Malaysian Journal of Chemistry, 2023, Vol. 25(4), 155-164

Phytochemical Study of the Leaves of *Muntingia calabura* and their Antibacterial Activity

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Muntingia calabura (Eleocarpaceae) is a medicinal plant that has gained interest due to its various pharmacological activities. The aim of this work was to isolate chemical constituents from the leaves of *M. calabura* and evaluate their antibacterial activity. Initially, the dried leaves were extracted with 95% ethanol, followed by water/ethyl acetate (EA) partitioning. The EA crude extract was then subjected to continuous column chromatography over silica gel. Antibacterial activity was determined by micro broth dilution. Ten known flavonoids comprising three flavanones {(2*S*,3*S*) 3,5-dihydroxy-7-methoxyflavanone (**FA1**), (2*R*,3*R*) 3,5,7-trihydroxyflavanone (**FA2**), (2*S*) 7,8-dihydroxyflavanone (**FA3**)}, three chalcones {2',4'-dihydroxy-3'-methoxychalcone (**C1**), 2',4'-dihydroxychalcone (**C2**), 2',3',4'-trihydroxychalcone (**C3**)} and four flavones {3,5-dihydroxy-7,8-dimethoxyflavone (**F1**), 3,5,7-trihydroxy-8-methoxyflavone (**F2**), 5-hydroxy-3,7-dimethoxyflavone (**F3**), 5,7-dihydroxyflavone (**F4**)} were obtained from this work. The structures were identified using spectroscopic methods and compared with literature data. Among the isolates, compounds **FA1**, **FA3**, and **C3** were isolated for the first time from *M. calabura*. Antibacterial activity showed that compounds **C1** and **C2** exhibited moderate activity with a MIC value of 32.5 µg/ml against *Bacillus cereus*. This study can serve as reference guide for drug development and highlights the potential of *M. calabura* leaves as an antibacterial agent.

Keywords: Antibacterial activity; chalcone; flavanone; flavone; *Muntingia calabura*

Received: March 2023; Accepted: May 2023

Historically, natural products are the most significant sources of new leads for pharmaceutical development, and these are mostly from plants [1, 2]. Around 80 % of people in developing countries still rely on traditional medicines as their primary healthcare source. Medicinal plants are vital therapeutic aids to alleviate illness, and approximately 85 % of traditional medicines consist of plant extracts [3]. The interest in herbal medicine and its products has increased, and this has stimulated scientists to explore and understand the pharmacologically-active constituents in medicinal plants [4].

Secondary metabolites such as polyphenols, flavonoids, quinones and tannins are sources of plant-based natural products used in medicine. They are known for their potent cytotoxic, antimicrobial and antioxidant activities [5]. Plants have produced many important drugs, from morphine that was discovered in the early nineteenth century to the recently discovered paclitaxel and artemisinin [4]. McChesney et al. [1] reported that around 10,000 of the world's plants were documented in medicinal use, however only roughly 150-200 of such agents are incorporated in western medicine. This indicates that there could be many more compounds in plants that may be useful for drug development. In addition, Mahmood et al. [3] reported that there were many medicinal plants yet to be investigated for possible valuable pharmaceutical application.

Muntingia calabura, known as 'kerukup siam' in Malay, is a common plant in Malaysia which has recently gained medicinal plant status [3]. It is a flowering plant from the Elaecarpaceae family and the only species from the genus *Muntingia*. It is a fast-growing tree that can reach a height of around 7.5–12 m with its branches spreading nearly horizontally. *M. calabura* was reported to have medicinal values according to Peruvian folklore medicine [6]. Various parts of this tree were documented in Southeast Asia and tropical America as having medicinal uses [7, 8]. The flowers are used to treat headaches, and as an antispasmodic, antidyspeptic and diaphoretic in the Philippines. In Colombia, flower infusions are consumed as a tranquillizer and tonic. The roots of this species are used as an abortifacient in Malaysia and as an emmenagogue in Vietnam [7]. The barks are boiled in water and used to reduce swelling in the hands and feet. Its leaves are used as a tea-like beverage to reduce swelling of the prostate gland or to relieve gastric ulcers. In addition, the leaves are also used to alleviate colds and headaches [6].

Research into the leaves, stem-bark and roots of *M. calabura* from Thailand, Philippines, Peru, Taiwan and Malaysia has revealed the presence of several flavanones, flavones and chalcones. The isolated compounds were evaluated for their cytotoxic activity [9, 10], antiplatelet effects [11], antibacterial [12], antinociceptive [13] and anti-inflammatory [14] activities. Surjowardojo *et al.* [15] concluded that *M. calabura* leaf extract had potential as an antimicrobial agent and that its bioactive constituents were epigallocatechin gallate and genistein. Buhian *et al.* [5] conducted phytochemical screening of the leaves and stems of *M. calabura* and showed that the ethanol extract of its leaves and stems had potential as antibacterial agents.

Previous research has provided only limited information about the antibacterial activity of the compounds in *M. calabura*. Therefore, in this study we attempt to perform a phytochemical investigation of *M. calabura* leaves and evaluate their antibacterial activity. From this study, three flavonoids were isolated for the first time from these leaves, while the chalcones obtained exhibited moderate activity against *Bacillus cereus* and *Bacillus subtilis*.

EXPERIMENTAL

Chemicals and Materials

Solvents (industrial grade) were used without further purification. 95% ethanol (QRec, Malaysia), hexane (Euro-chemo Pharma, Malaysia), ethyl acetate (Euro-Chemo Pharma, Malaysia), chloroform (Merck, Germany), dichloromethane (QRec, Malaysia), and Silica gel 60 (Carl Roth Kieselgel, 230-240 mesh) were used for column chromatography. Silica gel 60 F254 TLC aluminium sheets (Merck) was used for thin layer chromatography. Muller Hinton Agar (MHA, Microbiology grade, Merck, Germany), Muller Hinton Broth (MHB, Microbiology grade, Merck, Germany), Gentamicin (Bio Basic, Canada), DMSO (AR grade, Merck) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, AR grade, System) were used for antibacterial activity tests.

Instrumentation

NMR spectra were determined on a JOEL JNM-ECX 400 MHz spectrometer (Japan). UV-vis spectra were measured using a Shimadzu UV-1700 spectrophotometer (Japan) in a quartz cuvette (10 mm) in MeOH. IR spectra were determined using a Perkin-Elmer Spectrum RX1 IR spectrometer (United States) with KBr pellets. Mass spectra (HRMS, ESI) were determined using Agilent Technologies G6520B Accurate-Mass-Q-TOF LC-MS, United States. The specific rotation of chiral compounds was obtained on a Jasco 43 Europe P-2000 digital polarimeter, Japan. Melting points were determined using a Stuart SMP 10 Melting Point Instrument (United Kingdom) and were uncorrected. The bacterial suspension was adjusted to give a final organism density of 0.5 McFarland scale using a Thermo Scientific, Genesys10S UV-vis spectrophotometer (United States).

Plant Materials

The *Muntingia calabura* leaves were collected in Kampar, Perak in July 2011. They were identified by a plant botanist, Prof. Dr. Rusea Go, from Universiti Putra Malaysia. A voucher specimen (RG 6084) was deposited at the Herbarium Biology Department, Faculty of Science, Universiti Putra Malaysia (UPM).

Microbial strains

The antibacterial activity of the isolated compounds were evaluated against Gram-positive and Gram-negative bacteria strains i.e *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 13061, *Staphylococcus aureus*, ATCC 6528, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella* Typhimurium ATCC 14028 respectively. Bacteria were grown in Mueller Hinton broth at 37 °C and maintained in a nutrient agar at 4 °C until use.

Extraction and Isolation

3 kg of dried *M. calabura* leaves were extracted with 95 % ethanol (4 x 4 L) at room temperature for one week. The obtained ethanol crude extract was partitioned with water (4 x 1 L)/ethyl acetate (4 x 1 L) in a 1:1 ratio. The ethyl acetate crude extract (198.13 g) was then subjected to column chromatography (8 x 50 cm) over silica gel. 326 fractions were obtained by eluting with hexane, while gradually increasing the polarity with ethyl acetate (98:2, 94:6, 90:10, 75:25, 70:30, 50:50, 0;100), followed by increasing the polarity of ethyl acetate in ethanol (99:1, 90:10, 75:25, 50:50, 20:80, 0:100). The obtained fractions were chromatographed by TLC. Fractions that showed similar TLC profiles were combined to give seventeen major fractions (MF1-MF17).

From the 326 fractions, solids were obtained after some fractions were evaporated. Fraction 47 (1.67 g) was obtained as solid and further fractionated over a silica gel column (2 cm x 50 cm) eluted with hexane:acetone (95:5) to give compound **FA1** (50 mg) and with hexane:acetone (94:6) to give compound **C1** (103 mg). Fraction 52 was obtained in solid form (1.78 g) and was chromatographed on a silica gel column (5 x 50 cm) eluted with hexane, gradually increasing in polarity with acetone to give 34 subfractions. Subfraction F52-5 (0.49 g) was further fractionated with hexane:chloroform (3:7) to obtain compound **F1** (15.6 mg), and hexane:chloroform (1:9) to obtain compound **C2** (45 mg). Fractions 53-56 gave a yellow solid and afforded compound **F2** (1.1 g). Fraction 57 was obtained as orange crystals and yielded compound **C3** (108 mg). A compound

of light-yellow crystals, **F3** (1.19 g) was obtained from fraction 63.

MF5 (4.97 g) was chromatographed on a silica gel column (4 x 50 cm), eluted with hexane, gradually increasing in polarity with acetone (98:2, 96:4, 92:8, 90:10, 80:20, 70:30, 60:40, 50:50, 0:100) to give 32 subfractions. Subfraction MF5-22 yielded compound **F1** (32.5 mg). Subfractions MF5-20 to MF5-25 were combined (2.99 g) and further purified using a silica gel column (4 x 50 cm) eluted with hexane:dichloromethane (55:45) to give an additional amount of compound **C1** (23.1 mg) and with hexane: dichloromethane (15:85) to give compound **C2** (112.6 mg).

MF6 (4.21 g) was eluted with hexane and gradually increasing in polarity with acetone, and fractioned over a silica gel column (5 x 50 cm) to afford 100 subfractions. Subfraction MF6-8 eluted with hexane:acetone (96:4) yielded compound **F3** (20.2 mg) and was developed with hexane:acetone 50:50 to give compound **C1** (258.7 mg).

MF8 (10.84 g) was subjected to a silica gel column (5 x 50 cm) eluted with dichloromethane: hexane (90:10) to obtain a white compound **FA2** (153.5 mg) and a yellow compound **F4** (646.3 mg). MF11 (2.20 g) was chromatographed on a silica gel column (3 x 50 cm) and developed with hexane, gradually increasing in polarity with acetone. Compound **F4** (36.5 mg) was obtained with a solvent mixture of hexane:acetone (88:12). MF11 eluted with hexane:acetone (82:18) to yield a pale-yellow compound **FA3** (7.6 mg).

(2*S*, 3*S*) 3,5-dihydroxy-7-methoxyflavanone (**FA1**): light yellow solid; Yield: 50 mg (0.02 %); $[\alpha]_D$: -23.5˚(c 0.1 , MeOH) (Lit. $[\alpha]_D$: -28.1 [16]); M.p.: 175-177 °C (Lit. 176-179 °C [17]); IR υ_{max} 3467 (OH stretch), 2924 (CH_3 stretch), 1635 (C=O stretch), 1574 (C=C stretch), and 1134 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 342.3, 289.6; ^1H ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data: see Table 1. HRMS, ESI/TOF (m/z): 286.0849 [M^+]; 286.0841 Calc. [M^+] for $C_{16}H_{14}O_5$.

(2*R*, 3*R*) 3,5,7-trihydroxyflavanone (**FA2**): white solid; Yield: 153.5 mg (0.07 %); $[\alpha]_D$: +7.6° (c 0.1, MeOH); M.p.: 172-174 °C (Lit. 171-174 °C [18]); IR υ_{max} 3420 (OH stretch), 1636 (C=O stretch), 1618 (C=C stretch), and 1136 (C-O stretch) cm^{-1}; UV λ_{max}, nm:332, 292; ^1H (acetone-d_6, 400 MHz): δ = 4.63 (1H, d, *J* = 11.6 Hz, H-3), 5.15 (1H, d, *J* = 11.6 Hz, H-2), 5.95 (1H, d, *J* = 2.4 Hz, H-6), 5.98 (1H, d, *J* = 2.4 Hz, H-8), 7.40 (3H, m,, H-3', 4', 5'), 7.56 (2H, m, H-2',6'), 11.68 (1H, s, 5-OH). ^{13}C-NMR (acetone-d_6, 100 MHz): 83.5 (C-2), 72.3 (C-3), 197.1 (C-4), 164.2 (C-5), 96.3 (C-6), 167.1 (C-7), 95.2 (C-8), 163.1 (C-9), 100.6 (C-10), 137.4 (C-1'), 128.0 (C2',6'), 128.3 (C-3',5'). HRMS, ESI/TOF (m/z): 272.0690 [M^+]; 272.0684 Calc. [M^+] for $C_{16}H_{14}O_4$.

(2*S*) 7,8-dihydroxyflavanone (**FA3**): pale yellow solid; Yield: 7.6 mg (0.003 %); $[\alpha]_D$: +14.3˚(c 0.1, MeOH); M.p.: 162-164 °C (Lit. 164-166 °C [19]); IR υ_{max} 3431 (OH stretch), 2359, 2341 (CH_3 stretch), 1637 (C=O stretch), 1617 (C=C stretch), and 1182 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 349.5, 293.3; ^1H (acetone-d_6, 400 MHz) and ^{13}C-NMR (acetone-d_6, 100 MHz) data: see Table 2. HRMS, ESI/TOF (m/z): 256.0742 [M^+]; 256.07356 Calc. [M^+] for $C_{16}H_{14}O_4$.

2',4'-dihydroxy-3'-methoxychalcone (**C1**): orange solid; Yield: 361.7 mg (0.18 %); M.p.: 127-128 °C (Lit. 126-128 °C [20]); IR υ_{max} 3436 (OH stretch), 2941 (CH_3 stretch), 1634 (C=O stretch), 1593 (C=C stretch), and 1157 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 339.30; ^1H ($CDCl_3$, 400 MHz): δ = 4.01 (3H, s, O-CH_3), 6.58 (1H, d, *J* = 9.2 Hz), 7.42 (3H, m, H-3, 4, 5), 7.58 (d, *J* = 15.3 Hz, H-α), 7.62 (1H, d, *J* = 9.2 Hz, H-6'), 7.65 (2H, m, H-2,6), 7.90 (1H, d, *J* = 15.3 Hz, H-β), 9.27 (1H, s, 4'-OH), 13.57 (1H, s, 5-OH). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ = 134.8 (C-1), 128.7 (C-2, 6), 129.1 (C-3, 5), 130.9 (C-4), 120.3 (C-α), 144.8 (C-β), 192.7 (C=O), 115.1 (C-1'), 157.9 (C-2'), 134.4 (C-3'), 155.5 (C4'), 106.7 (C5'), 126.4 (C-6'), 60.9 (3'-OCH_3). HRMS, ESI/TOF (m/z): 270.090[M^+]; 270.089 Calc. [M^+] for $C_{16}H_{14}O_4$.

2',4'-dihydroxychalcone (**C2**): yellow needle crystals; Yield: 225.2 mg (0.11%); M.p.: 145-146 °C (Lit. 148-149 °C [21]); IR υ_{max} 3413 (OH stretch), 1635 (C=O stretch), 1495 (C=C stretch), and 1145 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 345, 321, and 263; ^1H (acetone-d_6, 400 MHz): δ = 6.36 (1H, d, *J* = 2.4 Hz, H-3'), 6.46 (1H, dd, *J* = 9.2, 2.1 Hz, H-5'), 7.44 (3H, m, H-3, 4, 5), 7.83 (1H, d, *J* = 15.9 Hz), 7.85 (2H, m, H-2,6), 7.92 (1H, d, *J* = 15.9 Hz, H-β), 8.15 (1H, d, *J* = 9.2 Hz), 13.45 (1H, s, 5-OH). ^{13}C-NMR (acetone-d_6, 100 MHz): δ = 135.1 (C-1), 129.0 (C-2, 6), 128.9 (C-3, 5), 130.7 (C-4), 144.7 (C-β), 120.3 (C-α), 192.0 (C=O), 113.6 (C-1'), 166.9 (C-2'), 102.9 (C-3'), 165.1 (C-4'), 108.1 (C-5'), 132.8 (C-6'). HRMS, ESI/TOF (m/z): 240. 0796 [M^+]; 240.0786 Calc. [M^+] for $C_{15}H_{12}O_3$.

2',3',4'-trihydroxychalcone (**C3**): orange needle crystals; Yield: 108 mg (0.05 %); M.p.: 173-175 °C; IR υ_{max} 3436 (OH stretch), 1636 (C=O stretch), 1572 (C=C stretch), and 1171 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 345, 300, and 231; ^1H (acetone-d_6, 400 MHz) and ^{13}C-NMR (acetone-d_6, 100 MHz) data: see Table 3. HRMS, ESI/TOF (m/z): 256.0736 [M^+]; 256.0736 Calc. [M^+] for $C_{15}H_{13}O_4$.

3,5-dihydroxy-7,8-dimethoxyflavone (**F1**): yellow solid; Yield: 62.5 mg (0.03 %); M.p.: 199-

202 °C; IR υ_{max} 3435 (OH stretch), 2973, 2935 (CH_3 stretch), 1652 (C=O stretch), 1619 (C=C stretch), and 1180 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 328, 271; ^1H ($CDCl_3$, 400 MHz): δ = 3.92 (3H, s, 8-OCH_3), 3.96 (3H, s, 7-OCH_3), 6.56 (1H, s, H-6), 6.74 (1H, s, 3-OH), 7.51 (3H, m, H-2', 4', 6'), 8.17 (2H, m, H-3', 5'), 11.61 (1H, s, 5-OH). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ = 145.5 (C-2), 136.6 (C-3), 175.6 (C-4), 152.6 (C-5), 90.8 (C-6), 159.5 (C-7), 132.3 (C-8), 151.5 (C-9), 104.6 (C-10), 130.8 (C-1'), 127.7 (C-2', 6'), 128.7 (C-3', 5'), 130.8 (C-4'), 56.4 (7-OCH_3), 61.0 (8-OCH_3). HRMS, ESI/TOF (m/z): 314.0798 [M^+]; 314.07904 Calc. [M^+] for $C_{17}H_{14}O_6$.

3, 5, 7-trihydroxy-8-methoxyflavone (**F2**): yellow solid; Yield: 1100 mg (0.55 %); M.p.: 205-207 °C (Lit. 203-205 °C [21]); IR υ_{max} 3393 (OH stretch), 3066 (aromatic CH stretch), 2946 (CH_3 stretch), 1654 (C=O stretch), 1625 (C=C stretch), and 1172 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 367, 309 and 273; ^1H (acetone-d_6, 400 MHz): δ = 3.93 (3H, s, 8-OCH_3), 6.30 (1H, s, H-6), 7.51 (1H, m, H-4'), 7.57 (2H, m, H-3', 5'), 8.28 (2H, m, H-2', 6'), 8.35 (1H, s, 3-OH), 9.47 (1H, s, 7-OH), 11.77 (1H, s, 5-OH). ^{13}C-NMR (acetone-d_6, 100 MHz): δ = 145.2 (C-2), 137.2 (C-3), 176.3 (C-4), 156.5 (C-5), 98.4 (C-6), 157.0 (C-7), 127.8 (C-8), 149.1 (C-9), 103.5 (C-10), 131.4 (C-1'), 127.6 (C-2', 6'), 128.7 (C-3', 5'), 130.1 (C-4'), 61.1 (8-OCH_3). HRMS, ESI/TOF (m/z): 300.0643 [M^+]; 300.0639 Calc. [M^+] for $C_{16}H_{12}O_6$.

5-hydroxy-3,7-dimethoxyflavone (**F3**); light yellow crystals; Yield: 1210 mg (0.61 %); M.p.: 142-144 °C (Lit. 143-144 °C [8]); IR υ_{max} 3436 (OH stretch), 2939 (CH_3 stretch), 3066 (aromatic CH stretch), 1651 (C=O stretch), 1606 (C=C stretch), and 1167 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 342.9, 305.9 (sh) and 266.6; ^1H ($CDCl_3$, 400 MHz): δ = 3.90 (6H, s, 3-OCH_3, 7-OCH_3), 6.35 (1H, d, *J* = 2.4 Hz), 6.44 (1H, d, *J* = 2.4 Hz), 7.51 (3H, m, H-3', 4', 5'), 8.06 (2H, m, H-2',6'), 12.57 (1H, s, 5-OH). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ = 156.0 (C-2), 139.7 (C-3), 179.0 (C-4), 162.1 (C-5), 98.0 (C-6), 165.6 (C-7), 92.2 (C-8), 156.9 (C-9), 106.2 (C-10), 130.5 (C-1'), 128.4 (C-2',6'), 128.7 (C-3',5'), 131.0 (C-4'), 60.4 (3-OCH_3), 55.9 (7-OCH_3). HRMS, ESI/TOF (m/z): 298.0841 [M^+]; 298.0841 Calc. [M^+] for $C_{17}H_{14}O_5$.

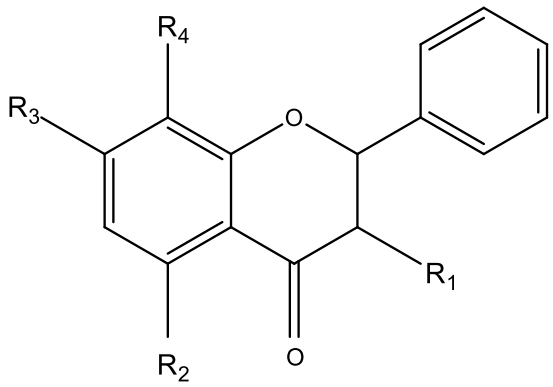
5,7-dihydroxyflavone (**F4**): yellow solid; Yield: 668.6 mg (0.33 %); M.p.: 286-287 °C (Lit. 283-285 °C [21]); IR υ_{max} 3436 (OH stretch), 1651 (C=O stretch), 1613 (C=C stretch), and 1169 (C-O stretch) cm^{-1}; UV λ_{max}, nm: 316.4 and 269.3; ^1H (DMSO-d_6, 400 MHz): δ = 6.17 (1H, d, *J* = 1.8 Hz, H-6), 6.47 (1H, d, *J* = 1.8 Hz, H-8), 6.91 (1H, s, H-3), 7.53 (3H, m, H-3',4',5'), 8.03 (2H, m, H-2',6'), 12.78 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ = 163.6 (C-2), 105.6 (C-3), 182.3 (C-4), 161.9 (C-5), 99.5 (C-6), 164.9 (C-7), 94.6 (C-8), 157.9 (C-9), 104.5 (C-10), 131.2 (C-1'), 126.9 (C-2',6'), 129.6 (C-3',5'), 133.5 (C-4'). HRMS, ESI/TOF (m/z): 254.0576 [M^+]; 254.0579 Calc. [M^+] for $C_{15}H_{10}O_4$.

Antibacterial Activity

The 96-well microbroth dilution assay was conducted by modifying the method as described by Eloff [22]. The minimum inhibitory concentration (MIC) values were determined in triplicate. Test compounds were dissolved in dimethyl sulphoxide (DMSO) to obtain a concentration of 1 mg/ml. 50 µl of the test compound and 50 µl of Muller Hinton broth were dispensed into a 96 well plate. Serial two-fold dilution of the test compounds was performed to obtain a range of concentrations (250 to 1.95 µg/ml). Several wells acted as sterility controls (100 µl MHB), growth controls (50 µl MHB + 50 µl of inoculum) and negative controls (50 µl MHB + 50 µl DMSO 10%). Gentamicin at 1000 µg/ml was used as a positive control. The bacterial suspension was adjusted to give a final organism density of 0.5 McFarland scale (1 x 10^5 CFU/ml) using a spectrophotometer with a 1 cm light path. The absorbance at 625 nm was expected to be 0.08 to 0.10. 50 µl bacteria suspensions were inoculated into all the wells. The final volume in each well was 100 µl. After that, the plates were sealed with parafilm and incubated for 24 h at 37 °C. After incubation, 20 µl of 0.4 mg/ml of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) was added into each well and the plate was further incubated for 20 mins. The MIC value was determined as the lowest concentration of test compounds in the well that did not form a purple colour.

RESULTS AND DISCUSSION

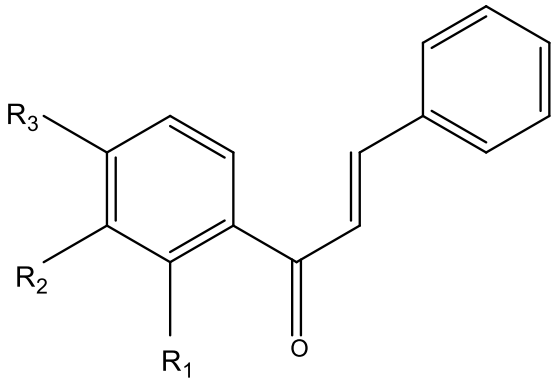
Ten known flavonoids were isolated from the leaves of *Muntingia calabura*. There were three flavanones {(2*S*,3*S*) 3,5-dihydroxy-7-methoxyflavanone (**FA1**), (2*R*,3*R*) 3,5,7-trihydroxyflavanone (**FA2**), (2*S*) 7,8-dihydroxyflavanone (**FA3**)}, three chalcones {2',4'-dihydroxy-3'-methoxychalcone (**C1**), 2',4'-dihydroxychalcone (**C2**), 2',3',4'-trihydroxychalcone (**C3**)} and four flavones {3,5-dihydroxy-7,8-dimethoxyflavone (**F1**), 3,5,7-trihydroxy-8-methoxyflavone (**F2**), 5-hydroxy-3,7-dimethoxyflavone (**F3**), 5,7-dihydroxyflavone (**F4**)}. The structures of the compounds are shown in Figure 1. Among them, compounds **FA1**, **FA3** and **C3** were isolated for the first time from *M. calabura* leaves. The structures were elucidated using spectroscopic methods and compared with literature data.



FA1: R_1=R_2=OH; R_3=OCH_3; R_4= H

FA2: R_1=R_2=R_3=OH; R_4= H

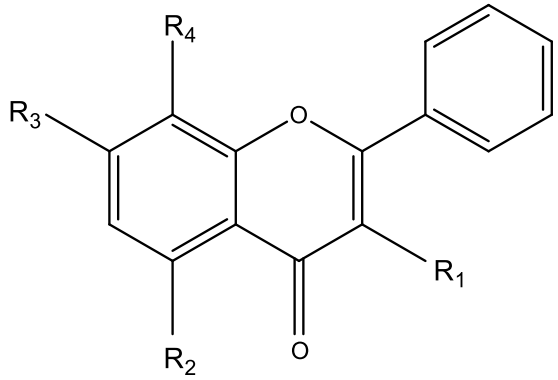
FA3: R_1=R_2=H; R_3=R_4=OH



C1: R_1=R_3=OH; R_2=OCH_3

C2: R_1=R_3=OH; R_2= H

C3: R_1=R_2=R_3=OH



F1: R_1=R_2=OH; R_3=R_4=OCH_3

F2: R_1=R_2=R_3=OH; R_4= OCH_3

F3: R_1=R_3=OCH_3; R_2=OH; R_4=H

F4: R_1=R_4=H; R_2=R_3=OH

Figure 1. Structures of compounds isolated from the leaves of *Muntingia calabura*.

Structure Elucidation

Compound **FA1**, (2*S*,3*S*) 3,5-dihydroxy-7-methoxy-flavanone, was a light-yellow solid. Its specific rotation was negative as $[\alpha]_D$: -23.5° (Lit value, $[\alpha]_D$: -28.1 [16]). The structure was confirmed as 2*S*, 3*S* as the coupling constant was the same as in the reported data [16]. According to Ekalu and Habila [23], the chemical shift of flavonoids is unique, which makes characterisation easier. The HRESIMS gave a pseudo molecular ion peak at m/z = 287.0922 $[M+H]^+$ which gave $C_{16}H_{14}O_5$ (found 286.0849; calculated 286.0841). The UV absorption peaks were 342.3 nm and 289.6 nm, representing bands I and II which are characteristic of flavanone absorptions. Band I is the absorption due to a B-ring cinnamoyl system and band II involves absorption due to A-ring benzoyl system [24]. The IR spectrum showed absorption bands at 3467 (OH stretch), 2924 (CH_3 stretch), 1635 (C=O stretch), 1574 (C=C stretch) and 1134 cm^{-1} (C-O stretch). The ^1H NMR spectrum of **FA1** showed characteristic peaks of 3-hydroxyflavanone or the flavanonol moiety as a doublet at δ_H 5.10 (J = 11.6 Hz) corresponding to H-2, and a doublet at δ_H 4.55 (J = 11.6 Hz) indicating H-3 [25]. Two doublets of aromatic protons at δ_H 6.06 (J = 2.4 Hz) and δ_H 6.11(d, J = 2.4 Hz) corresponded to H-8 and H-6 which is a meta coupling proton and one methoxy group at δ_H 3.84 (3H). Two aromatic multiplets at δ_H 7.46 (3H, m) and δ_H 7.53 (2H, m) indicate an unsubstituted benzene ring B. There was one chelated hydroxide group at δ_H 11.19 (5-OH). The 3-OH group was not shown in the

spectrum due to hydrogen-deuterated proton exchange. In the ^{13}C NMR spectrum, there were 13 signals which corresponded to 15 carbons of the flavonoid skeleton. There was a carbonyl carbon at δc 195.9 and a methoxy carbon at δc 55.9. Three signals at δc 168.9, δc 163.7 and δc 162.9 were oxygen-linked aromatic carbons corresponding to C-5, C-7, and C-9 respectively. Peaks at δc 94.8 and δc 95.6 were characteristic of the C-8 and C-6 resonances of a 3-hydroxyflavanone. Inspection of the ^{13}C NMR spectrum showed that the peaks at δ 127.5, δ 128.8 and δ 129.4 represented C-2',6', C-3',5' and C-4', respectively, of an unsubstituted B-ring. In the DEPT spectrum, there were 6 quaternary carbons, 9 methine carbons and one methoxy carbon. In the HMBC spectrum, the correlation of the methoxy group at δ 3.84 (7-OCH_3) to C-7 (δ_C 168.9) was confirmed, as well as the correlation of H-6 (δ_H 6.06) to C-7 (δ_C 168.9) and the correlation of H-8 (δ 6.11) to C-7 (δ 168.9). The 5-OH proton signal at δ_H 11.19 showed correlations with the carbons at δc 95.6 (C-6), δc 100.9 (C-10), and δc 162.9 (C-5). Table 1 shows the ^1H and ^{13}C spectral data of compound **FA1**. This compound was reportedly isolated from *Heliotropium huascoense* and induces the expression of cytokines which are vital to control viral infection in invertebrates [16, 26]. It has also shown *in vitro* antiviral activity against the infectious salmon anaemia virus.

Compound **FA3** was isolated as a white solid. Its specific rotation was positive as [α]D: +14.3°. Su *et al.* [27] reported that most flavanones obtained from natural sources had the 2*S* absolute configuration. The absolute configuration for this compound was also determined as 2*S*. The HRESIMS gave a pseudo molecular ion peak at m/z = 257.0815 $[M+H]^+$ which gave $C_{15}H_{12}O_4$ (found 256.0742; calculated 256.07356). The UV absorptions were 349.5 nm and 293.3 nm. The IR spectrum showed absorption bands at 3431 (OH stretch), 2359, 2341 (CH_3 stretch), 1637 (C=O stretch), 1617 (C=C stretch), and 1182 (C-O stretch) cm^{-1}. The ^1H NMR of compound **FA3** showed characteristic signals of the flavanone moiety seen as a doublet of doublets at δ_H 5.53 (*J* = 3.0, 12.8 Hz) and further upfield, a doublet of doublets at δ_H 3.07 (*J* = 12.8, 16.5 Hz) and another at 2.74 (*J* = 3.0, 16.8 Hz), corresponding to H-2, H-3α, and H-3β respectively. Two aromatic multiplets at δ_H 7.44 (3H, m) and δ_H 7.57 (2H, m) indicate an unsubstituted benzene ring B. The presence of hydroxy groups was revealed by the

Table 1. ^1H (400 MHz), and ^{13}C (100 MHz) spectral data for **FA1**.

	FA1		Reference [16]
Position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	5.10 d (11.6)	83.5	5.07 d (11.9)
3	4.54 d (11.6)	72.5	4.54 d (11.9)
4	-	195.9	-
5	-	168.9	-
6	6.06 d (2.4)	95.6	6.05 d (2.3)
7	-	163.7	-
8	6.11 d (2.4)	94.8	6.11 d (2.3)
9	-	162.9	-
10	-	100.9	-
1'	-	136.1	-
2', 6'	7.53 m	127.5	7.54 m
3', 5'	7.44 m	128.8	7.44 m
4'	7.44 m	129.4	7.44 m
5-OH	11.19 s	-	11.18 s
7-OCH_3	3.83 s	55.9	3.80 s

band at 3431(br) cm^{-1} in the IR spectrum. The hydroxy groups did not appear as exchangeable deuterated protons in the ^1H NMR spectrum. The doublets at δ_H 6.58 and δ_H 7.28, both with coupling constants J = 8.5 Hz indicating they were ortho couplings, were assigned as H-6 and H-5. In the ^{13}C NMR spectrum of **FA3**, δc 150.7 (C-7), δc 132.8 (C-8) and δc 151.9 (C-9) were oxygenated aromatic carbons. δ_C 80.3 and δ_C 44.3 were assigned as C-2, C-3α and C-3β, respectively. The DEPT spectrum showed that these were methylene carbons. The structure of **FA3** was confirmed by HMQC and HMBC spectra and compared with literature data [19]. This compound was isolated from the seeds of *Alpinia Katsumadai* Hayata and reported to have exhibited cytotoxic activity against cancer cell lines A549 and K562 with IC_{50} values of 0.20 mmol/mL and 0.056 mmol/mL, respectively [19]. Table 2 shows the summary NMR spectral data for compound **FA3**.

Compound **C3** was isolated as orange needle-shaped crystals. The HRESIMS showed a pseudo molecular ion peak at m/z = 257.0809 $[M+H]^+$ which gave $C_{15}H_{13}O_4$ (found 256.0736; calculated 256.0736). The UV absorption peaks obtained were 345 nm, 300 nm and 231 nm. The IR spectrum showed absorption bands at 3436 (OH stretch), 1636 (C=O stretch), 1572 (C=C stretch) and 1171 cm^{-1} (C-O stretch). In the ^1H NMR spectrum of **C3**, the presence of doublets at δ_H 7.85 (d, J = 15.6 Hz) and δ_H 7.93 (d, J =15.6 Hz) indicated the existence of trans olefin H-α and H-β protons, respectively. The singlets at δ_H 13.47 were due to the chelated 2'-OH. Multiplets at δ_H 7.45 (3H, m, H-3, H-4, and H-5) and δ_H 7.84 (2H, m, H-2 and H-6) were due to the monosubstituted B-ring. In the ^{13}C NMR spectrum of **C3**, olefinic carbons signals were observed at δc 120.8 and δc 143.9, and were assigned to C-α and C-β, respectively. A carbonyl carbon signal was observed at δc 192.6. Seven aromatic C-H peaks were observed at δc 107.6 (C-5'), δc 122.6 (C-6'), δc 128.8 (C-2,6), δc 129.0 (C-3,5) and δc 130.6 (C-4). Three oxygenated carbon peaks were observed at δc 153.5, δc 132.5 and δc 152.2, corresponding to C-2', C-3' and C-4', respectively. In the HMBC spectrum, 4'-OH showed correlations with C-5' (δc 107.6), C-3' (δc 132.5) and C-4' (δc 152.2), which confirmed the hydroxyl group at C-4'. Based on all the spectra including DEPT and HMQC, compound **C3** was confirmed as 2',3',4'-trihydroxychalcone. Table 3 shows the summary data for compound **C3**.

Table 2. ^1H (400 MHz), and ^{13}C (100 MHz) spectral data for **FA3**.

Position	**FA3**		Reference [19]
	δ_H (J in Hz)	δ_C	δ_C
2	5.53 dd (12.8, 3.0)	80.3	80.4
3α	3.07 dd (16.5, 12.8)	44.3	44.1
3β	2.74 dd (16.5, 3.0)	44.3	44.1
4	-	189.7	197.3
5	7.28 d (8.5)	109.8	96.2
6	6.58 d (8.5)	118.0	97.1
7	-	150.7	168.4
8	-	132.8	165.4
9	-	151.9	164.6
10	-	114.9	103.3
1'	-	139.6	140.4
2', 6'	7.57 m	126.6	127.3
3', 4', 5'	7.44 m	128.6	129.6

Table 3. ^{1}H (400 MHz), and ^{13}C (100 MHz) spectral data for **C3**.

	C3	
Position	δ_H (*J* in Hz)	δ_C
1	-	135.0
2,6	7.84 m	128.8
3,5	7.45 m	129.0
4	7.45 m	130.6
β	7.93 d (15.6)	143.9
α	7.85 d (15.6)	120.8
C=O	-	192.5
1’	-	113.8
2’	-	153.5
3’	-	132.5
4’	-	152.2
5’	6.50 d (9.2)	107.6
6’	7.72 d (9.2)	122.6
2’-OH	13.47 s	-
3’-OH	-	-
4’-OH	8.86 s	-

Antibacterial Activity

All the compounds were tested for antibacterial activity using the microbroth dilution method, except compounds **FA1** and **FA3** as they had low yields. The compounds were evaluated against three Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*). The results of the antibacterial screening are shown in Table 4.

Table 4.: MIC values of test compounds

Compounds	*B. cereus*	*B. subtilis*	*S. aureus*	*E. coli*	*S.* Typhimurium	*P. aeruginosa*
	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)
FA2	125	>250	>250	>250	>250	>250
C1	31.25	62.5	62.5	>250	>250	>250
C2	31.25	125	62.5	>250	>250	>250
C3	62.5	125	62.5	>250	>250	>250
F1	125	>250	>250	>250	>250	>250
F2	125	>250	>250	>250	>250	>250
F3	125	>250	>250	>250	>250	>250
F4	125	>250	>250	>250	>250	>250
Gentamicin	3.90	3.90	3.90	15.625	15.625	15.625

According to Kuete *et al.* [28], the antimicrobial activity of pure compounds can be classified as significant (MIC < 10 µg/ml), moderate (10 < MIC ≤ 100 µg/ml) or weak (MIC > 100 µg/ml). Based on this classification, the chalcone compounds **C1**, **C2**, and **C3** exhibited moderate activity against *B. cereus* and *S. aureus*, with MIC values ranging from 31.25 to 62.5 µg/ml. Whereas, flavanone **FA1** and flavone compounds **F1-F4** showed weak activity against *B. cereus* and *S. aureus* with MIC values > 100 µg/ml. For *B. subtilis*, only **C1** showed moderate activity with an MIC value of 62.5 µg/ml, while the rest of the compounds exhibited weak activity with MIC values > 250 µg/ml. All the compounds exhibited weak activity against the Gram-negative bacteria *E. coli*, *S.* Typhimurium, and *P. aeruginosa* with MIC values > 250 µg/ml. Gentamicin inhibited all the Gram-positive bacteria at 3.125 µg/ml and all the Gram-negative bacteria at 15.625 µg/ml. This is the first report on the antibacterial activity of compound **C3**.

Xie *et al.* [29] reported that the antibacterial activity of flavonoids depends on the substitutions on the aromatic rings. Chalcones with hydroxyl groups, especially at the 4'-position, have proved to induce and enhance antibacterial activity. Hydroxy groups at the 2- and 4- positions of ring B in chalcone have also been shown to exhibit antibacterial activity. On the other hand, Cushine and Lamb [30] reported that the presence of methoxy groups in flavonoids greatly decreased its antibacterial activity.

CONCLUSION

In conclusion, ten known flavonoids were isolated from the leaves of *Muntingia calabura*; these consisted of three flavanones, three chalcones and four flavones. Among the isolated compounds, two flavanones, namely (2*S*,3*S*) 3,5-dihydroxy-7-methoxyflavanone (**FA1**), and (2*S*) 7,8-dihydroxyflavanone (**FA3**), and one chalcone, 2',3',4'-trihydroxychalcone (**C3**), were isolated for the first time from the leaves of *M. calabura*. The three chalcones **C1**, **C2** and **C3** exhibited moderate antibacterial activity against *B. cereus*, with MIC values ranging from 31.25 to 62.5 µg/ml. These results indicate the potential of *M. calabura* extracts for drug development and as a source of antibacterial agents. The chalcones with moderate antibacterial activity may also be synthetically modified to produce compounds with higher antibacterial activity.

ACKNOWLEDGEMENTS

This work was supported by the Universiti Tunku Abdul Rahman Research Fund, UTARRF (grant number: IPSR/RMC/UTARRF/C2-11/C03 & IPSR/RMC/UTARRF/2018-C1/C01).

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