Bioactive Compounds Isolated from Indonesia Epiphytic Plant of Sarang Semut and Their Antibacterial Activity against Pathogenic Oral Bacteria

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A new phenolic compound (1), a steroid (2), a new steroid glycoside (4), triterpenoids (3 & 6) and a new sesquiterpenoid (5) has been isolated from the ethyl acetate extract of Sarang Semut (Myrmecodia pendants). The structures of the new and known compounds were established on the basis of extensive IR, NMR and Masssa spectral data. The bioactivity evaluation was conducted using the inhibition zone of compounds (mm) using Kirby-Bauer method at concentrations of 1000 and 5000 ppm for compound 1 against pathogenic oral bacteria Enterococcus faecalis, 8.55 and 8.05 mm, respectively. Compound 2-3 against Streptococcus mutans were 9.00 and 8.45 mm (2) 10.24 and 9.35 mm (3), respectively. Compound 5 against Porphyromonas gingivalis were 11.5 and 10.8 mm, respectively.

Keywords: Sarang Semut; antibacterial activity; Enterococcus faecalis; Streptococcus mutans; Porphyromonas gingivalis

Today the herbal or natural product have become more popular due to their high antibacterial activity, biocompatibility, anti-inflammatory and anti-oxidants properties [1]. Herbal remedies have a long history of use and tooth problems. In many traditional cultures, the use of herbal “chewing sticks” taken from plants, shrubs or trees with high antimicrobial activity are common [2]. Herbs may be good alternatives to current treatments for oral health problems but there is lack of information about the effect of herb in oral tissues, mechanism of effect, and side effects [2]. So the more research is required to explore these traditional medicines.

Sarang Semut (Myrmecodia pendants) is widely used in West Papua as herb with broad range of therapeutic values [3]. This plant is a member of Rubiaceae family with five Genus, however, only two of which have association with ants. They are Myrmecodia (forty five species) and Hypnophytum (twenty six species). From those species, only Hypnophytum formicarum, Myrmecodia pendants and Myrmecodia tuberosa are considered to have medicinal values [4]. Research data from previous analysis result of crude extract of Sarang Semut showed that the extract has antioxidant activity [5-6]. Ethyl acetate fraction of M. pendants (50 µg mL-1) showed the highest activity in lymphocytes proliferation thus sarang semut tubers are potential to be development as immunomodulatory agents [3]. Previous study by Soekmanto et al. (2010) have proved that the extract of M. pendants has anticancer activity in both human cervix (HeLa) and canine mammary tumor (MCM) cell lines with IC50 27.61 ppm (HeLa) and 54.57 ppm (MCM-B2), respectively [4]. This effect may be the result of phenolic compounds especially flavonoids contained in extract [4-7]. Triterpenoids have been isolated from Sarang Semut of Papua has capability to inhibit the growth of ovarian cancer cell lines (SKOV-3) with IC50 of 481µg mL-1 for 48 hours [8]. However, the antibacterial activity of the Sarang Semut and its active components against oral periodontal pathogens has not been evaluated. Thus, this study mainly aimed to isolate and elucidate antibacterial agents from the Sarang Semut against periodontal oral pathogen. Our chemical investigation isolated one new phenolic compound (1), a steroid (2), a new steroid glycoside (4) and two triterpenoids (3 and 6) and new sesquiterpenoid (5) from the Sarang Semut. Their structures were elucidated as dibenzo-p-dioxin-2,8-dicarboxylic acid (1), xxxxx (2), xxxx (3), 6'-O-tridecanoyl-3-O-β-D-glucosyl-sitosterol (4), phloroglucinol coupled sesquiterpene (5) and betulin (6) (Fig 1.) by spectroscopic data analyses (IR, ES-MS, 1D-NMR, and 2D-NMR). This report describes their isolation, structural elucidation, and antibacterial activity against Enterococcus faecalis 29212, Streptococcus mutans 25175 and Porphyromonas gingivalis ATCC 33277.

The ethyl acetate of Sarang Semut was subjected to multiple chromatographic steps, using silica gel G60 and ODS RP-18 to afford terpenoid (1-6) (Figure 1).

Compound 1 was obtained as a white crystal. Its molecular formula was C24H32O3, based on the [M+H]+ ion peak in mass spectrometry (m/z 272.5866). Hydroxyl group of compound 1 was showed peak at 3247.8 cm⁻¹ in IR spectrum. Based on the 13C-NMR spectrum, all carbons of compound 1 are seven carbon signals (δc 170.4, 151.6, 146.1, and 123.3). Carboxyl group was showed at δc 170.4 (13C-NMR) and peak at 1673.3 cm⁻¹ (IR spectrum). DEPT data showed three methyne (δc 123.9, 117.8 and 115.8) and four quaternary carbons (δc 170.4, 151.6, 146.1 and 146.1) but HMBC data showed each signal of methyne carbons correlated to two protons. So that, all signal carbons of compound 1 is double (contain two carbons). Because it, compound 1 deduced was arranged by two same of fragment structure. The 1H nuclear magnetic resonance (NMR) spectrum of compound 1 (in CD3OD) showed resonances of three aromatic protons at δH 7.44 (d, J = 1.95 Hz, H-4), 7.42 (d, J = 1.95 Hz, H-6), and 6.80 (dd, J = 7.8 Hz, H-5), forming an ABX spin system [9]. One subtituent group is carboxylic group that correlation with carbons at δc 123.3, and the other substituent group is oxygen that showed deshielded at δc 151.6 and 146.1 (oxyegenated carbon sp²). Analysis of spectrum HMBC creates fragment 3,4-dihydroxi
benzoic acid, and after compared the reference, we found chemical shift carbon and proton of compound 1 the same as 3,4-di hydroxy benzoic [10-12]. Based on UV, IR and NMR MS data, compound 1 is a dimer of 3,4-dihydroxy benzoic acid that was called dibenzo-p-dioxin-2,8-dicarboxylic acid.

Compound 4 was isolated as white amorphous powder. TLC analysis on Kiesel gel 60 F254 0.25 mm plate (Merck) in n-hexane-ethyl acetate = 40:60 (v/v), Rf = 0.25; ES-MS m/z 773.340 [M+H]+ calcd. for C43H60O2, together with seven degrees of unsaturation. The IR spectrum showed hydroxyl groups at 3404 cm\(^{-1}\), a carbon yl ester (1722 cm\(^{-1}\)) and gem-dimethyl (1465, 1379 cm\(^{-1}\)). The \(^1H\)- and \(^13C\)-NMR spectral data are shown in Table 1. The \(^13C\)-NMR spectrum showed forty eight carbon signals which, according to the DEPT 135\(^o\) spectrum, represented seven primary, twenty three secondary, fourteen tertiary, and four quaternary carbons. In the \(^13C\)-NMR spectrum showed twenty nine resonances attributed to a sitosterol skeleton, including double bond resonances at \(\delta\) 140.4 and 122.4, and a hydroxymethylene signal at \(\delta\) 79.7. Six carbon signals \([\delta\ 101.4, 76.1, 135.3, 163.4, 13\text{and } H\text{]}\) were due to \(\delta\)-glycose, and others \([\delta\ 175.0, 34.4, 32.1, 29.9-29.4, 25.1, 22.9, 14.3]\) attributed to fatty acid esters. The \(^1H\)-NMR spectrum shows signals for seven tertiary methyl groups at \(\delta\ 0.67 (3H, s)\), 0.80 (d, 3H, J = 6.5), 0.81 (d, 3H, J = 6.45), 0.84 (t, 3H, J = 1.3), 0.88 (m, 3H), 0.91 (d, 3H, J = 6.5), 1.00 (s, 3H), and an anomeric proton signal of \(\delta\)-glycose at \(\delta\ 4.37\) (d, 1H, J = 7.8), which indicated the \(\beta\)-linkage of \(\delta\)-glycose with sitosterol [13,14]. The \(^1H\)-NMR spectrum also exhibited the presence of a long chain fatty acid ester, according to signals at \(\delta\ 1.47\) (m, 2H) and a secondary methyl group at \(\delta\ 1.25\) (m, 2H), respectively, and also allowed the assignment of three hydroxyls at C-1’, C-3’ and C-5’. Consequently, the 2-methylenoyl substituent could only be placed at C-2’. Thus, a gravidol was established based on this analysis data above, which was also supported by comparison with the NMR data of grandinol [16]. The connections of the two structural fragments, quaternary carbons, and the other functional groups were mainly achieved by the HMBC spectrum. The correlations of H-14 with C-1, C-2 and C-6 suggested attachment of Me-14 to C-1. An isopropenyl group was attached to C-4 by the HMBC correlations of H-12 with C-4, C-11 and C-13 and H-13 with C-4, C-11 and C-12. Based on analysis of NMR data as well as by comparison with previously reported papers, the structure of compound 5 was therefore elucidated as phloroglucinol sesquiterpenoid (5). This is a new sesquiterpenoid reported for the first time from this plant.

### Table 1: NMR data (500 MHz for \(^1H\) and 125 MHz for \(^13C\), in CDCl\(_3\) for 4)

<table>
<thead>
<tr>
<th>Position</th>
<th>(^1H) NMR. (s, integral, m., J/Hz)</th>
<th>(^13C) NMR. (s, integral, m.)</th>
</tr>
</thead>
</table>

*The mass spectrum (ES-MS) was measured by using an Agilent 1100 LC/MSD trap TOF-MS equipped with a QuatPac C18 column*.

All these data suggested that compound 4 was a steroid glycoside with a long-chain fatty acid ester (Fig. 1). The complete structural determination was further achieved by analyses of HMOC and HMBC data. In the HMBC spectrum, the framework of \(\delta\)-glucose unit was also exhibited. The glucose unit was assigned to C-3 of the skeleton of sitosterol, according to the correlation from H-1' of \(\delta\)-glucose (\(\delta\) 4.37) to C-3 (\(\delta\) 79.7). A comparison of the NMR data of 4 with those of 4'-O-docosylamido-3-O-\(\beta\)-h-glucosyl-sitosterol [15] of revealed that the structures of the two compounds are closely related, the main differences are the position of fatty acid ester and number of fatty acid unit. In order to clarify the position of fatty acid ester unit, the HMBC experiment was carried out, proton at \(\delta\) 4.9 (2H, dd, 4.55, 4.55) was correlated to C-1' (\(\delta\) 175.0) confirmed the ester group attaching to C-6 of \(\delta\)-glycose (Fig. 1).

### Compounds

- **Compound 5** was isolated as pale brown oil. TLC analysis on Kiesel gel 60 F254 0.25 mm plate (Merck) in n-hexane-ethyl acetate = 80:20 (v/v), Rf 0.37; IR (KBr) \(\nu_{max}\) cm\(^{-1}\): 3415, 2973, 1628, 1451, 1381 and 1161; ES-MS m/z 447.700 [M+H]+ calcd. for C26H39O4; The IR spectrum of 5 indicated the presence of carbonyl at 1628 cm\(^{-1}\). From an inspection of 1D-NMR data and the HMOC spectrum, 5 was found to possess a 2-methylenaloyl side chain \([\delta\ 4.5\ (1H, m), 1.12\ (6H, m); \delta\ 166.5, 77.3, 29.4, 28.9, 8.0]\), a phloroglucinol unit \([\delta\ 5.8\ (1H, s); \delta\ 163.5, 164.3, 164.2, 109.0, 101.64, 95.0]\), one methylene \([\delta\ 2.62\ (1H, m), 2.56\ (1H, m); \delta\ 28.0]\),three tertiary methyl \([\delta\ 1.12\ (3H, s), 1.37\ (3H, J = 6.5), 1.31\ (3H, s); \delta\ 9.86, 16.8, 18.5]\), a terminal double bond \([\delta\ 4.50\ (1H, m), 4.60\ (1H, m); \delta\ 118.8, 149.5]\) as well as an oxygenated carbon at \(\delta\ 71.5\) The data suggested a phloroglucinol-coupled sesquiterpenoid for compound 5. The above accounted for seven out of eight double bond equivalents, which indicated the presence of one ring in compound 5. In the HMBC spectrum presence correlation of H-6" (\(\delta\ 5.8\)) with C-2' (\(\delta\ 101.6\)), C-4' (\(\delta\ 109.3\)) and C-5' (\(\delta\ 162.1\)) as well as H-10 (\(\delta\ 2.5\)) with C-3' (\(\delta\ 163.4\)), C-4' (\(\delta\ 109.3\)) and C-5' (\(\delta\ 162.1\)) located methyln at ring aromatic and the C-10 methylene at positions 6' and 4', respectively, as well as assigned the position of three hydroxyls at C-1', C-3' and C-5'. Consequently, the 2-methylenoyl substituent could only be placed at C-2'. Thus, a gravidol was established based on this analysis data above, which was also supported by comparison with the NMR data of grandinol [16]. The connections of the two structural fragments, quaternary carbons, and the other functional groups were mainly achieved by the HMBC spectrum. The correlations of H-14 with C-1, C-2 and C-6 suggested attachment of Me-14 to C-1. An isopropenyl group was attached to C-4 by the HMBC correlations of H-12 with C-4, C-11 and C-13 and H-13 with C-4, C-11 and C-12. Based on analysis of NMR data as well as by comparison with previously reported papers, the structure of compound 5 was therefore elucidated as phloroglucinol sesquiterpenoid (5). This is a new sesquiterpenoid reported for the first time from this plant.

### Compound 6

**Betulin** was isolated as white amorphous powder. TLC analysis on Kiesel gel 60 F254 0.25 mm plate (Merck) in n-
hexane-acetone = 80:20 (v/v), Rf 0.37; IR (KBr) νmax cm⁻¹: 3431, 2931, 1545, and 1051; ES-MS m/z: 443.500 [M+H]^+ calc'd for C35H58O3 [H^+]: 952.405. 1H-NMR (500 MHz, in CDCl₃) δ: 0.77 (s, 3H, H-27), 0.83 (s, 3H, H-5), 0.98 (s, 3H, H-24), 1.19 (s, 3H, H-26), 1.38 (m, 1H, H-9), 1.55 (m, 2H, H-7), 1.58 (m, 2H, H-15), 1.62 (m, 2H, H-11), 1.65 (m, 2H, H-12), 1.69 (s, 3H, H-30), 2.41 (m, 1H, H-19), 3.15 (dd, 1H, J = 4.5, 4.5 Hz, H-3), 3.91 (s, 2H, H-28), 4.5 (q, 1H, J = 1.3, 1.3, H-29) and 4.6 (d, 1H, J = 2.55, H-29). 13C-NMR (125 MHz, in CDCl₃) δ 15.5 (C-24), 16.5 (C-27), 17.1 (C-26), 18.4 (C-25), 19.2 (C-6), 19.6 (C-30), 24.7 (C-11), 27.2 (C-12), 28.5 (C-15), 28.9 (C-2), 29.5 (C-23), 30.9 (C-19), 34.8 (C-21), 35.9 (C-22), 36.8 (C-7), 39.8 (C-13), 39.9 (C-10), 41.1 (C-1), 42.2 (C-4), 43.0 (C-17), 44.2 (C-14), 44.4 (C-8), 49.5 (C-18), 49.7 (C-19), 53.5 (C-9), 57.3 (C-5), 62.3 (C-28), 79.9 (C-3), 110.2 (C-29) and 152.0 (C-20). Based on spectroscopic analyses and a comparison with the literatures, the known compound was identified as betulin (6) [19-21]. This is a new triterpenoid reported for the first time from this plant.

![Chemical structure of compounds 1-6](image)

**Figure 1: Chemical structure of compounds 1-6**

| Table 1: Antibacterial activity of compound 1-6 against E. faecalis ATCC 29212, S. mutans ATCC 25175 and P. gingivalis ATCC 33277 |
|---|---|---|---|---|
| Inhibition zone (mm) | E. faecalis 5000 | E. faecalis 1000 | P. gingivalis 5000 | S. mutans 5000 |
| 0 | 8.55 | 0 | 12.3 | 0 |
| 2 | 0 | 0 | 0 | 11.1 |
| 4 | 0 | 0 | 0 | 14.7 |
| 6 | 0 | 0 | 0 | 9.24 |
| 12 | 0 | 0 | 0 | 8.45 |
| 15 | 0 | 0 | 0 | 9.35 |
| 20 | 0 | 0 | 0 | 22.2 |

To evaluate the antibacterial activity of compounds, were conducted susceptibility test using disk diffusion method. Compound 1 was tested against *E. faecalis*, compound 2-3 were tested against *S. mutans* and compound 4-6 against *P. gingivalis*. Chlorhexidine was used as a positive control for each bacteria. Susceptibility of isolates (4-6) against *P. gingivalis* was determined on inhibition zone of isolates on growth bacteria. From the antibacterial results as showed in Table 1, All compounds have antibacterial activity although it is lower than chlorhexidine’s activity. The compound 1 can inhibit *E. faecalis* growth, the compound 2-3 give zone inhibition on *S. mutans* culture’s, and then the compound 5 was active against *P. gingivalis*, but the compounds 4 and 6 were inactive. When considering of the inhibition zone values at the micromolar level, it is possible to observe that chlorhexidine is about potent than compound 5. Nevertheless, several adverse effects are associated with regular use of chlorhexidine. The reinforces the great importances of compound 5 as a prototype or lead compound for the development of novel and safe bioactive compounds for control of periodontitis.

**Experimental**

**General:** NMR spectra recorded on a 500 MHz FT-NMR spectrometer (Varian ECA 500 JOEL, Japan) (500 (H) and 125 (13C)); δ in ppm rel. to TMS as internal standard, J in Hz. IR spectra were obtained from a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). ES-MS spectrometer (UPLC MS/MS TOF type, Waters); m/z. Column chromatography (CC): silica gel (SiO₂, 200-300 mesh; Merck, Darmstadt, Germany) and ODS was a LiChroprep RP-18 (Merck). TLC: Kiesel gel 60 F₂₅₄ and RP-18 F₂₅₄ (Merck). For antibacterial assay, laminar air flow, incubator Memmert, autoclave machine HVE-50 Hirayama jar and ELISA reader Diagnostic Automation Inc.

**Plant material:** Dried of Sarang Semut *Myrmecodia pendans* was collected from from Papua island, Indonesia and identified by Mr Joko Kusmoro (Padjadjaran University), Laboratory of Plants Taxonomi, Department of Biology, Faculty of Mathematic and Natural Science, Padjadjaran University, Sumedang, Indonesia.

**Extraction and isolation:** The air-dried tuber of Sarang Semut (1.5 kg) plants was extracted with 100% ethyl acetate (3x3 L) at 40°C on heating mantle of Soxhlet extractor. This method was chosen to yield thermostable compounds as similar with empirical experiences of local people who use it after boiling process. The extract was evaporated to yield a residue (20 g). Ethyl acetate extract was subjected to column chromatography on stationary phase silica gel 60 eluting with 10% gradient of n-hexane-ethyl acetate, to yield 11 fractions (A-K). Fraction H (0.45 g) was subjected to column chromatography on stationary phase silica gel 60 eluting with 5% gradient of n-hexane-ethyl acetate, to yield 21 fractions (H01-H21). Fraction H08 (71 mg) was subjected to column chromatography on stationary phase silica gel 60 eluting with 5% gradient of n-hexane-ethyl acetate, to yield 21 fractions (H01-H21). Fraction H15 (71 mg) was subjected to an RP-C18 column, eluting with isocratic solvent of methanol-water (90:10 v/v) to yield 6 (20 mg). Fraction F (2.10 g) was subjected to column chromatography on stationary phase silica gel 60 eluting with 2.5% gradient of n-hexane-ethyl acetate, to yield 20 fractions (F01-F20). Fraction F09 (196.2 mg) was subjected to an RP-C18 column, eluting with 2.5% gradient of methanol-water to yield compound 5 (5 mg).

**6′-O-tridecanoyl-3-O-β-D-glucosyl-sitosterol**

White amorphous powder.

IR (KBr): 3404, 2923, 1722, 1465, 1379, 1080 cm⁻¹.

1H NMR (500 MHz, CDCl₃): Table 1. 13C NMR (125 MHz, CDCl₃): Table 1.

**Antibacterial activity assay:** Bacterial strain *P. gingivalis* ATCC 33277, *E. faecalis* ATCC 29212 and *S. mutans* 25175 was used for the test, Muller Hinton broth and Muller Hinton agar as medium, chlorhexidine and fosfomicyn (were purchased from Merck Co. Ltd. and Sigma Aldrich) as positive control. The procedure is follow as...
reference in CLSI protocols [22]. Compounds (samples) were diluted with methanol-water (1:1), however, Chlorhexidine was the positive control were diluted with water. All of them (controls and samples) were performed out of concentration 5000 and 2000 μg/mL. Paper discs (6 mm) were impregnated with 20 μL of each sample and then discs loaded with natural products were placed onto the surface of the agar. Tests were performed in duplicate. The results are presented as inhibition of zone (mm) in Table 2.

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References