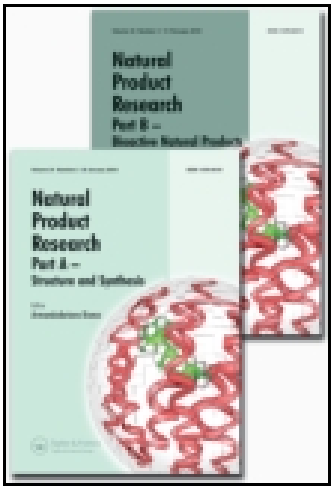

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Chemical constituents and antibacterial activity of Melastoma malabathricum L.

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Chemical constituents and antibacterial activity of *Melastoma malabathricum* L.

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The aqueous methanolic extracts of *Melastoma malabathricum* L. exhibited antibacterial activity when assayed against seven microorganisms by the agar diffusion method. Solvent fractionation afforded active chloroform and ethyl acetate fractions from the leaves and the flowers, respectively. A phytochemical study resulted in the identification of ursolic acid (**1**), 2α-hydroxyursolic acid (**2**), asiatic acid (**3**), β-sitosterol 3-*O*-β-*D*-glucopyranoside (**4**) and the glycolipid glycerol 1,2-dilinolenyl-3-*O*-β-*D*-galactopyanoside (**5**) from the chloroform fraction. Kaempferol (**6**), kaempferol 3-*O*-α-*L*-rhamnopyranoside (**7**), kaempferol 3-*O*-β-*D*-glucopyranoside (**8**), kaempferol 3-*O*-β-*D*-galactopyranoside (**9**), kaempferol 3-*O*-(2″,6″-di-*O*-*E*-*p*-coumaryl)-β-*D*-galactopyranoside (**10**), quercetin (**11**) and ellagic acid (**12**) were found in the ethyl acetate fraction. The structures of these compounds were determined by chemical and spectral analyses. Compounds **1**–**4**, the flavonols (**6** and **11**) and ellagic acid (**12**) were found to be active against some of the tested microorganisms, while the kaempferol 3-*O*-glycosides (**7**–**9**) did not show any activity, indicating the role of the free 3-OH for antibacterial activity. Addition of *p*-coumaryl groups results in mild activity for **10** against *Staphylococcus aureus* and *Bacillus cereus*. Compounds **2**–**5**, **7** and **9**–**12** are reported for the first time from *M. malabathricum*. Compound **10** is rare, being reported only once before from a plant, without assignment of the double bond geometry in the *p*-coumaryl moiety.

Keywords: *Melastoma malabathricum*; Sendudok; pentacyclic triterpenoids; acylated kaempferol glycosides; antibacterial activity

1. Introduction

Melastoma malabathricum L. (Melastomataceae, Malay name: Sendudok) is a small shrub widely used in the traditional medicine of Malaysia and Indonesia for treating diarrhoea, dysentery, leucorrhoea, ulcers and wounds (Burkill, 1966; Perry, 1980). The methanolic extract of the aerial parts was reported to have a moderate antiviral activity against HSV-1, a significant activity against Poliovirus and a cytotoxic effect

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($IC50 \leq 25\,\mu g\,ml^{-1}$) on murine cell lines (Lohezic-Le Devehat, Bakhtiar, Bezivin, Amoros, & Boustie, 2002). The ethanolic extract of the stem bark and leaves was found to be potentially antinociceptive, acting at both the peripheral and central levels in mice (Sulaiman, Somchit, Israf, Ahmad, & Moina, 2004). The flowers were shown to contain antioxidant and cytotoxic flavonoids (Susanti et al., 2007). Previous phytochemical studies have revealed the presence of malvidin 3,5-diglucoside (Lowry, 1976), 4′-methylpeonidin 7-*O*-*β*-*D*-glucoside (Mohandoss & Ravindran, 1993), kaempferol (Das & Kotoky, 1988; Mohandoss & Ravindran, 1993; Susanti et al., 2007), kaempferol 3-*O*-*β*-*D*-xyloside (Mohandoss & Ravindran, 1993), kaempferol 3-*O*-glucoside, kaempferol 3-*O*-(2″,6″-di-*O*-*p*-*trans*-coumaroyl) glucoside and naringenin (Susanti et al., 2007), *p*-hydroxybenzoic acid and gallic acid (Das & Kotoky, 1988) in the flowers, *β*-sitosterol and melastomic acid in the roots (Manzoor-I-Khuda, S.A. Chowdhury, Reza, & A.K. Chowdhury, 1981), quercetin 3-*O*-*α*-*L*-rhamnosyl-(1→2)-*β*-*D*-galactoside (Dinda & Saha, 1988), 1-octyl docosanoate, 11-methyl-1-tritriacontanol (Dinda & Saha, 1986) in the aerial parts, 32-methyl-1-tritriacontanol, *β*-sitosterol, ursolic acid (Das & Kotoky, 1988) and several tannins (Yoshida, Nakata, Hosotani, Nitta, & Okuda, 1992a, 1992b) in the dried leaves. The extensive use of *M. malabathricum* in traditional medicine led us to carry out further phytochemical investigations of this plant, guided by antibacterial activity assay. In this article we report the isolation of 12 compounds from *M. malabathricum*, some of them possessing antibacterial activity. Nine of the isolated compounds are reported for the first time in this plant.

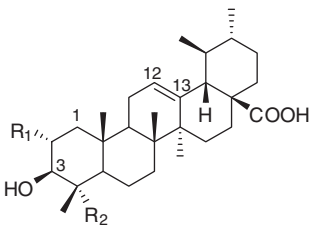
2. Results and discussion

2.1. *Extraction and isolation*

The aqueous methanolic extracts of the fresh leaves and flowers of *M. malabathricum* L. were evaluated for antibacterial activity by the agar diffusion method. Guided by this activity, solvent fractionation and isolation were carried out by silica gel and Sephadex column chromatography using different solvent systems. The isolated structures were confirmed by comparison of their spectral data (UV, IR, MS, ^{1}H- and ^{13}C-NMR) with literature data (Collins, Pilotti, & Wallis, 1992; Furuya, Orihara, & Hayashi, 1987; Kriwacki & Pitner, 1989; Li, Elsohly, Hufford, & Clark, 1999; Mabry, Markham, & Thomas, 1970; Markham, Ternai, Stanley, Geiger, & Mabry, 1978; Sakakaibara, Kaiya, Fukuda, & Ohki, 1983; Seo, Tomita, & Tori, 1981; Ternai & Markham, 1976; Yamagishi et al., 1988), 2D NMR, chromatographic methods (GC–MS, TLC, PC) and chemical analyses.

The chloroform fraction (fr-2) of the aqueous methanolic extract of the leaves (ext-1) gave ursolic acid (**1**), 2*α*-hydroxyursolic acid (**2**), asiatic acid (**3**), *β*-sitosterol 3-*O*-*β*-*D*-glucopyranoside (**4**) and glycerol 1, 2-dilinolenyl-3-*O*-*β*-*D*-galactopyranoside (**5**). The ethyl acetate fraction (fr-3) of the aqueous methanolic extract of the flowers (ext-2) afforded kaempferol (**6**), kaempferol 3-*O*-*α*-*L*-rhamnopyranoside (**7**), kaempferol 3-*O*-*β*-*D*-glucopyranoside (**8**), kaempferol 3-*O*-*β*-*D*-galactopyranoside (**9**), kaempferol 3-*O*-(2″,6″-di-*O*-*E*-*p*-coumaryl)-*β*-*D*-galactopyranoside (**10**), quercetin (**11**) and ellagic acid (**12**). The triterpenoids (**2**–**4**), glycolipid (**5**), flavonoids (**7**, **9**–**11**) and ellagic acid (**12**) are reported for the first time from *M. malabathricum*.

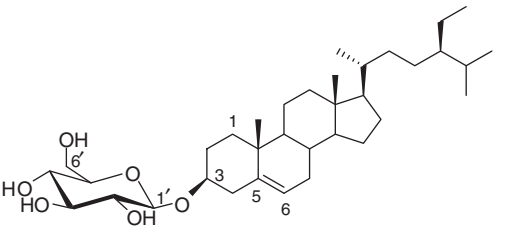
Compound (**10**) is uncommon, being reported only once before in a related species (Chou & Liao, 1982), but the configuration of the olefinic function of the *p*-coumaryl moiety was not specified (CAS Registry Number: 84461-47-2). In this study the olefinic protons of the two *p*-coumaryl groups were observed as two pairs of doublets at 7.69 and 6.38 ppm (3‴, 2‴), and 7.39 and 6.05 ppm (3⁗, 2⁗) with a coupling constant (*J*) 15.9 Hz (Figure S1). The large value of *J* indicated the *E*-configuration of the *p*-coumaryl moiety (Silverstein, Bassler, & Morrill, 1991).



1: R_1 = H, R_2 = Me

2: R_1 = OH, R_2 = Me

3: R_1 = OH, R_2 = CH_2OH



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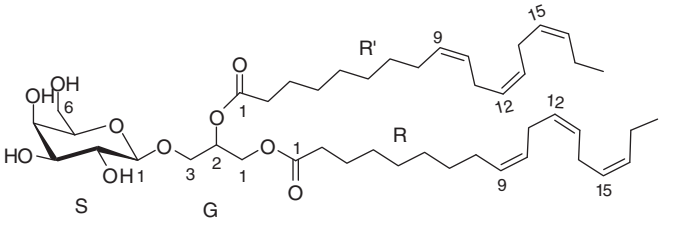

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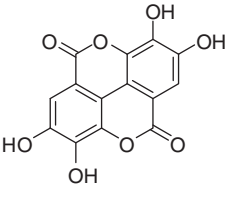

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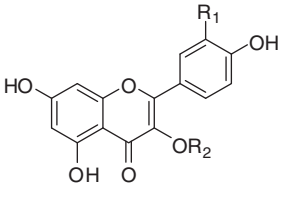


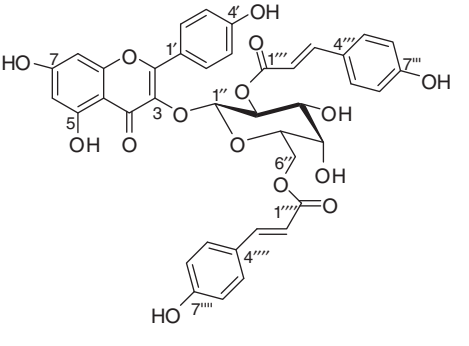
6: R_1 = R_2 = H

7: R_1 = H, R_2 = rha

8: R_1 = H, R_2 = glc

9: R_1 = H, R_2 = gal

11: R_1 = OH, R_2 = H



10

Scheme 1. Compounds **1** to **12**.

2.2. *Antibacterial activity*

The aqueous methanolic extracts of the leaves (ext-1) and flowers (ext-2) of *M. malabathricum* exhibited antibacterial activity when tested by the agar diffusion method (Table 1). From ext-1 an active precipitate (fr-1) was obtained which was extracted with chloroform to give fraction fr-2. Five compounds (**1**–**5**) were isolated from fr-2 of which **1**–**4** are triterpenoids active against some of the tested microorganisms, while **5** is an inactive glycolipid. Compound **3**, at concentrations ten times those of refampicin, showed activity against *Bacillus cereus* comparable to that shown by this reference antibiotic (Table 1). ext-2 was extracted with ethyl acetate to give fraction fr-3 from which seven compounds (**6**–**12**) were obtained. Among these are two flavonols (**6** and **11**) and ellagic acid (**12**), which are active compounds, and three kaempferol 3-*O*-glycosides (**7**–**9**), which did not show any activity indicating the role of the free 3-OH for antibacterial activity. The seventh compound is the diacylated kaempferol 3-*O*-glycoside (**10**). It appears that the addition of two *p*-coumaryl groups results in a mild activity for **10** against *Staphylococcus aureus* and *B. cereus* as shown in Table 1. The presence of *p*-coumaryl group has been seen before to dramatically increase the dermatitis activity of astragalin (Loggia, Negro, Bianchi, Romussi, & Tubaro, 1989). The results of extracts and pure compounds which possess partial activity are reported within parentheses in Table 1.

3. Experimental

3.1. *General*

Melting points were determined using a Gallenkamp apparatus. FTIR spectra were recorded on a Perkin–Elmer System 2000 spectrometer. EI–MS and GC–MS were recorded on a Hewlett Packard 5989 A. NMR spectra were recorded in deuterated methanol (Sigma Chem. Co.), chloroform (Aldrich) or pyridine (Merck), by a Bruker Avance 300 or a Bruker Avance 400 spectrometer operated at 300 and 400 MHz for ^{1}H NMR and 75 and 100 MHz for ^{13}C NMR, respectively. UV spectra of the flavonoids were measured in methanol, using a Jasco-V-530 UV/Vis spectrophotometer as described in Mabry et al. (1970). TLC was carried out on pre-coated silica gel 60 F_{250} plates (Merck). Column chromatography was carried out using silica gel (Riedel-deHaen 230-400 mesh, ASTM) and Sephadex LH-20 (Sigma-Aldrich). Paper chromatography was carried on Whatman No. 54. All solid compounds isolated were purified by recrystallisation in acetone (**1**, **2**, **3** and **4**), methanol (**6**, **8**, **9** and **12**) or acetone-chloroform (**7** and **10**). Compound **5** was purified by repeated CC.

3.2. *Plant material*

M. malabathricum was collected from Penang Island, Malaysia. The plant was identified by Professor Lai-Keng Chan, School of Biological Sciences, Universiti Sains Malaysia, where a voucher specimen (No. 10613) was deposited.

Table 1. Antibacterial activity of extracts and isolated compounds from *M. malabathricum*.

Sample	Conc (mg mL^{-1})	Zone of inhibition (diameter, mm, average of duplicate)						
		S. aureus	*B. subtilis*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. typhi*	*K. pneumoniae*
ext-1	40	15.0 (23.5)[a]	13.0	14.0 (27.0)	–[b]	09.0	15.5	13.0
	20	12.5 (18.0)	12.0	11.0 (25.0)	–	–	13.0	11.0
	10	10.0 (13.5)	11.0	08.0 (22.0)	–	–	12.0	09.0
	5	09.0 (11.0)	10.0	07.0 (19.0)	–	–	11.0	08.0
fr-1	40	10.0 (25.5)	14.5	10.0 (24.5)	12.0	12.0	13.0	10.0
	20	09.0 (20.0)	11.5	08.0 (21.5)	–	11.5	11.5	09.0
	10	08.0 (18.0)	11.0	07.0 (18.5)	–	11.5	10.5	–
	5	07.0	09.5	– (16.0)	–	09.5	09.0	–
fr-2	40	12.0 (19.5)	17.0	– (12.0)	12.0	12.5	13.0 (19.5)	12.5
	20	10.5	12.5	– (11.0)	11.0	11.5	11.5	11.0
	10	10.0	10.0	–	10.5	10.0	10.0	10.0
	5	08.0	08.5	–	–	08.0	09.0	09.0
1	2	10.5	10.5	07.0	–	–	11.0	–
	1	09.5	09.5	07.0	–	–	10.0	–
	0.5	08.5	09.0	07.0	–	–	10.0	–
	0.25	07.5	08.0	07.0	–	–	09.5	–
2	2	12.5	–	07.5	–	11.5	–	–
	1	11.0	–	07.5	–	10.5	–	–
	0.5	10.0	–	07.5	–	10.0	–	–
	0.25	0.90	–	07.5	–	09.0	–	–
3	2	11.0	–	13.5	–	–	–	09.0
	1	10.5	–	12.0	–	–	–	08.0
	0.5	10.0	–	11.5	–	–	–	–
	0.25	09.0	–	10.0	–	–	–	–
4	2	08.0	–	10.5	–	10.5	10.0	–
	1	07.0	–	–	–	09.0	09.0	–
	0.5	–	–	–	–	09.0	–	–
	0.25	–	–	–	–	09.0	–	–

(*continued*)

Table 1. Continued.

Sample	Conc (mg mL^{-1})	Zone of inhibition (diameter, mm, average of duplicate)						
		S. aureus	*B. subtilis*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. typhi*	*K. pneumoniae*
ext-2	20	15.5 (28.5)	16.5 (24.5)	13.0	19.5	19.5 (24.5)	18.0 (25.0)	14.0
	10	13.0 (24.5)	15.0 (22.0)	09.0	18.0	18.0 (22.0)	14.0 (20.5)	12.5
	5	11.0 (19.5)	11.5 (18.5)	07.0	15.5	16.0 (19.5)	12.0 (19.5)	11.5
	2.5	09.5 (15.5)	10.0 (14.5)	–	14.0	12.5	11.0 (16.0)	10.0
fr-3	20	16.0 (31.5)	18.0 (30.0)	11.0 (22.0)	18.5	17.5 (25.5)	– (29.5)	16.5 (36.0)
	10	14.0 (27.5)	16.0 (27.0)	10.0 (20.0)	15.5	16.0 (23.0)	– (25.0)	14.5 (32.0)
	5	12.0 (26.0)	15.0 (23.5)	08.0 (18.0)	14.0	14.0 (20.5)	– (22.0)	13.5 (28.0)
	2.5	10.5 (22.5)	10.0 (18.5)	07.0 (14.5)	12.5	13.0 (18.5)	– (19.0)	13.0 (24.0)
6	2	13.0	–	12.0	11.0	–	–	–
	1	12.5	–	12.0	–	–	–	–
	0.5	11.0	–	10.0	–	–	–	–
	0.25	10.0	–	09.0	–	–	–	–
10	2	09.0 (11.0)	–	11.0	–	–	–	9
	1	08.5 (10.0)	–	10.0	–	–	–	8
	0.5	07.5 (10.0)	–	09.5	–	–	–	–
	0.25	06.0 (09.0)	–	08.5	–	–	–	–
11	2	13.0 (21.0)	18.0	13.0	16.0	14.0	10.5	12.5
	1	11.0 (19.0)	16.0	12.0	14.0	12.0	10.0	10.5
	0.5	09.0 (16.0)	13.0	10.0	13.0	10.0	09.0	09.5
	0.25	08.0 (14.0)	10.0	08.0	11.0	08.0	–	09.0
12	2	15.0 (29.5)	25.5	– (21.5)	–	22.5	– (31.0)	– (36.0)
	1	15.0 (29.0)	24.5	– (20.0)	–	21.0	– (29.0)	– (32.0)
	0.5	14.0 (28.0)	22.0	– (19.0)	–	19.5	– (27.5)	– (28.0)
	0.25	13.0 (25.5)	20.0	– (18.0)	–	17.5	– (21.5)	– (24.0)
Ref[c]	0.2	30.0	16.0	14.0	15.0	10.0	14.0	14.0
	0.1	28.5	13.5	13.0	12.0	08.5	12.0	12.0
	0.05	27.5	11.5	12.0	11.0	07.5	10.0	10.0
	0.025	26.0	09.5	11.0	10.0	– (07.5)	09.0	09.0
Con[d]		–	– (09.0)	–	– (10.0)	– (07.5)	– (09.0)	– (07.0)

Notes: [a]Values within parentheses for partial activity. [b]–, no activity. [c]Ref, reference antibiotic (refampicin). [d]Con, control (DMSO), partial activity.

3.3. *Extraction and isolation*

Fresh leaves (3 kg) were homogenised in MeOH–H_2O (4 : 1) at room temperature. The slurry was filtered and the filtrate was concentrated under reduced pressure at a temperature <40°C to 1/10 of its original volume, using a rotary evaporator, to give the methanolic extract (ext-1) (193.5 g). When chilled and centrifuged, (ext-1) gave the precipitate (fr-1) (40.2 g). The precipitate (fr-1) was defatted with petroleum ether and extracted with chloroform (300 mL × 2 and 200 mL × 2). The chloroform extract (fr-2) (16.1 g) was fractionated by silica gel column chromatography (200 g), eluting with chloroform–acetone–methanol (18 : 6 : 1), chloroform–acetone–methanol (10 : 9 : 1) and acetone successively. The eluent was collected in 25 mL fractions. Fractions 15–19, which eluted with chloroform–acetone–methanol (18 : 6 : 1), yielded ursolic acid (**1**) (405 mg). Fractions 25–30 yielded 2α-hydroxyursolic acid (**2**) (96 mg). Fractions 46–50, which eluted with chloroform–acetone–methanol (10 : 9 : 1), yielded asiatic acid (**3**) (42.5 mg) and glycerol 1, 2-dilinolenyl-3-*O*-β-*D*-galactopyranoside (**5**) (1.35 g). Fractions 68–75, which eluted with acetone, yielded β-sitosterol 3-*O*-β-*D*-glucopyranoside (**4**) (111 mg).

The purple-magenta flowers (250 g) were defatted with petroleum ether and extracted with MeOH–H_2O (9 : 1) at room temperature. The solvent was removed under reduced pressure at a temperature <40°C to give a solid (ext-2) (13.0 g) which was extracted with ethyl acetate. The ethyl acetate extract (fr-3) (1.0 g) was fractionated on a Sephadex LH-20 column (20 g) using methanol. The eluent was collected in 20 ml fractions. Fractions 7–9 were chromatographed on a silica gel column (60 g) using dichloromethane–acetone–methanol (10 : 10 : 2), yielding kaempferol (**6**) (21 mg), kaempferol 3-*O*-α-*L*-rhamnopyranoside (**7**) (37 mg), kaempferol 3-*O*-β-*D*-glucopyranoside (**8**) (52 mg) and kaempferol 3-*O*-β-*D*-galactopyranoside (**9**) (8 mg). Fractions 10–12 precipitated ellagic acid (**12**) (14 mg) on concentration, while kaempferol 3-*O*-(2″,6″-di-*O*-*E*-*p*-coumaryl)-β-*D*-galactopyranoside (**10**) (41 mg) was recovered from the resulting solution. Fractions 13–15, on passing through a Sephadex LH-20 column eluting with methanol, yielded quercetin (**11**) (5 mg).

3.4. *Spectral data*

Kaempferol 3-*O*-(2″,6″-di-*O*-*E*-*p*-coumaryl)-β-*D*-galactopyranoside (**10**): Pale yellow powder, m.p. 194°C (dec) (CH_3COCH_3–$CHCl_3$); IR (KBr) ν_{max}: 3391, 1696, 1654, 1630, 1604, 1589, 1513, 1444, 1359, 1259, 1204, 1171, 1080, 982, 830 cm^{-1}; EI-MS *m*/*z* (rel. int.): 432 (0.2), 342 (0.7), 311 (2.4), 286 (42.0), 258 (4.6), 257 (5.3), 229 (6.6), 213 (6.2), 164 (35.8), 147 (41.2), 121 (28.3), 91 (19.5), 65 (12.4) and 44 (100); UV λ_{max} nm (MeOH): 268, 299, 313, 353, 366, (MeOH + NaOMe): 274, 310, 366, (MeOH + $AlCl_3$): 276, 315, 398, (MeOH + $AlCl_3$/HCl): 278, 317, 392, (MeOH + NaOAc): 275, 299, 314, 370 and (MeOH + NaOAc/H_3BO_3): 268, 301, 317, 365; ^{1}H NMR (methanol-d_4, 400 MHz) δ (ppm): 6.05 (*d*, 1.9, 1 H, H-6), 6.21 (*d*, 1.9, 1 H, H-8), 7.93 (*d*, 8.8, 2 H, H-2′,6′), 6.86 (*d*, 8.8, 2 H, H-3′,5′), 5.50 (*d*, 8.0, 1 H, H-1″), 5.37 (*dd*, 1.6, 8.0, 1 H, H-2″), 3.78-3.84 (*m*, 1 H, H-3″), 3.88 (*d*, 2.9, 1 H, H-4″), 3.78-3.84 (*m*, 1 H, H-5″), 4.37 (*dd*, 3.2, 11.5, 1 H, H-6″), 4.19 (*dd*, 4.0, 11.5, 1 H, H-6″), 6.38 (*d*, 15.9, 1 H, H-2‴), 7.69 (*d*, 15.9, 1 H, H-3‴), 7.46 (*d*, 8.6, 2 H, H-5‴,9‴), 6.81 (*d*, 8.6, 2 H, H-6‴,8‴), 6.05 (*d*, 15.9, 1 H, H-2⁗), 7.39 (*d*, 15.9, 1 H, H-3⁗), 7.26 (*d*, 8.6, 2 H, H-5⁗,9⁗) and 6.78 (*d*, 8.6, 2 H, H-6⁗,8⁗); ^{13}C-NMR (methanol-d_4, 75 MHz)

δ (ppm): 158.4 (C-2), 134.7 (C-3), 179.2 (C-4), 162.9 (C-5), 100.4 (C-6), 167.0 (C-7), 95.0 (C-8), 158.3 (C-9), 105.3 (C-10), 122.7 (C-1′), 132.1 (C-2′,6′), 116.3 (C-3′,5′), 161.5 (C-4′), 101.1 (C-1″), 74.0 (C-2″), 73.2 9 C-3″), 70.6 (C-4″), 75.0 (C-5″), 64.2 (C-6″), 168.8 (C-1‴), 115.2 (C-2‴), 147.0 (C-3‴), 127.2 (C-4‴), 131.2 (C-5‴,9‴), 116.8 (C-6‴,8‴), 161.3 (C-7‴), 168.8 (C-1⁗), 114.5 (C-2⁗), 146.7 (C-3⁗), 127.0 (C-4⁗), 131.2 (C-5⁗,9⁗), 116.8 (C-6⁗,8⁗) and 161.3 (C-7⁗) (see also Supplementary Figures S1–S6).

3.5. *Acid hydrolysis of compounds 4 and 5*

Four mg of **4** and 10 mg of **5** were separately placed in a 50 mL round bottom flask. Ten millilitres of 2 M HCl in methanol were added and the mixture was refluxed on a water bath (80°C) for three hours, and then cooled and diluted with 5 mL of water. The aglycones were extracted with chloroform (5 mL × 3) while the aqueous layer gave the monosaccharides after evaporation under reduced pressure. The sugars were identified by comparison with authentic monosaccharides on PC using solvent systems butanol–acetic acid–water (4 : 1 : 5, top layer) and butanol–toluene–pyridine–water (5 : 1 : 3 : 3), and silica gel TLC, using butanol–acetic acid–diethyl ether–water (9 : 6 : 3 : 1).

3.6. *Acid hydrolysis of the flavonoids 7–10*

Three milligrams of each compound were dissolved in 150 μL MeOH and 150 μL 2 M H_2SO_4 was added. After letting it stand for 20 min at 100°C, the MeOH was evaporated off and 1 mL of water was added. The aqueous layer was extracted 3 times with 300 μL Et_2O to afford the aglycone, while the aqueous layer was neutralised with $CaCO_3$, collected using a Pasteur pipette and concentrated to give the sugars. The sugars were identified by comparison with authentic monosaccharides on silica gel TLC, using the solvent systems *n*-butanol–acetic acid–diethyl ether–water (9 : 6 : 3 : 1) and ethyl acetate–acetic acid–methanol–water (13 : 4 : 3 : 3).

3.7. *Trans-esterification of compound 5 and 10*

Three to nine milligrams of each compound were placed in a 5 mL round bottom flask. 0.5 mL of 2 M methanolic sodium hydroxide was added and the mixture was refluxed for 5 min on a water bath (80°C). 0.5 mL of BF_3 solution (10% in methanol) was then added and the reflux was continued for 2 min, followed by the addition of 1 mL of heptane and further refluxing for 1 min. The mixture was then cooled, transferred into a small test tube and after addition of a small amount of a saturated solution of sodium chloride, gently swirled and allowed to separate. The organic layer, which contained the methyl ester, was collected by a Pasteur pipette. The methyl esters were identified by comparison of their retention times and mass spectra with those of authentic methyl linolenate and methyl (*E*)-*p*-coumarate by TLC and GC–MS analyses.

3.8. *Antibacterial activity*

Antibacterial activity was carried out by the agar diffusion method (Barry, 1991). Different concentrations of the test materials were prepared in DMSO and 5 mm diameter wells in the agar were filled with 60 μL of each sample. Refampicin was used as the reference compound. Seven microorganisms were used: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCTC 8236, *Bacillus cereus* ATCC 10876, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 17853, *Salmonella typhi* (lab. strain) and *Klebsiella pneumoniae* (lab. strain). The microorganisms were provided by the Microbiology Laboratory, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Malaysia. The experiments were carried out in duplicate.

Supplementary material

Additional spectral data relating to this article is available online, alongside Figures S1–S6.

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