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Chemical Constituents of *Tetradium sambucinum* (Bl.) Hartley

Ismiarni Komala[1], Mawardi Rahmani[1,*], Gwendoline Ee Cheng Lian[1], Hazar Bebe Mohd Ismail[2], Mohd Aspollah Sukari[1] and Asmah Rahmat[3]

[1] Department of Chemistry, Universiti Putra Malaysia, 43400 UPM, Serdang, Malaysia.
* mawardi@fsas.upm.edu.my. Telephone (+ 603) 89466794, Facsimile (+ 603) 89435380.
[2] Centre for Foundation Studies in Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
[3] Department of Health Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Malaysia.
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ABSTRACT Detailed extraction and separation of the aerial parts of *Tetradium sambucinum* (Bl.) Hartley have led to the isolation of decarine, rutaecarpine, aurantiamide acetate and 7-hydroxycoumarin. The structures of the compounds were established by one and two-dimensional ^{1}H- and ^{13}C-NMR as well other spectroscopic methods. The free radical scavenging and cytotoxic activities were assessed by DPPH assay and against two cancer cell lines, respectively.

ABSTRAK Pengekstrakan dan pemisahan bahagian atas *Tetradium sambucinum* (Bl.) Hartley telah berjaya mengasingkan dekarina, rutaekarpina, aurantiamida asetat dan 7-hidroksikoumarin. Struktur semua sebatian ini telah ditentukan dengan kaedah ^{1}H dan ^{13}C-NMR satu dan dua dimensi dan kaedah spektroskopi lain. Aktiviti antioksidan dan sitotoksik masing-masing telah ditentukan dengan menggunakan DPPH dan dua jenis sel kanser

(*Tetradium sambucinum*, *Evodia sambucina*, decarine, rutaecarpine, aurantiamide acetate, 7-hydroxycoumarin)

INTRODUCTION

Tetradium sambucinum (Bl.) Hartley (Rutaceae) is a small tree with aromatic smelling leaves and distributed in poorly drained primary and secondary forests of Malaysia and Sumatra at altitudes between 30 - 1400 m. This species was formerly identified as *Evodia sambucina* (Bl.) Hook but had been reassigned by Hartley as *Tetradium sambucinum* [1]. There is no record on the medicinal uses of the plant, but fruits of some related species have been used for the treatment of dysentery and also as antipyretic. The stem bark and leaves of the plant were collected in Padang Panjang, West Sumatera, Indonesia in December 2003. A voucher sample (number E11) was deposited in the Herbarium, Andalas University, West Sumatera, Indonesia. There have been no previous reports on detailed investigations of this plant.

MATERIALS AND METHODS

Figure 1 shows the structure of the four compounds (numbered **1** - **4**) isolated from *Tetradium sambucinum* (Bl.) Hartley.

The dried and powdered stem bark of *Tetradium sambucinum* (1.2 kg) was consecutively extracted twice with solvents of increasing polarity beginning with petroleum ether followed by chloroform and methanol at room temperature. The solvents were removed under reduced pressure to yield three dark viscous solids weighing 6.0, 15.2 and 35.0 g respectively. The chloroform extract (15 g) was separated by silica gel VLC and eluted with petroleum ether, chloroform and methanol of increasing polarity to give 4 major fractions. Fraction 4 was further chromatographed on a silica gel CC, eluted with petroleum ether, chloroform and methanol to afford brown needle-shaped crystals identified as decarine **(1)** (76 mg; 0.51%), m.p. 245 – 246 °C.

The dried and powdered leaves of Tetradium sambucinum (1.1 kg) were similarly extracted with solvents of increasing polarity (petroleum ether, chloroform and methanol). The chloroform extract (20 g) was separated by VLC on silica gel and eluted with petroleum ether, chloroform and methanol of increasing polarity to give 60 fractions. Fractions 25 - 29 were subjected to silica gel CC eluted with 10 % increase of chloroform in petroleum ether to yield white needle-shaped crystals of rutaecarpine **(2)** (20 mg; 0.1%) m.p. 264 - 265°C. Fractions 43 - 53 were subjected to another silica gel CC eluted with hexane and ethyl acetate in 10% increasing polarity to yield 3 fractions A, B and C. Fraction A was separated using Sephadex LH 20 eluted with methanol to give white crystals of aurantiamide acetate **(3)** (5 mg; 0.25%), m.p. 182 - 185°C. Fraction C was also separated using Sephadex LH 20 column and eluted with methanol to yield white crystals of 7-hydroxycoumarin **(4)** (4 mg; 0.02%), m.p. 223 - 225°C.

Decarine **(1)**: $C_{19}H_{13}NO_4$, brown needles, m.p. 245 - 246°C ([2], m.p. 243°C); UV λ_{max} nm ($CHCl_3$): 230.0, 246.8, 276.2, 324.4, 383.0; IR ν_{max} cm^{-1} (KBr disc): 3438, 2924, 2854, 2778, 2564, 1624, 1592, 1534, 1500, 1470, 1398, 1350, 1286, 1220; EIMS m/z (% intensity): 319 ([M^+], 100), 304 (86.5), 276 (77.7), 218 (11.5), 137 (26.9); ^{1}H-NMR (400 MHz, $CDCl_3$ and CD_3OD) δ: 9.63 (1H, s, H-8), 8.63 (1H, s, H-1), 8.34 (1H, d, 9.17 Hz, H-6), 8.31 (1H, d, 8.25 Hz, H-12), 7.85 (1H, d, 9.17 Hz, H-5), 7.54 (1H, d, 8.25 Hz, H-11), 7.33 (1H, s, H-4), 6.14 (2H, s, OCH_2O), 4.10 (3H, s, OCH_3); ^{13}C-NMR (100 MHz, $CDCl_3$ and CD_3OD) ppm: 148.4 (C-3), 148.0 (C-2), 146.9 (C-10), 145.6 (C-8), 142.3 (C-9), 139.2 (C-14), 129.5 (C-4a), 128.6 (C-12a), 127.6 (C-1a), 127.1 (C-5), 122.8 (C-11), 121.5 (C-8a), 120.5 C-13), 118.8 (C-12), 118.1 (C-6), 104.4 (C-4), 101.6 C-1), 101.2 (OCH_2O), 62.0 (OCH_3).

Rutaecarpine **(2)**: $C_{18}H_{13}N_3O$, white needle-shaped crystals, m.p. 264 - 265°C ([3], m.p. 259 - 260°C); λ_{max} nm ($CHCl_3$): 224.0, 243.2, 344.6, 362.2; IR ν_{max} cm^{-1} (KBr disc): 3344, 2918, 2850, 1722, 1654, 1604, 1550, 1492, 1472, 1378, 1342, 1292, 1230, 1168; EIMS m/z (% intensity): 287 ([M^+], 88.4), 286 (100), 143 (28.1), 129 (31.0); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 9.60 (1H, s, NH), 8.32 (1H, d, 8.24 Hz, H-4), 7.72 (1H, dd, 1.84 8.24 Hz, H-2), 7.68 (1H, d, 8.24 Hz, H-1), 7.63 (1H, d, 8.24 Hz, H-9), 7.43 (1H, t, 8.24 Hz, H-3), 7.38 (1H, d, 8.24 Hz, H-12), 7.32 (1H, t, 8.24 Hz, H-11), 7.18 1H, t, 8.24 Hz, H-10), 4.59 (2H, t, 7.32, 6.44 Hz, H-7), 3.25 (2H, t, 7.32, 6.44 Hz, H-8); ^{13}C-NMR (100 MHz, $CDCl_3$) ppm: 161.5 (C-5), 147.2 (C-1a), 145.2 (14a), 138.4 (12a), 134.4 (C-2), 127.2 (C-4), 126.9 (C-13a) 126.3 (C-1), 126.2 (C-3), 125.6 (C-11), 125.4 (C-9a), 121.0 (C-4a), 120.6 (C-10), 120.0 (C-9), 118.6 (C-8a), 112.2 (C-12), 41.1 (C-7) and 19.6 (C-8).

Aurantiamide acetate **(3)**: $C_{27}H_{28}N_2O_4$, white crystal, m.p. 182 - 185°C ([4], m.p. 188°C); UV λ_{max} nm ($CHCl_3$) nm: 217; IR ν_{max} cm^{-1} ($CHCl_3$): 3296, 2920, 2854, 1728, 1632, 1534, 1376, 1322, 1256, 1044; EIMS m/z (% intensity): 444 [M^-] (0.6), 252 (31.9), 224 (20.5), 172 (9.2), 105 (100), 91 (42.5), 77 (76.1), 43 (23.5); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.72 (2H, d, 7.36 Hz, H-2'/6'), 7.53 (1H, t, 7.36, H-4'), 7.45 (2H, t, 7.36 Hz, H-3'/5'), 7.06-7.73 (10 H of benzyl protons), 6.76 (1H, d, 8.24, H-8), 5.94 (1H, d, 8.24 Hz, H-5), 4.76 (1H, m, H-7), 4.35 (1H, m, H-4), 3.93 (1H, dd, 4.60, 11.00, Hz, H-3), 3.80 (1H, dd, 4.60, 11.00 Hz, H-3), 3.22, (1H, dd, 6.40, 13.30, H-11), 3.05 (1H, dd, 8.30, 13.30 Hz, H-11), 2.75 (2H, t, 6.40 Hz, H-10), 2.03 (3H, s, H-1); ^{13}C-NMR (100 MHz, $CDCl_3$) ppm: 170.80 (C-2), 170.14 (C-6), 167.05 (C-9), 136.62 (C-1'"), 136.54 (C-1"), 133.55 (C-1'), 131.92 (C-4'), 128.72 (C-3'/5'), 128.60- 136.62 (10 C of benzyl carbon), 127.01 (C-2'/6'), 64.52 (C-3), 54.94 (C-7), 49.37 (C-4), 38.40 (C-11), 37.37 (C-10), 20.81 (C-1).

7-Hydroxycoumarin **(4)**: $C_9H_6O_3$, white needles, m.p 223 - 225°C ([5], m.p. 223 - 225°C); UV λ_{max} nm: 221 and 323; IR ν_{max} cm^{-1} (NaCl cell): 3418, 2922, 2850 and 1732, 1612, 1464, 1376, 1232, 1126; EIMS m/z (% intensity): 162 ([$M]^+$, 41.0), 149 (11.0), 134 (31.1), 113 (19.5), 71 (19.8), 43 (100); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7. 64 (1H, d, 9.60 Hz, H-4), 7.36 (1H, d, 8.70 Hz, H-5), 6.83 (1H, d, 2.60 Hz, H-8), 6.80 (1H, dd, 2.60, 8.70 Hz, H-6), and 6.26 (1H, d, 9.60 Hz, H-3); ^{13}C-NMR (100 MHz, $CDCl_3$) ppm: 161.4 (C-7), 160, 2 (C-2), 155.8 (C-8a), 143.5 (C-4), 129.2 (C-5), 113.1 (C-6), 113.0 (C-3) and 113.0 (C-4a), 103.3 (C-8).

DPPH free radical scavenging activity: The technique using 96 micro-well plates was used [6]. A solution of DPPH (5 mg) was prepared by dissolving in methanol (2 ml) and the solution was kept in the dark at 4°C. Similarly, a stock solution of the compound to be tested was

prepared at 1 mg/ml I methanol. The standard used in this assay was vitamin C. The stock solution was serially diluted in 96 micro-well plates to varying concentrations. Then 5µl of methanolic DPPH solution was added to each well, shaken to ensure thorough mixing before being placed in the dark and wrapped with aluminium foil. After 30 minutes, the optical density of the solution was read using an ELISA Reader at a wavelength of 517 nm and percentage of inhibition was calculated. All tests and analyses were run in triplicate and averaged.

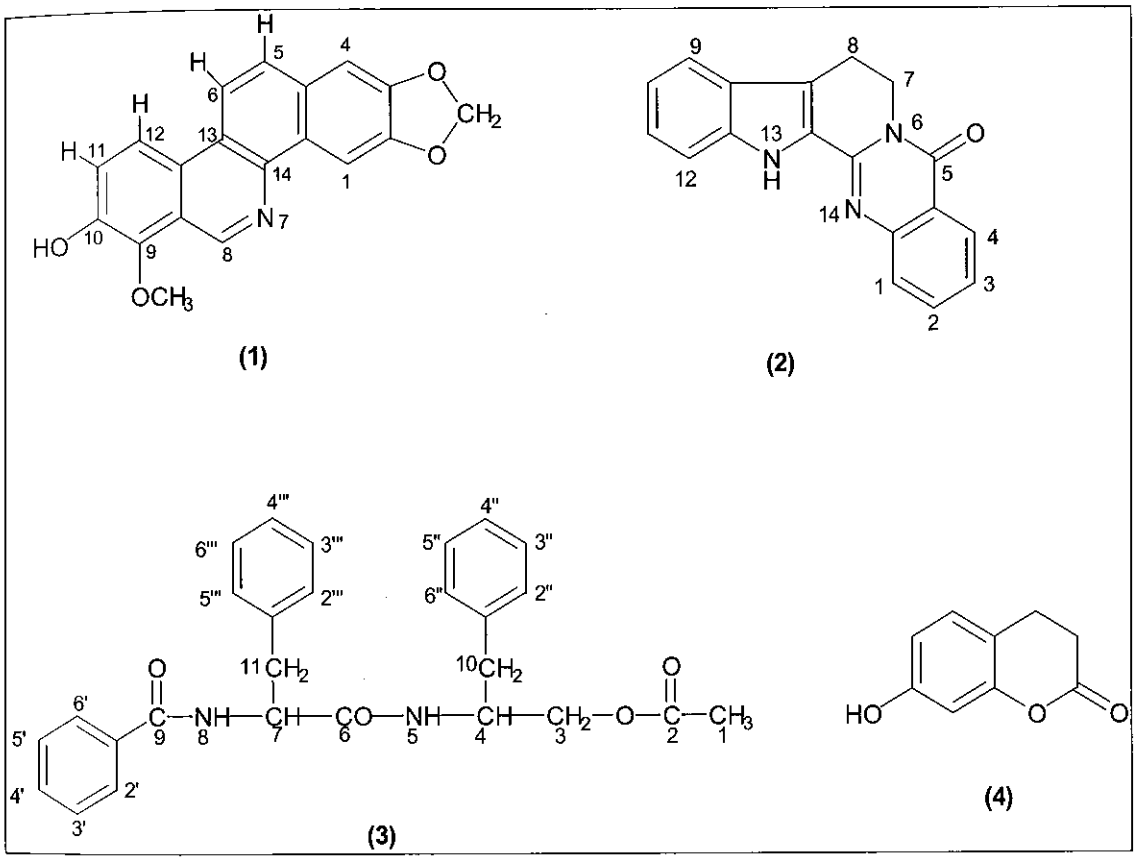

Figure 1. Compounds isolated from *Tetradium sambucinum* (Bl.) Hartley - **(1)**, Decarine; **(2)**, Rutaecarpine; **(3)**, Aurantiamide acetate and **(4)**, 7-Hydroxycoumarin

RESULTS AND DISCUSSION

Decarine **(1)** was obtained as brown needle crystals with m.p. 245 - 246°C ([2], m.p. 243 °C). The UV spectrum displayed absorptions at 230.4, 246.8, 276.2, 324.4 and 383.0 nm suggested the presence of phenantridine alkaloid moiety. A strong and broad absorption at 3438 cm^{-1} in the IR spectrum indicated the presence of hydroxyl group in the molecule. The EIMS spectrum showed the presence of a molecular ion peak as the base peak at m/z 319 corresponding to the molecular formula $C_{19}H_{13}NO_4$. The ^{1}H-NMR spectrum showed the presence of seven aromatic protons, one methylenedioxy and one methoxyl group. The three sharp singlets, integrated for one proton each occurred at δ 9.63, 8.63 and 7.33 were assigned to H-8, H-1 and H-4, respectively. Two sets of *ortho*-coupled doublets were observed at δ 7.85 and 8.34 (J = 9.17 Hz) assigned to H-5 and H-6 and the other at δ 7.54 and 8.31 (J = 8.25 Hz) assigned to H-11 and H-12, respectively. Hence, the molecule has the characteristic skeleton of benzophenantridine moiety with the substitution occurred at C-2, C-3, C-9 and C-10 [7]. The ^{13}C-NMR spectrum indicated the presence of nineteen carbons, one OCH_3, one OCH_2O, seven C-H aromatic and 10 quaternary carbons further supported the molecular formula suggested. The assignments of all protons and carbons were established by COSY, HSQC, HMBC spectral data and

comparison with the literature values [2]. Decarine was previously reported to occur from *Zanthoxylum decaryi*.

Rutaecarpine **(2)** was obtained as white needle-shape crystals with melting point 264 - 265°C. The UV spectrum displayed absorptions at 224.0, 243.2, 344.6 and 362.2 nm. Prominent peaks at 3344 and 1654 cm^{-1} were observed in the IR spectrum due to the presence of NH and carbonyl groups, respectively. The EIMS showed the molecular ion peak at m/z 287 which corresponded to the molecular formula $C_{18}H_{13}N_3O$. The ^{1}H-NMR spectrum indicated the presence of one low field NH signal (δ 9.60), eight aromatic protons made up of two sets of signals and four aliphatic protons. The COSY spectrum indicated that the signals at δ 8.32 (1H, d, 8.24 Hz), 7.43 (1H, t, 8.24 Hz), 7.72 (1H, dd, 1.84, 8.24 Hz) and 7.68 (1H, d, 8.24) formed a spin system and were assigned to the aromatic protons H-4, H-3, H-2 and H-1, respectively. The second aromatic spin system was also made up of four protons, H-9, H-10, H-11 and H-12. The *ortho*-coupled protons at δ 7.63 (1H, d, 8.24 Hz) and δ 7.18 (1H, t, 8.24 Hz) were due to coupling of H-9 and H-10, respectively. The other *ortho*-coupled H-11 and H-12 gave resonances at δ 7.32 (1H, t, 8.24 Hz) and 7.38 (1H, d, 8.24 Hz), respectively. The DEPT, HSQC and HMBC spectra further supported the proton and carbon connectivities of **(2)**. These resonances were in accordance with the structure of rutaecarpine, which has been isolated previously from other species in Rutaceae family [8], [9].

Aurantiamide acetate **(3)** was obtained as white needles, with melting point 182 - 185 ^{0}C ([4], m.p. 188°C). The UV spectrum displayed an absorption band at 217 nm. The IR spectrum displayed characteristic peaks for the amide CO and acetate groups at (3296 and 1632) cm^{-1} and (1728 and 1256) cm^{-1}, respectively [10]. The EIMS displayed the molecular ion peak at m/z 444, consistent with the molecular formula $C_{27}H_{28}N_2O_4$.

The ^{1}H-NMR spectrum clearly indicated the presence of fifteen aromatic protons derived from the three aromatic rings, two protons of the CO-NH groups, two protons of unsaturated methine, six protons of the three methylene groups and three protons for a methyl group. The five aromatic protons, H-2'/6', H-4' and H3'/5', occurred at δ 7.72 (2H, d, 7.36 Hz), 7.53 (1H, t, 7.36 Hz) and 7.45 (2H, t, 7.36 Hz), respectively. In addition, the ten integrated aromatic protons at the region δ 7.07-7-30 were assigned to the other two phenyl protons. The two CO-NH groups which appeared as two doublets at δ 5.94 and 6.76 (J = 8.24 Hz) were due to H-5 and H-8, respectively. The quartet and multiplet signals at δ 4.35 and 4.76 each integrated for one proton were assigned to H-4 and H-7, respectively. One of the benzylic methylene protons, H-11, occurred at two different chemical shifts δ 3.05 (dd, 8.30 Hz, 13.30 Hz) and 3.22 (dd, 6.40 Hz, 13.30 Hz). Meanwhile the other methylene benzylic proton H-10 was observed as triplet at δ 2.75 (J= 6.40 Hz). The methylene protons at H-3 was observed to occur as doublet of doublet at δ 3.80 and 3.93 (J= 4.60 Hz, 11.00 Hz). The low field chemical shift appearance of these protons compared to other methylene protons was due to the inductive effect of the adjacent acetoxy group. The acetoxy methyl group occurred as three protons singlet at δ 2.03. The COSY spectrum clearly exhibited the various correlations of the protons along the amide chain. The methylene protons next to the acetoxy group H-3 showed correlation with methine proton H-4, which in turn correlate with H-10 and H-5. Similarly, the methine proton H-7 showed correlation with H-8 and H-11.

The ^{13}C-NMR spectrum indicated the presence of an acetoxy methyl C-1 at δ 20.81. The two methylene benzylic carbons of C-10 and C-11 occurred at δ 37.4 and 38.4, respectively. While the other methylene carbon C-3 occurred at low field δ 64.52 due to electronegative effect. The two methine carbons at δ 49.4 and 54.9 were assigned to C-4 and C-7, respectively. The three benzene ring carbons occurred as overlap signals in the region δ 126 - 136. The confirmation of this structure was carried out by analysis of the HMBC spectrum, which clearly exhibited the ^{13}C-^{1}H long range ^{2}J and ^{3}J correlations of H-8 to C-9; H-5 to C-6; H-7 to C-6 and C-11. The protons of methyl group (H-1) showed cross peaks only to C-2 supported that this compound has the acetoxy methyl groups. The methylene proton of H-10 showed the correlation to C-4 and C-1'', supported that the methylene group occurred adjacent of the benzyl groups. This pattern correlations were also observed at methylene protons of H-11, which exhibited the cross peak correlations to C-6, C-7 and C-1'''. The methine protons of H-2' and H-6' showed correlation to the carbonyl functional group of C-

9 which supported it occurrence adjacent to the benzoyl group. The complete assignments of these spectral data are summarized in Table 1. Based on this analysis and comparison with literature values [4], [10-11], the compound **(3)** was assigned as aurantiamide acetate, previously isolated from *Piper aurantiacum*, *Melicope bonwickii* and the red algae *Acantophora spicifera*.

The simple 7-hydroxycoumarin **(4)** was obtained as white needles with a melting point 224 - 225^{0}C ([5], m.p. 223 - 225°C) and under UV light the spot in the TLC plate was observed as a bright blue fluorescent spot. The UV spectrum showed the absorption bands at 221 and 323 nm, which were characteristic of 7-hydroxycoumarin. The IR spectrum supported the presence of carbonyl and hydroxyl functional groups, with the absorption bands at 1732 cm^{-1} 3418 cm^{-1}. EIMS spectrum showed a molecular ion peak at m/z 162, which corresponded to the molecular formula $C_9H_6O_3$.

Table 1. ^{1}H and ^{13}C NMR, COSY, HMBC Spectral Data of **(3)**.

	δ H (ppm)	δ C (ppm)	COSY	HMBC	δ H [9]	δ C [9]
1	2.03 (3H, s)	20.81		C-2	2.03	20.76
2		170.80				170.75
3	3.80 (1H, dd, 4.60, 11.00 Hz)	64.52	H-4		3.84	64.57
3	3.93 (1H, dd, 4.60, 11.00, Hz)	64.52	H-4		3.95	64.57
4	4.35 (1H, m)	49.37	H-3, H-5, H-10		4.20-4.47	49.46
5	5.94 (1H, d, 8.24 Hz)		H-4	C-6	6.1	
6		170.14				170.31
7	4.76 (1H, m)	54.94	H-8, H-11	C-6, C-11	4.78	49.46
8	6.76 (1H, d, 8.24 Hz)		H-7	C-9	6.85	
9		167.05				167.13
10	2.75 (2H, t, 6.4 Hz)	37.37	H-4		3.07	37.43
11	3.05 (1H, dd, 8.30, 13.30 Hz)	38.40	H-7		3.22	37.43
11	3.22 (1H, dd, 6.40 13.30 Hz)	38.40	H-7		2.74	37.43
1'		133.55				133.66
2'/6'	7.72 (2H, d, 7.36 Hz)	127.01	H-3'/5', H-4'	C-9	7.12-7.78	128.56
3'/5'	7.45 (2H, t, 7.36 Hz)	128.72	H-2'/6'		7.12-7.78	128.61
4'	7.53 (1H, t, 7.36, Hz)	131.92	H-2'/6'		7.12-7.78	131.88
1"		136.54				136. 60
1"'		136.62				136. 70
10 H	7.06-7.73	128.60- 136.62			7.12-7.78	127.05-136.70

CHEMOTAXONOMIC SIGNIFICANCE

Decarine **(1)** is a phenantridine alkaloid, which has also been found to occur in the genera *Zanthoxylum* L, *Phellodendron Rupr.*, *Toddalia Juss*, and *Fagaropsis Mildb* [7]. The finding of this type of alkaloid further supported the indication that *Tetradium* has philogenetic relation to *Zanthoxylum* and *Phellodendron* in a linear sequence [1]. This further supports Hartley's decision to reassign the plant as *Tetradium sambucinum*. Rutaecarpine **(2)**, an indoloquinozoline alkaloid, has been reported previously to occur in *Evodia officinalis* [9]. Hartley has also reassigned this species as *Tetradium ruticarpum* [1]. The occurrence of coumarin, such as 7-hydroxycoumarin, is quite common in Rutaceae. The dipeptide, aurantiamide acetate **(3)**, originally isolated from *Piper aurantiacum* but was subsequently isolated also from *Melicope hayesii* and red algae (*Acantophora spicifera*) [4], [5], [10]. The identification of this compound further supported the link between *Tetradium*, *Euodia* and *Melicope* [12].

Both compounds **(1)** and **(2)** displayed a low level of antioxidant activity in the DPPH assay with IC_{50} values of 75 and 95 μg/ml, respectively. However, compound **(1)** was found to be active against the Hela cell line with an IC_{50} value of 14.6 μg/ml.

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