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Prenylated anthronoid antioxidants from the stem bark of *Harungana madagascariensis*

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Abstract

Two new prenylated anthronoids, harunmadagascarins A and B, were isolated from the stem bark of *Harungana madagascariensis* along with six known compounds including two anthronoids: harunganol B and harungin anthrone, one benzophenone: methyl 3-formyl-2,4-dihydroxy-6-methyl benzoate and three pentacyclic triterpenes: friedelin, lupeol and betulinic acid. Harunmadagascarins A and B were characterized as 8,9-dihydroxy-4,4-bis-(3,3-dimethylallyl)-6-methyl-2,3-(2,2-dimethylpyrano)anthrone and 8,9-dihydroxy-4,4,5-tris-(3,3-dimethylallyl)-6-methyl-2,3-(2,2-dimethylpyrano)anthrone, respectively. The structures of these secondary metabolites were determined by spectroscopic means and comparison with the published data. Methyl 3-formyl-2,4-dihydroxy-6-methyl benzoate was isolated for the first time from a plant. Harunmadagascarins A and B, harunganol B and harungin anthrone exhibited significant antioxidant activity.

Keywords: *Harungana madagascariensis*; Hypericaceae; Prenylated anthronoids; Harunmadagascarins A and B; Antioxidant

1. Introduction

Harungana madagascariensis Lam. (Hypericaceae) is a perennial shrub that has been used in European herbal medicine to treat indigestion and poor pancreatic function (Prajapati et al., 2003) and in African folk medicine as a treatment for diarrhoea and dysentery (Prajapati et al., 2003; Berhaut, 1975). The family Hypericaceae is well known for the production of various phenolic compounds such as anthraquinones, xanthones, coumarins, biflavonoids and anthrone derivatives (Gunatilaka et al., 1984; Iinuma et al., 1995; Ritchie and Taylor, 1964a,b). Some of the compounds exhibit antihypoglycemic (Gunatilaka et al., 1984), antioxidant (Minami et al., 1995), cytotoxic (Nkengfack et al., 2002) and platelet aggregation inhibitory (Lin et al., 1993) activity. As part of our continuing search for compounds possessing medicinally useful properties (Dufall et al., 2003), a hexane-soluble extract of the stem bark of this plant was found to exhibit significant antioxidant effects, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydraxyl (DPPH) free radical (Lee et al., 1998). In this paper, we describe the isolation and the structural elucidation of two new compounds: harunmadagascarin A (**1**) and B (**2**) as well as the antioxidant activity of these new compounds as compared with the known compounds **3** and **4**.

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2. Results and discussion

The hexane soluble fraction of the methanolic extract of the finely powdered stem bark of *H. madagascariensis* was subjected to flash column chromatography. The less polar fraction afforded two new anthronoids named harunmadagascarins A (**1**) and B (**2**), along with the known harungin anthrone (**3**), harunganol B (**4**), methyl 3-formyl-2,4-dihydroxy-6-methyl benzoate (**5**), friedelin (**6**), lupeol (**7**) and betulinic acid (**8**). The known compounds were identified by comparison of their spectral data with the published ones (Ritchie and Taylor, 1964a,b; Iinuma et al., 1995; Pulgarin and Tabacchi, 1989) (Fig. 1).

Compound (**1**), harunmadagascarin A was obtained as orange crystals, reacting positively with $FeCl_3$ (methanol; green colour). The molecular formula was determined as $C_{30}H_{34}O_4$ by HREIMS $[M]^+$ *m/z* 458.2437, in conjunction with the NMR spectra. This formula indicated the degree of unsaturation as 14. The IR spectrum showed an inter- and intra-molecularly hydrogen-bonded hydroxyl (2922), a hydrogen-bonded carbonyl (1634), olefinic carbons (1594), aromatic double bonds (1443) and carbonyl (1726). The UV bands at λ_{max} 202, 231, 274 and 383 nm indicated an aromatic chromophore system which, in conjunction with the IR spectral data, suggested the anthronoid skeleton (Ritchie and Taylor, 1964a,b). In the ^1H NMR spectrum, the typical AB spin system at δ 6.94 and 6.66 (*d*, $J = 1.0$ Hz, 1H each) established the presence of two aromatic protons with *meta* coupling. The presence of two γ,γ-dimethylallyl groups attached to a saturated carbon was inferred from $^1H/^{13}C$ NMR spectra which displayed one two-proton triplet at δ_H 4.54 (2H, *t*, $J = 6.8$ Hz)/δ_C 118.3, two two-proton multiplets at δ_H 2.91 (2H, *dd*, $J = 6.8$; 14.3 Hz)/δ_C 40.5 and δ_H 2.58 (2H, *dd*, $J = 6.8$; 14.3 Hz)/δ_C 40.5, and two six-proton singlet at δ_H 1.46 (6H, *s*)/δ_C 18.2 and δ_H 1.43 (6H, *s*)/δ_C 25.6. This finding suggested clearly the symmetrical position of the two γ,γ-dimethylallyl groups. Furthermore, the presence of one aromatic methyl group and a set of spin systems for the 2,2-dimethylpyrano group can be deduced from a three proton singlet at δ 2.40, a six proton singlet at δ_H 1.42 and two protons at δ 6.57 and 5.35 (*d*, $J = 10.0$ Hz, 1H each). This was confirmed by the presence of a signal for an oxymethine at δ 80.7 in the ^{13}C NMR spectrum. Two chelated hydroxyls can also be observed as two one-proton signals at δ 16.98 and 9.97. The downfield chemical shift of the highly chelated hydroxyl is characteristic for 8,9-dihydroxyanthrone derivatives (Ritchie and Taylor, 1964a,b; Monache et al., 1980). The methyl group at δ 2.40 was located at position 6, in agreement with biogenetic considerations (Billen et al., 1988). Thus, the partial structure of compound **1** was expanded to a 8,9-dihydroxy-6-methylanthrone. In the heteronuclear multiple-bond connectivity (HMBC) spectrum (Fig. 2), the aromatic methyl proton (δ 2.40) caused cross peaks with two aromatic carbons at 111.7 and 117.8, indicating that the two protons with *meta* coupling are located at positions 5 and 7. Furthermore, C-5 was correlated to the aromatic proton at 7.07 (C-10), which in turn also showed cross peaks with a saturated carbon at δ 48.8. This finding clearly indicates that the remaining aromatic proton is located at position 7 and the two γ,γ-dimethylallyl groups at position 4. Thus, the 2,2-dimethylpyrano fragment can be located at positions 2 and 3. This was also confirmed by the HMBC spectrum in which the vinyl proton signal at 6.57 (H-17) showed long range

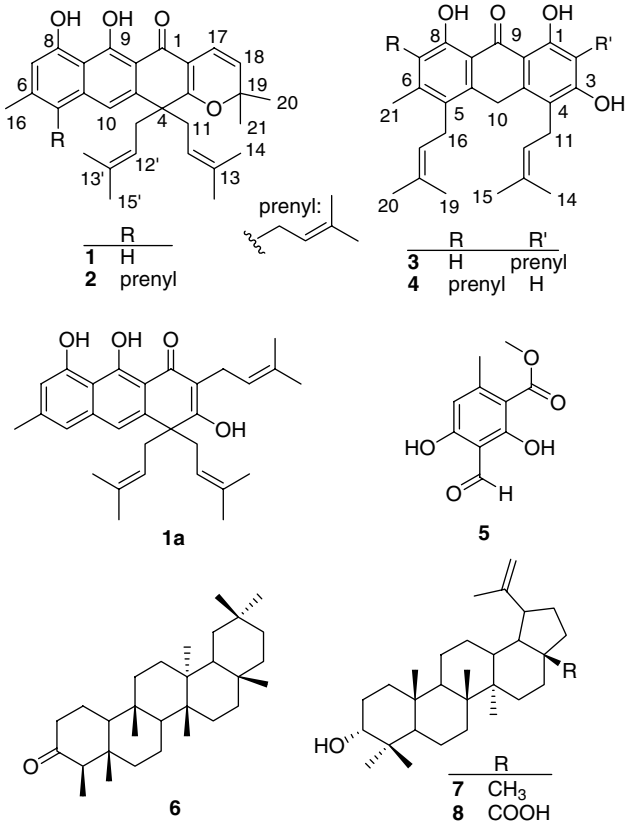

Fig. 1. Structures of secondary metabolites isolated from *H. madagascariensis*.

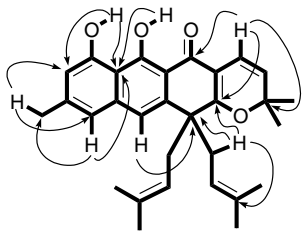

Fig. 2. Selected HMBC correlations for compound **1**.

coupling with the carbonyl group at δ 186.9, one oxygenated aromatic carbon signal at δ 174.0 and an oxymethine at δ 80.7.

From the above evidence, the structure of harunmadagascarin A was finally characterized as 8,9-dihydroxy-4,4-bis-(3,3-dimethylallyl)-6-methyl-2,3-(2,2-dimethyl pyrano)anthrone.

Harunmadagascarin B (**2**) was obtained as a red pigment from hexane/EtOAc solution. It reacted positively with $FeCl_3$(methanol; green colour). The HREIMS $[M]^+$ *m/z* 526.3029 (calcd. 526.3082) together with the NMR spectra suggested the molecular formula $C_{35}H_{42}O_4$. The UV–Vis spectrum showed bands at 204, 233, 272 and 422 nm and it was assumed from the ^{1}H NMR spectrum that **2** would be an anthrone derivative (Monache et al., 1980; Pinheiro et al., 1984). In addition, the ^{1}H NMR spectrum showed two signals (*s*, 1H each) assignable to hydroxyl groups at δ 16.92 and 10.04 which suggested the presence of a Harunganin type structure (Ritchie and Taylor, 1964a,b). Like compound **1**, the ^{1}H NMR spectra showed resonance signals for two prenyl groups at δ 2.60 (*dd*, J = 14.2, 6.9, H_{11}), 2.90 (*dd*, J = 14.2, 6.9, $H_{11'}$), 4.56 (*br t*, J = 6.9, H_{12}), 1.44 and 1.43 (*s*, 6 CH_3). Comparison of the ^{1}H NMR data of compound **1** and those of **2** (Table 2) showed the signals corresponding to one additional prenyl group in **2** at δ 3.55 (2H, *d*, J = 6.1), 5.01 (1H, *t*, J = 6.1), 1.64 and 1.87 (3H each, *br s*) These results were confirmed by mass spectral (*m/z* 526) and ^{13}C NMR data (Table 3) with the signals occurring at δ 27.5 (CH_2), 123.6 (=CH), 131.1 (quaternary carbon), 18.1 and 25.5. This group was located at position 5 on the basis of HMBC spectra (Fig. 3). The assignment of all the quaternary carbons (Table 3), as well as of the different groups, was possible from the HMBC correlation experiment (Table 2) and by comparison of measured values with those reported for ferruginin B (**1a**) (Nicoletti et al., 1982).

On the basis of the above results, the structure of harunmadagascarin B (**2**) is therefore confirmed as 8,9-dihydroxy-4,4,5-tris-(3,3-dimethylallyl)-6-methyl-2,3-(2,2-dimethylpyrano)anthrone. To the best of our knowledge, this is the first time a tetraprenylated anthrone is reported.

The results of the antioxidant tests of the isolated compounds **1**–**4** are summarized in Table 1. Compounds **1** and **4**, with IC_{50} values of 60.97 and 64.76 μM, respectively, exhibited relatively strong activity as free radical scavengers in the DPPH assay (reference antioxidants 3-*t*-butyl-4-hydroxyanisole showed IC_{50} 44.2 μM). Compounds **2** and **3,** with IC_{50} value of 155.39 and 92.10 μM, respectively, exhibited less potent antioxidant activity than compounds **1** and **4**.

Table 1
Antioxidant activity of compounds **1**–**4**

Compounds	DPPH Radical IC_{50} ± SEM (μM)
1	60.97 ± 3.2
2	155.39 ± 2.5
3	92.10 ± 4.5
4	64.76 ± 5.5
3-*t*-Butyl-4-hydroxyanisole (standard)	44.20 ± 1.2

Table 2
^{1}H NMR data for harunmadagascarin A (**1**) and B (**2**) (δ (ppm), $CDCl_3$, J in Hz, 400 and 500 MHz, respectively) and ^2J, ^3J gradient HMBC correlations for **1** and **2**

H	**1**		**2**	
	δ_H	HMBC(H → C)	δ_H	HMBC(H → C)
5	6.66 (*d*, J = 1.0)	C-7, C-8a, C-10	–	
7	6.94 (*d*, J = 1.0)	C-5, C-8a, C-16	6.68 (*s*)	C-5, C-8a, C-16
8-OH	9.97 (*s*)	C-7, C-8, C-8a	10.04 (*s*)	C-7, C-8, C-8a
9-OH	16.98 (*s*)	C-8a, C-9, C-9a	16.92 (*s*)	C-8a, C-9, C-9a
10	7.07 (*s*)	C-4, C-5, C-8a	7.30 (*s*)	C-4, C-5, C-8a
11, 11′	2.58 (*dd*, J = 14.3, 6.8)	C-3, C-13, C-13′	2.60 (*dd*, J = 14.1, 6.9)	C-3, C-13, C-13′
	2.91 (*dd*, J = 14.3, 6.8)	C-3, C-13, C-13′	2.90 (*dd*, J = 14.2, 6.9)	C-3, C-13, C-13′
12, 12′	4.54 (*br t*, J = 6.8)	C-4, C-14, C-15	4.56 (*br t*, J = 6.9)	C-4, C-14, C-15
17	6.57 (*d*, J = 10.0)	C-1, C-3, C-19	6.58 (*d*, J = 10.0)	C-1, C-3, C-19
18	5.35 (*d*, J = 10.0)	C-2, C-19	5.36 (*d*, J = 10.0)	C-2, C-19
14, 14′	1.46 (*s*)[a]	C-15, C-15′	1.44 (*s*)[a]	C-15, C-15′
15, 15′	1.43 (*s*)[b]	C-14, C-14′	1.43 (*s*)[b]	C-14, C-14′
CH_3-6	2.40 (*s*)	C-5, C-6, C-7	2.39 (*s*)	C-5, C-6, C-7
$C(CH_3)_2$	1.42 (*s*)	C-18	1.43 (*s*)	C-18
22	–		3.55 (*d*, J = 6.1)	C-6, C-10a, C-24
23	–		5.01 (*t*, J = 6.1)	C-5, C-24
25	–		1.64 (*br s*)	C-23, C-26
26	–		1.87 (*br s*)	C-23, C-25

[a,b] Within the same column assignments may be reversed.

Table 3
^{13}C NMR data for harunmadagascarin A (**1**) and B (**2**) (δ (ppm), $CDCl_3$, 100 and 125 MHz, respectively) and ferruginin B (**1a**) (δ (ppm), dioxan-*d*, 25.2 MHz) (Nicoletti et al., 1982)

C	1	2	1a
1	186.9	186.9	192.5
2	110.3	110.4	117.0
3	174.0	174.0	182.2
4	48.8	49.0	50.7
4a	141.8	139.1	142.9
5	117.8	125.1	123.5
6	141.8	139.5	141.5
7	111.7	112.9	113.0
8	157.2	155.5	159.0
8a	111.0	111.7	109.9
9	163.5	163.7	164.6
9a	108.2	107.9	117.7
10	115.0	112.4	115.9
10a	138.3	136.5	139.8
11,11′	40.5	40.6	43.1
12,12′	118.3	118.4	120.3
13,13′	134.4	134.2	135.2
14,14′	18.2*	18.8^{+}	21.9
15,15′	25.6*	25.6^{+}	26.6
16	19.1	20.8	19.1
17	114.9	114.9	26.8
18	123.6	123.6	118.9
19	80.7	80.7	134.1
20	28.7	28.7	22.9
21	28.7	28.7	26.6
22	–	27.5	–
23	–	123.6	–
24	–	131.1	–
25	–	18.1	–
26	–	25.5	–

*,+ Signals with the same signs in the column may be interchanged.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a Yanaco melting point apparatus and are uncorrected. IR spectra were recorded on JASCO 302-A spectrophotometer in KBr disks. UV spectra were obtained on a Hitachi UV 3200 spectrophotometer. EIMS (ionization voltage 70 eV) were measured on a Varian MAT 311 A mass spectrometer and HREIMS were measured on a JEOL HX 110 mass spectrometer. 1D and 2D NMR spectra were run on Bruker AMX 400 and AMX 500 MHz NMR spectrometers. The chemical shifts are given in ppm (δ), relative to TMS as internal standard and coupling constants are in Hz. Column chromatography was carried out on silica gel (70–230 mesh, Merck) and flash silica gel (230–400 mesh, Merck). TLC was performed on Merck precoated silica gel 60 F_{254} aluminium foil and spots were detected using ceric sulfate spraying reagent.

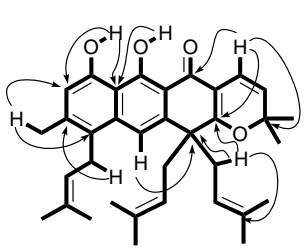

Fig. 3. Selected HMBC correlations for compound **2**.

3.2. Plant material

H. madagascariensis Lam. stem bark was collected at Bandjoun, Cameroon in March 2004. The plant was identified by Mr. Nana of the National Herbarium, Yaounde (Cameroon) where a voucher specimen (HNC 32358) was deposited.

3.3. Extraction, isolation and characterization

Air-dried and finely powdered stem bark (2.3 kg) was macerated in MeOH for 48 h. Filtration and evaporation yielded a crude MeOH extract (250.7 g) which was re-extracted with hexane follow by EtOAc. The removal of the solvent yielded 50 g of hexane-soluble extract and 80.3 g of non-soluble extract. The hexane extract was subjected to column chromatography over silica gel 60 eluting with pure hexane and followed by hexane–EtOAc mixture with increasing polarity. A total of 150 fractions of ca. 200 ml each were collected and combined on the basis of TLC analysis leading to 5 series (I–V). Further purification of these series was achieved by column chromatography and PTLC.

Series I (10.4 mg), upon examination by TLC (hexane/EtOAc) contained a complex mixture and was not investigated. Series II (15.5 g) obtained with hexane–ethyl acetate (95/5), was purified successively by CC and PTLC to yield compounds **1** (12.5 mg), **2** (7.5 mg) and **5** (20.3 mg). Series III (9.4 g) was subjected to repeated column chromatography. Elution of the column with hexane–ethyl acetate (49/1), yielded compound **5** (9.7 mg) again together with **3** (8.6 mg) and **6** (17.3 mg). Series IV gave compound **7** (35.6 mg), while series V gave compound **8** (25.8 mg).

3.4. 8,9-Dihydroxy-4,4-bis-(3,3-dimethylallyl)-6-methyl-2,3-(2,2-dimethylpyrano)anthrone (harunmadagascarin A) (*1*)

Orange crystals from hexane; m.p.: 149 °C; UV λ_{max} nm (MeOH) (log ε): 202 (4.54), 231 (4.45), 274 (4.41), 383 (4.12); IR ν_{max} (KBr) cm^{-1}: 3433, 2971, 2922, 2855, 1726, 1634, 1594, 1443, 1383, 1354, 1137; ^1H NMR (400 MHz, $CDCl_3$): see Table 2; ^{13}C NMR (100 MHz, $CDCl_3$): See Table 3; HR EIMS *m/z* 458.2437 (calcd. for $C_{30}H_{34}O_4$, 458.2456); EIMS *m/z* (rel. int.) 458 (8), 390 (39), 389 (100), 375 (15), 348 (7), 347 (34),

334 (17), 331 (13), 319 (28), 81 (6), 69 (26), 67 (9), 55 (11), 53 (12).

3.5. 8,9-Dihydroxy-4,4,5-tris-(3,3-dimethylallyl)-6-methyl-2,3-(2, 2- dimethylpyrano)anthrone (harunmadagascarin B) (2)

Orange crystals from MeOH; m.p.: 122.5 °C; UV λ_{max} nm (MeOH) (log ε): 204 (4.22), 233 (4.20), 272 (4.35), 422 (3.79); IR ν_{max} (KBr) cm^{-1}: 3448, 2958, 2923, 2854, 1629, 1598, 1443, 1385, 1260, 1097, 1026, 875, 802; ^1H NMR (500 MHz, $CDCl_3$): see Table 2; ^{13}C NMR (125 MHz, $CDCl_3$): see Table 3; HR EIMS *m/z* 526.3029 (calcd. for $C_{35}H_{42}O_4$, 526.3082); EIMS *m/z* (rel. int.) 526 (8), 458 (38), 457 (100), 401 (19), 390 (11), 389 (39), 387 (17), 371 (10), 330 (8), 69 (19).

3.6. 1,3,8-Trihydroxy-2,4,5-tris-(3,3-dimethylallyl)-6-methylanthrone (harungin anthrone) (3)

Brown crystals from hexane; m.p.: 170.6 °C; UV λ_{max} nm (MeOH) (log ε): 200 (4.26), 203 (4.02), 320 (3.42), 365 (3.62); IR ν_{max} (KBr) cm^{-1}: 3465, 2970, 2921, 1598, 1474, 1441, 1378, 1293, 1201, 1168, 1095, 819, 776; ^1H NMR (400 MHz, $CDCl_3$): 13.21 (1H, *s*, 1-OH), 12.57 (1H, *s*, 8-OH), 6.73 (1H, *s*, 7-H), 6.30 (1H, *s*, 3-OH), 5.25 (1H, *t*, *J* = 5.8 Hz, 17-H), 5.02 (1H, *t*, *J* = 5.3 Hz, 23-H), 4.86 (1H, *t*, *J* = 5.1 Hz, 12-H), 4.03 (2H, *s*, 10-H), 3.46 (2H, *d*, *J* = 7.1 Hz, 22-H); 3.35 (2H, *d*, *J* = 6.5 Hz, 11-H), 3.31 (2H, *d*, *J* = 6.1 Hz, 16-H), 2.31 (3H, *s*, 21-H), 1.84, 1.81, 1.79, 1.76, 1.70, 1.68 (3H each, 6*s*, 14-H, 15-H, 19-H, 20-H, 25-H, 26-H); ^{13}C NMR (100 MHz, $CDCl_3$): 17.9 (C-14), 25.6 (C-15), 17.9 (C-19), 25.5 (C-20), 17.9 (C-25), 25.8 (C-26), 20.9 (C-21), 21.8 (C-22), 24.3 (C-11), 27.3 (C-16), 29.2 (C-10), 109.3 (C-9a), 111.9 (C-2), 113.6 (C-8a), 116.8 (C-4), 117.0 (C-7), 121.1 (C-12), 121.2 (C-17), 121.3 (C-23), 128.2 (C-5), 133.1 (C-13), 133.7 (C-18), 135.9 (C-24), 138.5 (C-4a), 138.7 (C-10a), 145.8 (C-6), 159.9 (C-3), 160.7 (C-1), 160.8 (C-8), 192.7 (C-9); EIMS *m/z* (rel. int.) 460 (13), 404 (81), 361 (35), 349 (79), 348 (74), 333 (43), 319 (15), 305 (82), 293 (100), 280 (17), 276 (10), 264 (9), 249 (6), 202 (8), 189 (8), 165 (12), 69 (15).

3.7. 1,3,8-Trihydroxy-4,5,7-tris-(3,3-dimethylallyl)-6-methyl-anthrone (harunganol B) (4)

Yellow crystals from hexane–ethyl acetate; m.p.: 200 °C; UV λ_{max} nm (MeOH) (log ε): 204 (3.80), 260 (3.28), 278 (3.14), 313 (3.10), 368 (3.33); IR ν_{max} (KBr) cm^{-1}: 3377, 2922, 1603, 1440, 1381, 1270, 1162, 816, 781; ^1H NMR (400 MHz, $CDCl_3$): 12.97 (1H, *s*, 8-OH), 12.86 (1H, *s*, 1-OH), 6.33 (1H, *s*, 3-OH), 5.73 (1H, *s*, 2-H), 5.07 (1H, *br s*, 23-H), 5.05 (1H, *br s*, 12-H), 4.89 (1H, *t*, *J* = 6.4 Hz, 17-H), 4.05 (2H, *s*, 10-H), 3.44 (2H, *d*, *J* = 6.4 Hz, 22-H); 3.35 (4H, *br s*, 11-H, 16-H), 2.29 (3H, *s*, 21-H), 1.81, 1.80, 1.79, 1.71, 1.68, 1.67 (3H each, *s*, 14-H, 15-H, 19-H, 20-H, 25-H, 26-H); ^{13}C NMR (100 MHz, $CDCl_3$): 16.5 (C-21), 17.9 (C-14), 17.9 (C-19), 18.0 (C-25), 24.2 (C-11), 25.0 (C-16), 25.6 (C-15), 25.6 (C-20), 25.7 (C-26), 27.8 (C-22), 29.5 (C-10), 101.9 (C-2), 110.2 (C-9a), 112.8 (C-8a), 116.4 (C-4), 120.8 (C-12), 121.5 (C-17), 122.2 (C-23), 126.8 (C-7), 128.1 (C-5), 131.8 (C-24), 132.9 (C-18), 134.6 (C-13), 135.6 (C-10a), 141.5 (C-4a), 144.5 (C-6), 159.0 (C-8), 160.6 (C-3), 163.5 (C-1), 192.8 (C-9); EIMS *m/z* (rel. int.) 460 (19), 404 (44), 389 (13), 361 (100), 349 (68), 336 (18), 335 (47), 306 (17), 305 (48), 293 (35), 280 (12), 165 (9), 69 (56), 67 (11), 55 (47).

3.8. Methyl 3-formyl-2,4-dihydroxy-6-methyl benzoate (5)

White powder from ethanol; m.p.: 146.5 °C; ^1H NMR (400 MHz, $CDCl_3$): 12.85 (1H, *s*, 4-OH), 12.39 (1H, *s*, 2-OH), 10.32 (1H, *s*, CHO), 6.27 (1H, *s*, 5-H), 3.94 (3H, *s*, OCH_3), 2.51 (3H, *s*, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): 25.3 (CH_3), 52.5 (OCH_3), 103.9 (C-1), 108.5 (C-3), 112.3 (C-5), 152.2 (C-6), 166.9 (C-4), 172.3 (C-1′), 194.0 (CHO).

3.9. Experimental DPPH free radical scavenging assay

Test samples were allowed to react with stable free radical, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) for half an hour at 37 °C. The concentration of DPPH was kept as 300 μM. The test samples were dissolved in DMSO while the DPPH solution was prepared in ethanol. After incubation, the decrease in absorption was measured at 515 nm using multiplate reader (Spectra MAX-340). Percent radical scavenging activity by the samples was determined in comparison with a DMSO treated control group (Lee et al., 1998).

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