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A Dihydrochalcone from *Syzygium samarangense* with Anticholinesterase Activity

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Cholinesterase inhibitors are known to be useful in the treatment of neurodegenerative disorders such as Alzheimer's disease, senile dementia, and ataxia. These had also been found to improve long term memory processes by enhancing cholinergic activity. Herbs rich in flavonoids were shown to have therapeutic effects on neurodegenerative diseases. Flavonoids isolated from *Syzygium samarangense*, locally known in the Philippines as "makopa", identified from spectral data as 7-hydroxy-5-methoxy-6,8-dimethylflavanone (1), 2'-hydroxy-4',6'-dimethoxy-3'-methylchalcone (2), 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (3), 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (4), and 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone (5), were tested for anticholinesterase activity against two cholinesterases, acetylcholinesterase (3.1.1.7), and butyrylcholinesterase (3.1.1.8). Compounds 2 and 3 were hydrogenated to yield 2'-hydroxy-4',6'-dimethoxymethoxy-3'-methyldihyrdochalcone (6) and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl-dihydrochalcone (7), respectively. Compound 7 exhibited 98.5% inhibitory activity against acetylcholinesterase at 0.25 mM concentration. When tested against butyrylcholinesterase, it exhibited 68.0% inhibitory activity at 0.20 mM concentration and its IC50 was determined to be 127 μM. The IC50 of physostigmine, the positive control, was 0.041 μM and 0.857 μM against acetylcholinesterase and butyrylcholinesterase, respectively. The rest of the compounds did not exhibit significant inhibition of the cholinesterases. This is the first report of anticholinesterase activity of compound 7, a dihydrochalcone.

Key words: acetylcholinesterase, butyrylcholinesterase, flavonoids, neurodegenerative disorders

INTRODUCTION

Enzyme assays are used to discover bioactive compounds that interfere with a specific enzyme-mediated biological reaction (Devlin 1997). Anticholinesterases are being utilized in the treatment of neurodegenerative diseases such as Alzheimer's disease (McGleenon et al. 1999; Rosenthal 2002), senile dementia (Eager and Harvey 1995), and ataxia, as well as improving long term memory processes by enhancing cholinergic activity (Nochi et al. 1995). There are three main classes of cholinesterase inhibitors considered as potential drugs for the treatment of Alzheimer's disease, and these are grouped into the following: physostigmine and its analogues (Teoh et al. 2001), 1,2,3,4-tetrahydroacridines, and benzylamines. Some specific examples of cholinesterase inhibitors are tacrine or tetrahydroaminoacridine (THA) (Badia et al. 1998), huperzin A and huprine X (Camps et al. 2000), donepezil or E2020 (Dooley and Lamb 2000),

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rivastigmine (Kennedy et al. 1999), galantamine (Lilienfeld and Parys 2000) and eptastigmine (Mant et al. 1998). Other examples are the pesticides malathion and sevin (Foye 1995), and the nerve gases sarin and soman (Quinn 1987).

A number of rare C-methylated flavonoids were isolated from the leaves of *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Amor et al. 2004), locally known in the Philippines as 'makopa'. The flavonoids that were isolated from *S. samarangense* belong to the chalcone, dihydrochalcone, and flavanone subclasses. As part of the continuous search for cheap and alternative medicines and of a collaborative effort on finding new bioactive compounds, these flavonoids were tested for anticholinesterase activity against acetylcholinesterase (3.1.1.7) and butyrylcholinesterase (3.1.1.8) enzymes.

MATERIALS AND METHODS

All the solvents used for extraction, isolation, and purification were technical grade and distilled before use. Normal phase column chromatography (NPCC) with gradient elution was the technique generally employed in the isolation of the flavonoids using silica gel, type-60 (70-230 mesh, Merck). Thin layer chromatography (TLC) was used to monitor separation with GF-254 aluminum plates (Merck). TLC plates were visualized under UV lamp set at 254 and 365 nm. Spray reagent used was 65% $CeSO_4$. H_2SO_4. FT-IR spectrum was recorded with a JASCO A-302 IR spectrophotometer or a Bio-RAD FTS 40-A spectrophotometer. EI-MS was recorded with a Finnigan MAT 312 equipped with a Masspec Data system at 70 eV. The 1D and 2D NMR spectra were recorded with a Bruker AM 300 MHz (75 MHz for ^{13}C) FT NMR or Bruker AM 500 MHz (125 MHz for ^{13}C) spectrometer with Aspect 3000 and X-32 data, and a JEOL Lambda 400 MHz (100 MHz for ^{13}C) NMR spectrometer. The melting point was recorded with a Yanaco micro melting point apparatus or a Fisher-Johns melting point apparatus. The recorded melting points were all uncorrected.

Plant material. Leaves of the plant were sampled from Parañaque, Metro Manila. This was authenticated and a voucher specimen with accession No. 14258 was submitted to the Dr. Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines in Diliman, Quezon City.

Extraction and isolation. The ground air-dried leaves of *S. samarangense* (2.9 kg) were extracted at room temperature with methanol and the resulting crude extract subsequently partitioned between H_2O:hexane (1:6 v/v), from which the hexane extract was obtained and concentrated (140 g oily residue) in vacuo. This was subjected to fractionation by NPCC on silica gel (1:10 m/m) employing gradient elution (10% increments) with hexane, dichloromethane-hexane, dichloromethane, methanol-dichloromethane, and finally with methanol, giving fractions 1-4.

Compound 2 (75 mg) crystallized out of fraction 1, eluted with hexane and 10% dichloromethane-hexane and was purified by recrystallization in 5% dichloromethane-hexane.

Sequential NPCC of fraction 2, eluted with 20% to 50% dichloromethane-hexane, on silica gel employing gradient elution (10% increments) with hexane and dichloromethane-hexane yielded compound 3 (3 g).

Sequential NPCC of fraction 3, eluted with 60% dichloromethane-hexane to 30% methanol-dichloromethane, on silica gel employing gradient elution (10% increments) with hexane, dichloromethane-hexane, dichloromethane, methanol-dichloromethane yielded compounds 4 (100 mg), 5 (3 mg), and 1 (50 mg).

7-Hydroxy-5-methoxy-6,8-dimethylflavanone (1)
M.p. 210-211°C.- UV λ_{max} (MeOH=284.4 nm; λ_{max} ($AlCl_3$, HCl)=278.8 nm; λ_{max} (NaOMe)=334.0 nm; λ_{max} (NaOAc, H_3BO_3)=278.4 nm.- FT-IR (KBr): ν_{max}=3281, 3010, 2933, 2837, 1648, 1582, 1467, 1428, 1305, 1223, 1208, 1111, 996, 934, 764, 699 cm^{-1}.- ^1H-NMR ($CDCl_3$, 300 MHz) [NOE, enhanced signal]: δ=2.14 (6-CH_3, 3H, s) [3.81, 5.37], 2.14 (6-CH_3, 3H, s) [3.81, 5.37], 2.83 (3-H, 1H, dd, J = 16.7, 3.2), 2.97 (3-H, 1H, dd, J = 16.7, 12.8), 3.81 (5-OCH_3, 3H, s) [2.14], 5.37 (7-OH, 1H, br s) [2.14, 2.14], 5.41 (2-CH, 1H, dd, J = 12.8, 3.2), 7.35 – 7.50 (H-2', H-3', H-4', H-5', H-6', 5H, br m).- ^{13}C-NMR ($CDCl_3$, 100 MHz) (DEPT) [HMQC] {HMBC}: δ=7.8 (6-CH_3, CH_3) [2.12], 8.2 (8-CH_3, CH_3) [2.14], 45.6 (C-3, CH_2) [2.83], 61.2 (5-OCH_3, CH_3) [3.81], 78.6 (C-2, CH) [5.41] {2.97}, 106.9 (C-8, C) {2.14}, 109.1 (C-10, C), 111.3 (C-6, C) {2.14}, 125.8 (C-4', CH) [7.2], 128.4 (C-5', CH) [7.2], 128.4 (C-3', CH) [7.2], 128.7 (C-2', CH) [7.2], 128.7 (C-6', CH) [7.2], 139.2 (C-1', C), 157.7 (C-9, C), 158.9 (C-5, C) {3.81}, 159.6 (C-7, C) {2.14}, 189.8 (C-4, C) {2.83}.- EIMS (70 eV, rel. int.%): $C_{18}H_{18}O_4$ 298.0 (67.82), 221.0 (14.94), 194.0 (100), 166.0 (20.13), 136.0 (27.87), 104.1 (11.98), 83.0 (35.30), 77.0 (21.71).

2'-hydroxy-4',6'-dimethoxy-5'-methylchalcone (2)
M.p. 145°C.- UV λ_{max} (MeOH=344.0 nm; λ_{max} (AlCl3, HCl)=374.6 nm; λ_{max} (NaOMe)=339.6 nm; λ_{max} (NaOAc, H3BO3)=349.8 nm.- FT-IR (KBr): ν_{max}=3130, 2941, 2860, 1625, 1563, 1428, 1332, 1223, 1142, 980, 872, 791, 749,

706 cm^{-1}.- ^{1}H-NMR ($CDCl_3$, 300 MHz) [NOE, enhanced signal]: δ=2.04 (3'-CH_3, 3H, s) [14.08], 3.90 (6'-OCH_3, 3H, s) [5.99], 3.95 (4'-OCH_3, 3H, s) [5.99], 5.99 (H-5', 1H, s) [3.95], 7.40 (H-3, H-4, H-5, 3H, br m), 7.60 (H-2, H-6, 2H, br m), 7.76 (C_β-H, 1H, d, J = 15.6), 7.89 (C_α-H, 1H, d, J = 15.6), 14.08 (2'-OH, 1H, s). The intensity of the signal at 14.08 decreased upon D_2O shake.- ^{13}C-NMR ($CDCl_3$, 100 MHz) (DEPT) [HMQC] {HMBC}: δ=7.3 (3'-CH_3, CH_3) [2.04], 55.6 (4'-OCH_3, CH_3) [3.95], 55.8 (6'-OCH_3, CH_3) [3.90], 86.3 (C-5', CH) [5.99], 106.1 (C-3', C) {2.04}, 106.3 (C-1', C) {5.99, 14.08}, 128.0 (C-4, CH) [7.89], 128.3 (C-2, CH) [7.60] {7.40}, 128.3 (C-6, CH) [7.60] {7.40}, 128.8 (C-3, CH) [7.40], 128.8 (C-5, CH) [7.40], 129.9 (C_α, CH) [7.40], 135.6 (C-1, C) {7.40, 7.76}, 141.9 (C_β, CH) [7.76], 161.1 (C-6', C) {3.90, 5.99}, 163.6 (C-4', C) {3.95, 5.99}, 164.2 (C-2', C) {2.04, 14.08}, 193.0 (C=O, C) {7.76}.- EIMS (70 eV, rel. int.%): $C_{18}H_{18}O_4$ HR-MS 298.11989 (calcd. 298.120500, 75.31), 281.0 (9.39), 270.0 (13.49), 221.1 (100), 195.1 (42.69), 179.1 (15.47), 165.0 (15.87), 136.0 (35.29), 103.1 (47.11), 91.0 (18.46), 77.0 (60.09), 51.0 (48.51).

2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (3)

M.p. 125-126°C.- UV λ_{max} (MeOH=335.8 nm; λ_{max} ($AlCl_3$, HCl)=368.8 nm; λ_{max} (NaOMe)=409.8 nm; λ_{max} (NaOAc, H_3BO_3)=421.4 nm.- FT-IR (KBr): ν_{max}=3335, 2945, 2860, 1629, 1548, 1424, 1359, 1312, 1231, 1169, 1115, 985, 911, 818, 760, 691 cm^{-1}.- ^{1}H-NMR ($CDCl_3$, 300 MHz) [NOE, enhanced signal]: δ=2.15 (3'-CH_3, 3H, s), 2.17 (5'-CH_3, 3H, s), 3.66 (6'-OCH_3, 3H, s) [2.17, 8.00], 5.88 (4'-OH, 1H, s), 7.41 (H-3, H-4, H-5, 3H, m), 7.63 (H-2, H-6, 2H, m), 7.84 (C_β-H, 1H, d, *J* = 15.7), 8.00 (C_α-H, 1H, d, *J* = 15.7), 13.69 (2'-OH, 1H, s). The intensity of the signals at 5.88 and 13.69 decreased upon D2O shake.- ^{13}C-NMR ($CDCl_3$, 100 MHz) (DEPT) [HMQC] {HMBC}: δ=7.6 (5'-CH_3, CH_3) [2.17], 8.2 (3'-CH_3, CH_3) [2.15], 62.3 (6'-OCH_3, CH_3) [3.66], 106.6 (C-1', C) {2.15, 13.69}, 109.0 (C-3', C) {2.15, 13.69}, 109.0 (C-5', C) {5.88}, 126.7 (C_α, CH) [8.00], 128.4 (C-3, CH) [7.41] {7.63}, 128.4 (C-5, CH) [7.41] {7.63}, 128.9 (C-2, CH) [7.63], 128.9 (C-6, CH) [7.63], 130.2 (C-4, CH) [7.41] {7.63}, 135.3 (C-1, C) {7.84}, 142.9 (C_β, CH) [7.84] {7.63, 8.00}, 158.8 (C-6', C) {3.66}, 159.3 (C-4', C) {2.17, 5.88}, 162.0 (C-2', C) {2.15, 13.69}, 193.4 (C=O, C) {8.00}.- EIMS (70 eV, rel. int.%): $C_{18}H_{18}O_4$ 298.1 (100), 221.0 (93.12), 194.0 (80.67), 166.1 (24.11), 136.0 (20.47), 103.1 (33.04), 83.0 (49.84), 77.0 (21.92), 69.1 (14.18).

2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (4)

M.p. 198-203o C.- UV λ_{max} (MeOH)=346.0 nm; λ_{max} ($AlCl_3$, HCl)=372.2 nm; λ_{max} (NaOMe)=389.8 nm; λ_{max} (NaOAc, H_3BO_3)=370.2 nm.- FT-IR (KBr): ν_{max}=3142, 2933, 2725, 1625, 1536, 1447, 1339, 1231, 1150, 1119, 976, 864, 795, 760, 699 cm^{-1}.- ^{1}H-NMR (C_3D_6O, 300 MHz) [NOE, enhanced signal]: δ=2.00 (3'-CH_3, 3H, s), 3.92 (6'-OCH_3, 3H, s) [6.15], 6.15 (H-5', 1H, s) [3.92], 7.44 (H-3, H-4, H-5, 3H, m), 7.71 (H-2, H-6, 2H, m), 7.74 (C_β-H, 1H, d, *J* = 15.6), 8.02 (C_α-H, 1H, d, *J* = 15.6), 14.51 (2'-OH, 1H, s) [-]. The intensity of the signal at 14.51 decreased upon D_2O shake.- ^{13}C-NMR (C_3D_6O, 125 MHz) (DEPT) [HMQC] {HMBC, $CDCl_3$, 400 MHz}: δ=7.36 (3'-CH_3, CH_3) [2.00], 56.1 (6'OCH_3, CH_3) [3.90], 91.5 (C-5', CH) [6.15], 104.4 (C-1', C) {2.00, 6.15}, 106.0 (C-3', C) {6.15}, 128.6 (C_α, CH) [8.00], 129.0 (C-2, CH) [7.7], 129.0 (C-6, CH) [7.7], 129.7 (C-3, CH) [7.4], 129.7 (C-5, CH) [7.4], 130.7 (C-4, CH) [7.4], 136.4 (C-1, C), 161.8 (C-4', C) {2.00}, 163.3 (C-6', C) {3.92}, 166.4 (C-2', C) {2.00}, 193.1 (C=O, C).- EIMS (70 eV, rel. int.%): $C_{17}H_{16}O_4$ 284.1 (100), 267.1 (41.15), 256.1 (37.33), 207.1 (99.1), 181.1 (62.20), 165.0 (42.87), 151.1 (37.39), 122.0 (55.53), 103.1 (51.14), 77.0 (47.71).

2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone (5)

M.p. 171-173°C.- UV λ_{max} (MeOH=292.4 nm; λ_{max} ($AlCl_3$, HCl)=313.8 nm; λ_{max} (NaOMe)=330.0 nm; λ_{max} (NaOAc, H_3BO_3)=295.4 nm.- FT-IR (KBr): ν_{max}=3331, 2956, 2875, 1648, 1613, 1571, 1428, 1305, 1204, 1119, 838, 787, 695 cm^{-1}.- ^{1}H-NMR ($CDCl_3$, 300 MHz): δ=2.05 (3'-CH3, 3H, s), 3.00 (C_β-H, 2H, t, *J* = 7.8), 3.32 (C_α-H, 2H, t, *J* = 7.8), 3.82 (6'-OCH_3, 3H, s), 5.41 (4'-OH, 1H, br s), 5.92 (H-5', 1H, s), 7.20 – 7.33 (H-2, H-3, H-4, H-5, H-6, 5H, br m), 14.29 (2'-OH, 1H, s); Intensity of the signals at 5.41 and 14.29 decreased upon D_2O shake.- ^{13}C-NMR (30% CD_3OD-$CDCl_3$,125 MHz) (DEPT) [HMQC] {HMBC}: δ=7.0 (3'-CH_3, CH_3) [2.05], 31.0 (C_δ, CH_2) [3.00] {3.32}, 45.7 (C_α, CH2) [3.32] {3.00}, 55.2 (6'-OCH_3, CH_3) [3.82], 90.2 (C-5', CH) [5.92], 104.4 (C-3', C) {2.05, 5.92, 14.29}, 104.9 (C-1', C) {5.92, 14.29}, 125.8 (C-4, CH) [7.26] {7.20-7.33}, 128.3 (C-2, CH) [7.26] {3.00}, 128.3 (C-3, CH) [7.26] {3.00}, 128.3 (C-5, CH) [7.26] {3.00}, 128.3 (C-6, CH) [7.26] {3.00}, 141.8 (C-1, C) {3.00, 3.32, 7.20-7.33}, 160.9 (C-6', C) {3.82, 5.92}, 161.9 (C-4', C) {2.05, 5.92}, 164.9 (C-2', C) {2.05, 5.92, 14.29}, 204.5 (C=O, C) {2.05, 3.00, 3.32}.- EIMS (70 eV, rel. int.%): $C_{17}H_{20}O_4$ 286.1 (71.38), 269.1 (6.84), 181.1 (100), 154.1 (54.11), 138.0 (6.97), 104.1 (5.42), 91.0 (22.51), 77.0 (6.75).

Hydrogenation of compounds 2 and 3

The hydrogenation procedure of Laswell and Hufford (1977) was adopted and done on a Parr hydrogenation apparatus model 3911 (shaker type hydrogenator) with 66CA 50 mL reaction bottle. Analytical grade

acetone (for **2**) or diethyl ether (for **3**) was used as solvent. Sample weighing 30 mg was placed in the reaction vessel together with 30 mL of solvent and 20 mg of catalyst, 10% palladium in carbon. Pressure was maintained at 30 psi and reaction time was 2 h. The mixture in the reaction bottle was filtered and the filtrate concentrated *in vacuo*. The crude product was chromatographed on silica gel and purified through a dropper column using isocratic elution with dichloromethane. Hydrogenation of **2** and **3** yielded **6** (9 mg) and 7 (12 mg).

2'-hydroxy-4',6'-dimethoxy-5'-methyldihydrochalcone (6)

M.p. 143-145°C.- UV λ_{max} (MeOH=283.4 nm; λ_{max} ($AlCl_3$, HCl)=339.8 nm; λ_{max} (NaOMe)=283.2 nm; λ_{max} (NaOAc, H_3BO_3)=282.8 nm.- FT-IR (KBr): ν_{max}=3026, 2945, 1613, 1599, 1413, 1281, 1212, 1139, 884, 791, 737, 699 cm^{-1}.- ^{1}H-NMR ($CDCl_3$, 500 MHz): δ=2.02 (3'-CH_3, 3H, s), 3.00 (C_β-H, 2H, t, J = 7.8), 3.33 (C_α-H, 2H, t, J = 7.8), 3.88 (6'-OCH_3, 3H, s), 3.89 (4'-OCH_3, 3H, s), 5.96 (H-5',1H, s), 7.20 – 7.30 (H-2, H-3, H-4, H-5, H-6, 5H, br m).- EIMS (70 eV, rel. int.%): $C_{18}H_{20}O_4$ 300.0 (59.99), 283.1 (12.77), 269.0 (11.52), 195.0 (100), 180.0 (15.97), 168.0 (57.85), 151.9 (13.66), 137.0 (9.76), 122.0 (10.39), 109.0 (14.54), 91.0 (40.62), 77.0 (18.70).

2',4'-dihydroxy-6'-methoxy-3',5'-dimethyldihydrochalcone (7)

M.p. 65-68°C.- UV λ_{max} (MeOH=281.2 nm; λ_{max} ($AlCl_3$, HCl)=305.0 nm; λ_{max} (NaOMe)=337.4 nm; λ_{max} (NaOAc, H_3BO_3)=340.0 nm.- FT-IR (KBr): ν_{max}=3323, 2945, 2864, 1606, 1567, 1420, 1355, 1308, 1223, 1146, 1108, 992, 826, 753, 702 cm^{-1}.- ^{1}H-NMR ($CDCl_3$, 300 MHz): δ=2.09 (3'-CH_3, 3H, s), 2.11 (5'-CH_3, 3H, s), 3.02 (C_β-H, 2H, t, J = 7.75), 3.40 (C_α-H, 2H, t, J = 7.75), 3.66 (6'-OCH_3, 3H, s), 5.32 (4'-OH, 1H, s), 7.16 – 7.31 (H-2, H-3, H-4, H-5, H-6, 5H, br m), 13.45 (2'-OH, 1H, s). Intensity of the signals at 5.32 and 13.45 decreased upon D_2O shake.- EIMS (70 eV, rel. int.%): $C_{18}H_{20}O_4$ 300.1 (64.16), 283.2 (8.68), 269.2 (32.68), 195.1 (100), 168.1 (59.01), 152.1 (33.38), 91.1 (55.87), 83.0 (25.30), 77.0 (21.08).

Anticholinesterase assay

Enzyme inhibitory activity was tested following the procedure from Nochi et al. (1995). All reagents for the cholinesterase inhibition assay were purchased from Sigma Chemical Co., acetylcholinesterase (3.1.1.7) from electric eel, and butyrylcholinesterase (3.1.1.8) from horse serum were the cholinesterases used.

Phosphate buffer-1 (0.1 M) for the enzyme and the test sample was prepared by dissolving 15.6 g of $Na_2HPO_4 \cdot 2H_2O$ in 750 mL distilled water. Since pH at 25°C should be 8.0, pH was adjusted accordingly by adding NaOH solution (100 mM/L). The solution was diluted to 1 L with distilled water. The resulting buffer solution-1 was stable as long as no microbial contamination occurs and was stored at 4°C.

Phosphate buffer-2 (0.1 M) for Ellman's reagent (Dithio-bisnitrobenzoic acid, DTNB) was prepared by dissolving 15.6 g of $Na_2HPO_4 \cdot 2H_2O$ in 750 mL water. pH at 25°C should be neutral. pH was adjusted accordingly by adding NaOH solution (100 mM/L). The solution was diluted to 1 L with deionized water. The buffer solution-2 is stored at 4°C and was stable as long as no microbial contamination occurs.

Buffered Ellman's reagent (DTNB, 0.1 M; $NaHCO_3$, 17.85 mmol/L) was prepared by dissolving 39.6 mg DTNB in 10 mL phosphate buffer-2 solution. 15 mg of $NaHCO_3$ was added and stored in a dark bottle at 4°C and was stable for 4 wk as long as kept in dark bottles.

Acetylthiocholine iodide (75 mmol/L) for the substrate was prepared by dissolving 108.35 mg acetylthiocholine iodide in 5 mL of distilled water and stored at 4°C. The solution was not kept for more than 7 days.

Acetylcholinesterase and butyrylcholinesterase enzymes were prepared by dissolving the enzyme in phosphate buffer-1 so that the concentration of the enzyme in the reaction mixture was about 0.0025 unit (U)/mL. This was kept in iced water bath at 5°C.

The test sample in triplicates was dissolved in the proper solvent to obtain the desired concentration. For water insoluble compounds, the effects of other solvents on the enzyme activity were checked prior to the experiment. Controls receive the same volume of solvent as the test samples.

The actual inhibitory assay involves the addition of 30 µL of test sample solution and 30 µL of enzyme stock solution to 2.81 µL of phosphate buffer-1. The mixture was incubated for 5 – 10 min at 25°C. A 100 µL of DTNB stock solution and 30 µL of substrate stock solution were then added and absorbance at 412 nm was recorded. The control used was physostigmine. The percent inhibition was calculated according to the following equation: % Inhibition = [1 – (Absorbance of test sample)/Absorbance of control)] x 100.

RESULTS AND DISCUSSION

The rare C-methylated flavonoids from *S. samarangense* were identified from spectral data as 7-hydroxy-5-methoxy-6,8-dimethylflavanone (1), 2'-hydroxy-4',6'-dimethoxy-3'-methylchalcone (2), 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (3), 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (4), and 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone (5) (Fig. 1). Since the chalcones (2-4) and dihydrochalcone (5) isolated were structurally related, the dihydrochalcones (6 and **7**) were prepared by reducing the Cα-Cβ double bonds of 2 and 3 to aid in structure activity relationship studies. These were identified from spectral data as 2'-hydroxy-4',6'-dimethoxymethoxy-3'-methyldihyrdochalcone (6) and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl-dihydrochalcone (**7**) (Fig. 1).

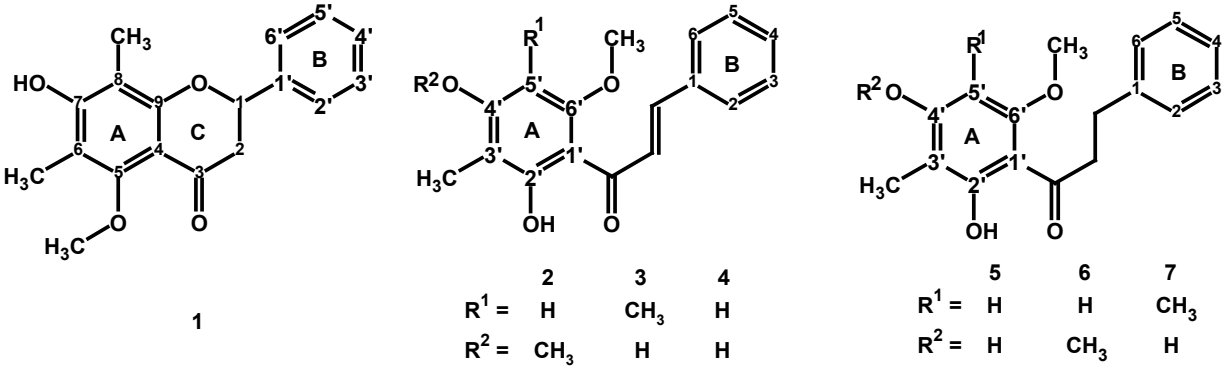

Figure 1. Structures of compounds **1**-**7** isolated from *Syzgium samaragense* (makopa). **1** - 7-hydroxy-5-methoxy-6,8-dimethylflavanone; **2** - 2'-hydroxy-4',6'-dimethoxy-3'-methylchalcone; 3 - 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone ; **4** – 2',4'-dihydroxy-6'-methoxy-3'methylchalcone;5 - 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone ; **6** - 2'-hydroxy-4',6'-dimethoxy-3'-methyldihydrochalcone; **7** - 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl- dihydrochalcone.

Only **7** showed an inhibitory activity against the acetylcholinesterase and butyrylcholinesterase enzymes with a 98.5% inhibition at a concentration of 0.25 mM and a 68% inhibition at a concentration of 0.20 mM, respectively. Physostigmine, the positive control, exhibited 100% inhibition at both concentrations. This data suggests that the observed activity of **7** is highly specific. That is, it is not the class of compound because **5** and **6** are also dihydrochalcones like **7** and yet are inactive. All the chalcone (2-4) and dihydrochalcone (**5**-**7**) test compounds have a C-2'-OH, a C-3'-CH_3, and a C-6'-OCH_3 in the A ring but only **7** is active. Compounds **3**, **4**, **5** and **7** all have a C-4'-OH, but again, only **7** is active. Compounds **3** and **7** both have a C-5'-CH_3, and yet only the latter is active. Therefore, both the C-5'-CH_3 and C_α-C_β are necessary for activity.

The active site of cholinesterases, specifically the acetylcholinesterase enzyme has been characterized and is believed to have three major domains (Quinn 1987). These are the esteratic site, which contains the active serine; the anionic site, which is about 4.7 Å from the esteratic site and binds the quaternary ammonium group of the substrate acetylcholine and, a hydrophobic region that is adjacent with or near the esteratic and anionic sites, which is believed to be important in binding aryl substrates and active site ligands (Quinn 1987). Quantitative structure activity relationship (QSAR) studies involving the three main classes of cholinesterase inhibitors point to the importance of hydrophobicity among the physostigmine and benzylamine derived inhibitors, electronic effects among benzylamine derivatives, and the contribution of steric effects (Recanatini et al. 1997). These observations were affirmed by structure activity relationship studies on the inhibitory action of phenserine, a physostigmine derivative, against acetylcholinesterase and butyrylcholinesterase (Yu et al. 2001).

What is apparent among the flavonoids from *S. samarangense* that were tested for cholinesterase inhibitory activity is their hydrophobic nature. It is also noted that there are structural similarities between compound **7** and the positive control, physostigmine (Fig. 2), such as the presence of a –C=O group and a –CH_3 and

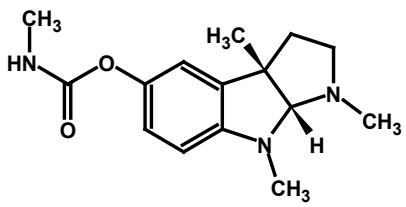

Figure 2. Structure of physostigmine.

a $-C_6H_6$ group near the –C=O group. The distinguishing structural characteristics of the active compound **7** are the C-5'-CH_3 and C_α-C_β groups, which probably enhances its hydrophobic nature. The C_α-C_β group in compound **7** is free to rotate and renders it flexible, which might contribute to a better enzyme-inhibitor interaction that results in its observed activity. It may be argued that compounds 5 and 6 also has this C_α-C_β group but they do not have the C-5'-CH_3. These suggestions, however, will have to be confirmed by kinetic and molecular modeling studies.

The IC_{50} of **7** against butyrylcholinesterase was determined to be 127 μM and that of physostigmine, the positive control, was 0.857 μM. No previous report of anticholinesterase activity for compound **7** was found in the literature. In addition, this is the first report of a flavonoid with anticholinesterase activity. Compound **7** was previously reported to exhibit prolyl endopeptidase inhibitory activity (Amor et al. 2004).

This finding adds to the number of bioactivities observed for dihydrochalcones in particular and flavonoids in general. Further investigation may be done to establish the mechanism of inhibitory action of **7** through enzyme kinetic experiments, and combined with molecular modeling studies may result in the development of a more potent compound, which may later be developed into a drug.

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