CHAPTER 2

Research Methodology

2.1 Instruments and Chemicals

Melting points were determined on a SANYO Gallenkamp melting point apparatus and optical rotations were measured in CH_3OH at the sodium D-line (590 nm) on a Bellingham & Stanley ADP220 polarimeter are uncorrected. The UV-Vis absorption spectra were measured in CH_3OH with a Perkin-Elmer UV-Vis spectrophotometer. The infrared (IR) spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer. The NMR spectra were recorded using either a 400 MHz Bruker or a 500 MHz Varian UNITY INOVA spectrophotometer. Chemical shifts are recorded in parts per million (δ) in Acetone-d_6 (δ_H 2.05 and δ_C (CO) 206.2 and (CH_3) 29.8), $CDCl_3$ (δ_H 7.26 and δ_C 77.0), CD_3OD (δ_H 3.31 and δ_C 49.0) and DMSO-d_6 (δ_H 2.50 and δ_C 39.5), with TMS as an internal reference. The MicroTOF data was obtained on a Bruker Daltonics mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel 60 GF_{254} (Merck). Column chromatography (CC) was carried out on Sephadex™ LH-20 with CH_3OH or silica gel (Merck) type 100 (62-400 μm) and type 60 (5-40 μm) for quick column chromatography (QCC). Solvents for extraction and chromatography were distilled prior to use.

2.2 Plant Materials

The leaves and stem bark of *Garcinia mckeaniana* Craib were collected from Doi Suthep National Park, Chiang Mai, Thailand in September 2013. This plant was identified by Mr. James F. Maxwell, Chiang Mai University Biology Herbarium, Chiang Mai University, Thailand, where a voucher specimen has been deposited (specimen no. Tonglau 1).

2.3 Extraction

Dried leaves of *G. mckeaniana* (0.7 kg) were cut into small pieces and extracted with acetone (8 L) over a period of 3 days at room temperature (3 times). The filtered acetone extract was concentrated under reduced pressure to yield the GML crude extract (95.6 g) as a dark brown gum.

The air dried stem bark of *G. mckeaniana* (2.5 kg) were chopped and extracted with acetone (8 L) over a period of 3 days at room temperature (3 times). Removal of the solvent under reduced pressure provided the GMB crude extract (56.6 g) as a dark brown gum.

2.4 Isolation

2.4.1 Isolation of *G. mckeaniana* Leaves

An acetone extract GML (95.6 g) was separated by QCC eluted with a gradient of hexanes - acetone - CH_3OH to give 8 fractions (A-H).

Fraction B (3.08 g, dark brown gum), was separated by QCC over silica gel eluting with a gradient of acetone-hexanes (100% hexanes to 100% acetone) to provide 5 subfractions (B1-B5). Subfraction B2 (739.0 mg) was isolated by Sephadex LH-20 with 100% CH_3OH to give 3 subfractions (B2A-B2C). Subfraction B2B (482.0 mg) was purified by CC eluting with 20% acetone/hexanes to give compound **GML1** (28.9 mg, yellow solid). Compound **GML2** (7.0 mg, yellow solid), was obtained from subfraction B4 (258.1 mg) by Sephadex LH-20 with 100% CH_3OH.

Fraction C (2.61 g, brown gum), was fractionated by Sephadex LH-20 with 100% CH_3OH to give 3 subfractions (C1-C3). Subfraction C2 (311.8 mg) was isolated by Sephadex LH-20 with 100% CH_3OH to afford 3 subfractions (C2A-C2C). Subfraction C2B (23.3 mg) was purified by FCC with 15% EtOAc/hexanes to yield compounds **GML5** (2.1 mg, yellow viscous oil) and **GML3** (7.1 mg, yellow viscous oil).

Fraction E (5.50 g, dark brown gum), was subjected to QCC eluted with a gradient of CH_2Cl_2-hexanes to 10% CH_3OH/CH_2Cl_2 to provide 3 subfractions (E1-E3).

Subfraction E2 (1.40 g) was separated by Sephadex LH-20 with 100% CH_3OH to give 5 subfractions (E2A-E2E). Subfraction E2B (188.2 mg) was repeated by Sephadex LH-20 with 100% CH_3OH to provide 3 subfractions (E2B1-E2B3). Subfraction E2B2 (69.3 mg) was purified by FCC with 20% acetone/hexanes to obtain compound **GML8** (17.5 mg, yellow viscous oil). Subfraction E2D (700.0 mg) was repeated by CC over Sephadex LH-20 with 100% CH_3OH to give 3 subfractions (E2D1-E2D3). Subfraction E2D2 (549.5 mg) was purified by FCC elution with 20% acetone/hexanes to afford compound **GML4** (37.7 mg, yellow viscous oil).

Fraction G (5.27 g, dark brown gum), was performed by FCC eluted with a gradient of CH_3OH-CH_2Cl_2 (100% CH_2Cl_2 to 10% CH_3OH/CH_2Cl_2) to provide 5 subfractions (G1-G5). Subfraction G2 (187.6 mg) was fractionated by Sephadex LH-20 with 100% CH_3OH to afford 3 subfractions (G2A-G2C). Subfraction G2B (34.4) was purified by FCC by elution with 30% EtOAc/hexanes to provide compound **GML6** (18.1 mg, yellow solid). Subfraction G4 (449.3) was repeated FCC by elution with 3% CH_3OH/CH_2Cl_2 to give 3 subfractions (G4A-G4C). Compound **GML7** (9.0 mg, yellow solid), was obtained from subfraction G4B (32.1 mg) by Sephadex LH-20 with 100% CH_3OH.

Compound **GML1** (Mckeanianone A): Yellow solid; mp 187-189 °C; UV (CH_3OH) λ_{max} nm (log ε): 217 (4.39), 295 (4.61), 337 (4.04), 384 (3.79); IR (neat) ν_{max}: 3418, 1649, 1610, 1463, 1285 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) and ^{13}C NMR (100 MHz, Acetone-d_6) spectral data see Table 2; HRESI-TOFMS *m/z* 479.2063 $[M+H]^+$(calcd for 479.2070, $C_{28}H_{31}O_7$).

Compound **GML2** (Bannaxanthone I): Yellow solid; mp 212-214 °C; UV (CH_3OH) λ_{max} nm (log ε): 217 (4.27), 295 (4.40), 338 (3.84), 383 (3.61); IR (neat) ν_{max}: 3444, 1715, 1650, 1611, 1437,1286 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data see Table 3.

Compound **GML3** (Bannaxanthone E): Yellow viscous oil; UV (CH_3OH) λ_{max} nm (log ε): 217 (4.27), 295 (4.40), 338 (3.84), 383 (3.61); IR (neat) ν_{max}: 3444, 1715, 1650, 1611, 1437,1286 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) data see Table 4.

Compound **GML4** (Mckeanianone B): Yellow viscous oil; UV (CH_3OH) λ_{max} nm (log ε): 218 (4.49), 292 (4.57), 338 (4.18), 379 (3.96) ; IR (neat) ν_{max}: 3447, 1704, 1650, 1611, 1446, 1288 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data see Table 5; HRESI-TOFMS *m/z* 517.1839 $[M+Na]^+$ (calcd for 517.1838, $C_{28}H_{30}O_8Na$).

Compound **GML5** (Mckeanianone C): Yellow viscous oil; UV (CH_3OH) λ_{max} nm (log ε): 217 (4.27), 295 (4.40), 338 (3.84), 383 (3.61); IR (neat) ν_{max}: 3418, 1736, 1650, 1597, 1448, 1244 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data see Table 6; HRESI-TOFMS *m/z* 559.1945 $[M+Na]^+$ (calcd for 559.1944, $C_{30}H_{32}O_9Na$).

Compound **GML6** (Mckeaniabiflavone): Yellow solid; mp 237-239 °C; UV (CH_3OH) λ_{max} nm (log ε): 218 (4.57), 269 (4.51), 331 (4.30); IR (neat) ν_{max}: 3444, 1650, 1608,1508, 1178 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) and ^{13}C NMR (100 MHz, Acetone-d_6) spectral data see Table 7; HRESI-TOFMS *m/z* found 553.1155 $[M+H]^+$ (calcd for 553.1135, $C_{31}H_{21}O_{10}$).

Compound **GML7** (Amentofavone): Yellow solid; mp 273-275 °C; UV nm (CH_3OH) λ_{max} nm (log ε): 217(4.55), 269 (4.45), 334(4.39); IR (neat) ν_{max}: 3442, 1650 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) and ^{13}C NMR (100 MHz, Acetone-d_6) spectral data see Table 8.

Compound **GML8** (Vomifoliol): Yellow viscous oil; $[\alpha]_D^{28}$: +62 (*c* 0.16, $CHCl_3$) (lit. $[\alpha]_D^{22}$: +41 (*c* 0.01, $CHCl_3$), UV (CH_3OH) λ_{max} nm (log ε): 214 (3.73), 238 (3.85); IR (neat) ν_{max}: 3444, 1650 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data see Table 9.

2.4.2 Isolation of *G. mckeaniana* Stem Bark

An acetone extract GMB was separated by QCC over silica gel eluting with a gradient from hexanes - CH_2Cl_2 - CH_3OH to give 11 fractions (A-K).

Fraction B (268.3 mg, brown viscous oil), was further purified by CC elution with 5% EtOAc/hexanes to give 3 subfractions (B1-B3). Subfraction B2 (21.3 mg) was purified by Sephadex LH-20 with 100% CH_3OH to afford compound **GMB1** (3.0 mg, yellow powder)

Fraction D (419.0 mg, brown viscous oil), was separated by CC with 10% acetone/hexanes to give 3 subfractions (D1-D3). Subfraction D2 (13.2 mg) was further purified by Sephadex LH-20 with 100% CH_3OH to give compound **GMB2** (10.0 mg, yellow solid)

Fraction F (1.02 g, dark brown gum), was separated by Sephadex LH-20 with 100% CH_3OH to give 3 subfraction (F1-F3). Subfraction F2 (109.6 mg) was further purified by Sephadex LH-20 with 100% CH_3OH to yield compound **GMB4** (9.3 mg, yellow viscous oil)

Fraction H (489.1 mg, brown viscous oil), was isolated by CC with 20% acetone/hexanes to give 5 subfractions (H1-H5). Subfraction H2 (87.4 mg) was fractionated by CC with 15% EtOAc/hexanes to afford 3 subfrations (H2A-H2C). Subfraction H2B (13.1 mg) was further purified by Sephadex LH-20 with 100% CH_3OH to give compound **GMB6** (6.7 mg, yellow solid). Compound **GMB5** (2.0 mg, yellow amorphous), was obtained from subfraction H4 (20.5 mg) by Sephadex LH-20 with 100% CH_3OH.

Fraction J (4.71 g, dark brown gum), was separated by CC with a gradient elution of 1-10% CH_3OH/CH_2Cl_2 to provide 9 subfractions (J1-J9). Subfraction J2 (329.6 mg) was isolated by CC eluting with 20% acetone/hexanes to give 3 subfractions (J2A-J2C). The second subfraction (9.2 mg) was purified by Sephadex LH-20 with 100% CH_3OH to obtain compound **GMB11** (3.7 mg, yellow solid). Subfraction J4 (762.6 mg) was subjected to QCC by elution with a gradient of 80% CH_2Cl_2/hexanes to 5% CH_3OH/CH_2Cl_2 to give 4 subfractions (J4A-J4D). Compound **GMB9** (2.0 mg, yellow solid), was obtained from subtraction J4B (99.0 mg) by Sephadex LH-20 with 100% CH_3OH. Subfraction J4C (145.6 mg) was fractionated by Sephadex LH-20 with 100% CH_3OH to provide 3 subfractions (J4C1-J4C3). Subfraction J4C3 (30.2 mg) was purified by FCC elution with 3% CH_3OH/CH_2Cl_2 to give compound **GMB8** (6.3 mg, yellow solid). Compound **GMB7** (4.8 mg, yellow solid), was received from subfraction J6 (341.1 mg) by purification with Sephadex LH-20 eluting 100% CH_3OH. Subfraction J8 (987.5 mg) was isolated by Sephadex LH-20 with 100% CH_3OH to provide 5 subfractions (J8A-J8E). Subfraction J8B (20.0 mg) was purified by Sephadex LH-20 with 100% CH_3OH to give 3 subfraction (J8B1-J8B3). Subfraction J8B2 (12.6 mg) was futher purified by FCC by elution with 30% acetone/hexanes to afford compound

GMB3 (6.7 mg, yellow solid). Subfraction J8D (374.9 mg) was fractionated by FCC eluting with 30% acetone/hexanes to give 3 subfractions (J8D1-J8D3). Subfraction J8D2 (12.4 mg) was purified by Sephadex LH-20 with 100% CH_3OH to provide compound **GMB10** (4.4 mg, yellow powder).

Compound **GMB1** (*R*-(–)-Mellein): Yellow powder; $[\alpha]_D^{28}$: –29 (*c* 0.03, CH_3OH) (lit. $[\alpha]_D^{22}$: –85.2 (*c* 0.0027, CH_3OH) (Efdi *et al*., 2007), UV (CH_3OH) λ_{max} nm (log ε): 217 (3.92), 246 (3.60), 313 (3.34); IR (neat) ν_{max}: 3387, 1675, 1618, 1463 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) data see Table 10.

Compound **GMB2** (Cotoin): Yellow solid; mp 127-128 °C; UV (CH_3OH) λ_{max} nm (log ε): 217 (4.13), 251 (3.78), 302 (3.91); IR (neat) ν_{max}: 3449, 1631 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data see Table 11; HRESI-TOFMS *m/z* 245.0810 $[M+H]^+$ (calcd for 245.0814, $C_{14}H_{13}O_4$).

Compound **GMB3** (2,3′,4,6-Tetrahydroxybenzophenone): Yellow solid; mp 248-250 °C; UV (CH_3OH) λ_{max} nm (log ε): 218 (4.25), 304 (4.01); IR (neat) ν_{max}: 3448, 1700, 1635, 1454 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) spectral data see Table 12.

Compound **GMB4** (Pancixanthone A): Yellow viscous oil; UV nm (CH_3OH) λ_{max} nm (log ε): 212 (4.58), 245 (4.78), 319 (6.73), 359 (3.88); IR (neat) ν_{max}: 3429, 1647, 1579, 1500 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data see Table 13.

Compound **GMB5** (Assiguxanthone A): Yellow amorphous; UV (CH_3OH) λ_{max} nm (log ε): 215 (4.57), 253 (4.83), 286 (4.27), 327 (4.49); IR (neat) ν_{max}: 3416, 1648, 1575, 1466 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) data see Table 14.

Compound **GMB6** (1,3,7-Trihydroxy-2-(3-methylbut-2-enyl)-xanthone): Yellow solid; mp 226-228 °C; UV (CH_3OH) nm λ_{max} (log ε): 239 (4.29), 262 (4.29), 313 (3.95), 376 (3.52); IR (neat) ν_{max}: 3423, 1646, 1610, 1477 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) data see Table 15.

Compound **GMB7** (Assiguxanthone B): Yellow solid; mp. 258-260 °C; UV (CH_3OH) λ_{max} nm (log ε): 215 (4.34), 242(4.41), 258 (4.41), 320 (4.18), 366 (3.98); IR (neat) ν_{max}: 3396, 1647, 1570, 1489 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) data see Table 16.

Compound **GMB8** (1,3,5-Trihydroxyxanthone): Yellow solid; mp 230-232 °C; UV (CH_3OH) λ_{max} nm (log ε): 216 (4.07), 244 (4.25), 312 (4.90), 367 (3.20); IR (neat) ν_{max}: 3444, 1653, 1577, 1497 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) data see Table 17.

Compound **GMB9** (1,3,7-Trihydroxyxanthone): Yellow solid; mp >300 °C (decompose); UV (CH_3OH) λ_{max} nm (log ε): 216 (4.17), 238 (4.24), 254 (4.30), 312 (3.98), 359 (3.94); IR (neat) ν_{max}: 3417, 1705, 1653, 1579, 1291 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) data see Table 17.

Compound **GMB10** (Norathyriol): Yellow powder; UV (CH_3OH) λ_{max} nm (log ε): 216 (4.29), 237 (4.32), 254(4.42), 311 (4.10), 363 (4.01); IR (neat) ν_{max}: 3444, 1635, 1304 and 1186 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) data see Table 17.

Compound **GMB11** (Montixanthone): Yellow solid; mp >270 °C (decompose); UV (CH_3OH) λ_{max} nm (log ε): 215 (4.31), 238 (4.38), 254 (4.46), 312 (4.16), 358 (4.07); IR (neat) ν_{max}: 3357, 1705, 1615, 1490 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) data see Table 17.

2.5 Biological Assays

2.5.1 Antimalarial Assay

The *in vitro* antimalarial activity against *Plasmodium falciparum* (TM4 and K1, multidrug resistant strains) was carried out using as described by Wangchuk, (Wangchuk *et al*., 2011),using chloroquine, cycloguanil and pyrimethamine as positive controls.

2.5.2 Cytotoxic Assays

The cytotoxicity assays against human oral cavity cancer (KB) and normal Vero cells from African green monkey kidney were evaluated using the sulforhodamine B (SRB) assay (Wangchuck *et al.*, 2011). The ellipticine and doxorubicine were used as positive controls.

2.5.3 Antibacterial Assay

Bacillus cereus TISTR688, *Escherichia coli* TISTR780, *Micrococcus luteus* TISTR884, *Pseudomonas aeruginosa* TISTR781, *Salmonella typhimurium* TISTR292, *Staphylococcus aureus* TISTR1466 and *Staph. Epidermidis* ATCC12229 were obtained from the Microbiological Research Center of the Thailand Institute of Scientific and Technological Research. The minimum inhibition concentrations (MICs) were determined by a two-fold serial dilution method using Mueller Hinton broth according to the Clinical and Laboratory Standards Institute recommendations (CLSI, 2012). Amphicilin and gentamicin were used as positive controls.