85 (C-19), 11.97 (C-18), 18.79 (C-26), 19.02 (C-21), 19.39 (C-19), 19.81 (C-27), 21.07 (C-11), 23.06 (C-28), 24.29 (C-25), 26.06 (C-16), 29.14 (C-23), 31.65 (C-2), 31.90 (C-7), 31.90 (C-8), 33.93 (C-22), 36.14 (C-20), 36.49 (C-10), 37.24 (C-1), 39.76 (C-12), 42.29 (C-4), 42.31 (C-13), 45.82 (C-24), 50.12 (C-9), 56.05 (C-17), 56.76 (C-14), 71.80 (C-3), 121.71 (C-6), 140.75 (C-5).

Compound IX (β-sitostenone). White solid, C_{29}H_{48}O; EIMS, m/z 412 (100), 370 (54), 289 (60), 229 (70), 124 (85); UV_{max}: 245 nm; IR (KBr) ν_{max} cm^{-1}: 3230 (OH), 2930 (C–H), 2852 (C–H), 1657 (C=O) and 1465 (CH₂). \(^1H\) NMR (δ_H, CDCl₃, 500 MHz): 5.70 (1H, s, H-4), 1.16 (3H, s, H-19), 0.90 (3H, d, J = 6.5 Hz, H-21), 0.83 (3H, d, J = 7.1 Hz, H-29), 0.82 (3H, d, J = 6.8 Hz, H-26), 0.79 (3H, d, J = 6.8 Hz, H-27), 0.68 (3H, s, H-18). \(^13\)C NMR (δ_c, CDCl₃, 125 MHz): 12.0 (C-18), 12.0 (C-29), 17.34 (C-26), 18.68 (C-21), 19.01 (C-19), 19.80 (C-27), 21.01 (C-11), 23.05 (C-28), 24.17 (C-15), 26.05 (C-25), 28.18 (C-16), 29.13 (C-23), 32.04 (C-2), 32.94 (C-6), 33.87 (C-7), 33.96 (C-22), 35.61 (C-1), 35.70 (C-8), 36.10 (C-20), 38.59 (C-10), 39.61 (C-12), 42.37 (C-13), 45.81 (C-24), 53.80 (C-9), 55.86 (C-17), 56.0 (C-14), 123.71 (C-4), 171.78 (C-5), 199.71 (C-3).
3.5. Bacteria strains
Four strains of Gram-positive bacteria: *E. faecalis* ATCC 29212; *S. aureus* ATCC 29213; *S. epidermidis* 2001 D44; and methicillin-resistant *S. aureus* (MRSA) 2001 D40 from National Taiwan University Hospital (Taipei, Taiwan) were used.

3.6. Antibacterial assay
The antibacterial activities of specimens were tested at each isolation stage by the standard broth dilution method described in the Manual of Clinical Microbiology (Jones, Barry, & Gavan, 1985). MHB (Mueller Hilton broth) powder was added into distilled water (22 g L$^{-1}$), after sterilising, mixed with the sample matched at specific concentrations (micrograms per millilitre). The culture of bacteria strains was added to each well to give a final colony of $5 \times 10^5$ CFU mL$^{-1}$. The microplate was incubated at 37°C overnight. MIC value was defined as the minimum inhibitory concentration of tested samples that completely inhibited the growth of the bacteria. Three replicates were carried out in each case. Two antibiotics, cefotaxime and gentamicin, were used as positive controls; MIC values of cefotaxime and gentamicin were in the standard range.

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References


