CHAPTER III

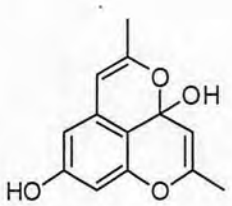
EXPERIMENTALS

3.1 Materials and Methods

All reagents were analytical grade and purchased from Fluka, or Aldrich Chemicals Co., Ltd. and were used as received without future purification. Solvents for reactions were AR grade. FT-IR spectra were recorded on a PerkinElmer Fourier Transform Infrared Spectrophotometer (PerkinElmer Inc., USA). Infrared spectra were recorded between 400 cm^{-1} to 4,000 cm^{-1} in transmittance mode. ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC spectra were obtained in CD_3OD, unlesss noted otherwise, at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei (Varian Company, USA). Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual solvent peak. Coupling constants (J) are reported in Hertz (Hz). Mass spectra were recorded by electron spray ionization mass spectroscopy (ESI-MS) on a Bruker Daltonics Mass Spectrometer: micrOTOF (Bruker Daltonics Ins., USA).

3.2 Experimental Procedures

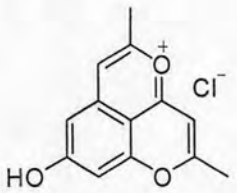
3.2.1 Extraction of Barakol (1)



Following a standard procedure [10] with slight modification, fresh young leaves and flowers of *Cassia siamea* (2 kg) were boiled with 0.5% (v/v) aqueous sulfuric acid (2.5 L) for 30 min and filtered. The filtrate was basified with a saturated sodium carbonate solution to pH 9 10. The resulting basic solution was divided into four portions and each was extracted with dichloromethane (200 mL × 4). The combined dichloromethane extracts were concentrated under reduced pressure until the volume of

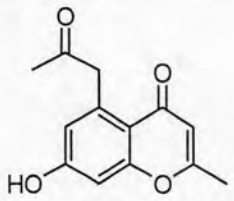
organic extract was one-fourth of the starting volume, and then equal volume of distilled water was added. The mixture was shaken vigorously, to give a yellow needle crystalline solid of **1** (9.7 g) that was collected by filtration. ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H), 2.36 (s, 3H), 6.05 (s, 1H), 6.29 (s, 1H), 6.30 (s, 1H), 6.41 (s, 1H). This ^{1}H-NMR data are consistent with those described in the literature.

3.2.2 Synthesis of Anhydrobarakol Chloride (3)



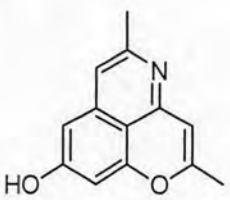
Following a published procedure with slight modification [8], to a solution of **1** (0.232 g, 1.00 mmol) in methanol (2.0 mL), concentrated hydrochloric acid (0.5 mL) was slowly added at room temperature. The mixture was stirred during the addition of the acid until the greenish-yellow needle crystalline solid was obtained. The mixture was cooled to 0 5 °C in an ice bath and the stirring was continued at this temperature for additional 15 min. The residual mixture was precipitated by adding THF (10.0 mL). The resulting solid was filtered and washed with THF to give **3** (0.147 g, 58%). ^{1}H NMR δ 2.50 (s, 3H), 2.72 (s, 3H), 7.04 (s, 1H), 7.08 (s, 1H), 7.15 (s, 1H), 7.16 (s, 1H); ^{13}C NMR δ 17.9, 20.1, 101.7, 102.1, 107.8, 108.1, 108.9, 135.0, 157.9, 159.3, 168.7, 170.2, 176.0. These ^{1}H- and ^{13}C-NMR data are consistent with those described in the literature.

3.2.3 Synthesis of 5-Acetonyl-7-hydroxy-2-methyl Chromone (27)



Following a published procedure with slight modification [8], a mixture of **1** (0.232 g, 1.00 mmol) and triethylamine (0.101 g, 1.00 mmol) in 5% v/v aqueous methanol (5.0 mL) was reacted at room temperature for 12 h. The mixture was concentrated under reduced pressure. The resulting residual crude was purified by siliga column chromatography using ethylacetate as eluent to obtain chromone **27** (0.075 g, 32%). ^{1}H NMR ($CDCl_3$/DMSO-d_6 (9:1)) δ 2.12 (s, 3H), 2.15 (s, 3H), 3.98 (s, 2H), 5.74 (s, 1H), 6.41 (s, 1H), 6.57 (s, 1H), 9.89 (s, −OH); ^{13}C NMR ($CDCl_3$/DMSO-d_6 (9:1)) δ 19.9, 29.9, 49.6, 102.1, 110.7, 114.2, 118.3, 137.6, 159.5, 161.2, 164.1, 178.9, 205.4. These ^{1}H- and ^{13}C-NMR data are consistent with those described in the literature.

3.2.4 Synthesis of Cassiarin A (4)



3.2.4.1 Synthesis of Cassiarin A (4) via Cyclization of 5-Acetonyl-7-hydroxy-2-methyl Chromone (27) with Ammonium Acetate

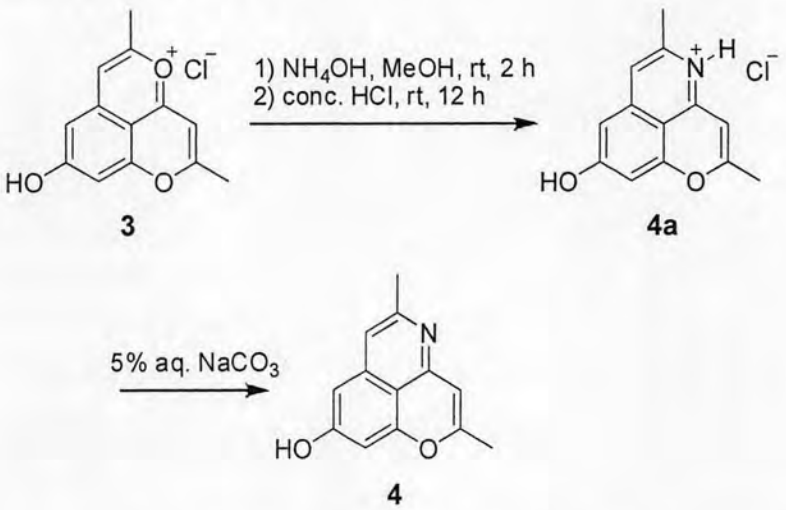
A chromone **27** (0.232 g, 1.00 mmol) and ammonium acetate (0.154 g, 2.00 mmol) were reacted in glacial acetic acid (5.0 mL) under reflux for 4−5 h (TLC monitoring; MeOH/CH_2Cl_2 (1:4)). The reaction mixture was diluted with distilled water (5.0 mL) and cooled to 0−5 °C in an ice bath. The resulting mixture was basified with a saturated sodium carbonate solution to pH 9−10 and the stirring was continued until the

precipitates were appeared. The resulting solid was filtered off and washed with water to afford **4** (0.124 g, 58%).

3.2.4.2 Synthesis of Cassiarin A (4) via Condensation of Barakol (1) with Ammonium Acetate and Ammonium Chloride

Brakol 1 (0.232 g, 1.00 mmol) and ammonium acetate (0.154 g, 2.00 mmol) or ammonium chloride (0.107 g, 2.00 mmol) were mixed in methanol (10.0 mL). The mixture was stirred and refluxed for 4–5 h (TLC monitoring; $MeOH/CH_2Cl_2$ (1:4)). The reaction mixture was concentrated under reduced pressure, and the residue was dissolved with distilled water (5.0 mL). The resulting solution was cooled in an ice bath and basified with saturated sodium carbonate solution to pH 9–10, and the stirring was continued until the solid were appeared. The resulting solid was filtered off and washed with water to afford **4** (0.122–1.43 g, 57–67%).

3.2.4.3 Synthesis of Cassiarin A (4) via Condensation of Anhydrobarakol Chloride (3) with Ammonium Hydroxide

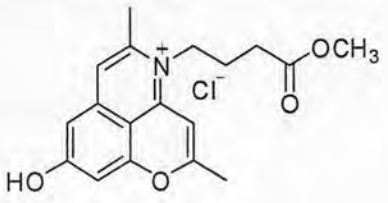


A solution of salt **3** (0.125 g, 0.501 mmol) in methanol (2.0 mL) was reacted with a 25% w/v aqueous ammonium hydroxide solution (0.5 mL) at room temperature for 2 h. The resulting mixture was further reacted with concentrated hydrochloric acid (0.5 mL)

and the stirring was continued at this temperature for 12 h. Methanol was removed under reduced pressure, and the residual solid was precipitated by adding THF (10.0 mL). The product was filtered and washed with THF to afford a pure compound **4a** as a light green solid (0.124 g, 99%). m.p. 327 °C (dec.); ^{1}H NMR δ 2.45 (s, 3H), 2.46 (s, 3H), 6.50 (s, 1H), 6.85 (d, J = 1.6 Hz, 1H), 6.89 (d, J = 1.6 Hz 1H), 7.06 (s, 1H); ^{13}C NMR δ 17.5, 19.1, 98.0, 101.1, 104.4, 109.4, 114.2, 138.1, 140.6, 148.3, 156.4, 166.1, 168.1; ESI-MS *m/z* 214.1 ($[M-Cl]^+$); HR-ESI-MS obsd 214.0882 ($[M-Cl]^+$), calcd 214.0863 ($[M-Cl]^+$), 249.0557 (M^+; M = $C_{13}H_{12}ClNO_2$).

To a solution of **4a** (0.125 g, 0.501 mmol) in water (5.0 mL), was added a 5% wt/v aqueous sodium carbonate solution (1.0 mL). The resulting mixture was stirred until a light green solid was obtained (approximately 5 min) and the stirring was continued for additional 15 min. The resulting solid was filtered and washed with water to give the **4** as a light greenish-yellow solid (0.096 g, 90%). m.p. 322 °C (dec.); ^{1}H NMR (DMSO-d_6) δ 2.13 (s, 3H), 2.28 (s, 3H), 6.06 (s, 1H), 6.42 (s, 1H), 6.45 (s, 1H), 6.80 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 19.8, 24.5, 99.1, 100.9, 106.1, 111.7, 112.8, 138.4, 150.5, 153.4, 155.3, 159.1, 160.9; ESI-MS *m/z* 214.1 ($[M+H]^+$); HR-ESI-MS obsd 214.0868 ($[M+H]^+$), calcd 214.0863 ($[M+H]^+$), 213.0790 (M^+; M = $C_{13}H_{11}NO_2$).

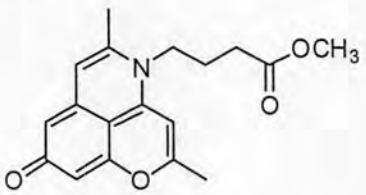
3.2.5 Synthesis of *N*-4-Methoxy-4-oxobutyl Cassiarin A Chloride (5a)



Following a procedure described for **4a**, a mixture of **3** (0.125 g, 0.501 mmol), methyl-4-aminobutyrate hydrochloride (0.154 g, 1.00 mmol) and triethylamine (0.101 g, 1.00 mmol) in methanol (2.0 mL) was reacted at room temperature for 2 h. The resulting mixture was further reacted with concentrated hydrochloric acid (0.5 mL) and the stirring was continued at room temperature for 12 h. Methanol was removed under reduced

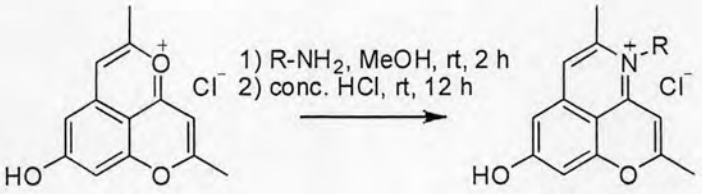
pressure, and the residual solid was precipitated by adding THF (10.0 mL). The product was filtered and washed with THF to obtain **5a** as a light yellow solid (0.152 g, 87%). m.p. 243 °C (dec.); ^{1}H NMR (D_2O/DMSO-d_6 (9.5:0.5)) δ 1.91−1.83 (m, 2H), 2.28 (s, 3H), 2.36 (s, 3H), 2.47 (t, J = 6.6 Hz, 2H), 3.55 (s, 3H), 3.98 (t, J = 8.0 Hz, 2H), 6.38 (d, J = 1.6 Hz, 1H), 6.48 (d, J = 2.0 Hz, 1H), 6.54 (s, 1H), 6.76 (s, 1H); ^{13}C NMR (D_2O/DMSO-d_6 (9.5:0.5)) δ 175.4, 169.1, 163.5, 155.0, 148.7, 142.6, 135.6, 116.7, 110.1, 103.8, 101.1, 97.1, 52.2, 47.7, 29.7, 22.1, 20.1, 19.2; ES-MS *m/z* 314.1 ([M−Cl]$^+$); HR-ESI-MS obsd 314.1382 ([M−Cl]$^+$), calcd 314.1387 ([M−Cl]$^+$), 349.1081 (M^+; M = $C_{18}H_{20}ClNO_4$).

3.2.6 Synthesis of Cassiarin B (5)



To a solution of **5a** (0.152 g, 0.43 mmol) in water (4.0 mL), was added a 5% (wt/v) aqueous sodium carbonate solution (1.0 mL). The mixture was stirred at room temperature for 5 min. The aqueous solution was extracted with chloroform (4 × 15.0 mL). The combined chloroform extracts were concentrated under reduced pressure until the volume of organic extract was reduced to approximately 1.0 mL. The resulting crude mixture was kept at the room temperature until **5** was obtained as a yellow solid (0.081 g, 60%). m.p. 138 °C (dec.); ^{1}H NMR δ 1.96−1.89 (m, 2H), 2.37 (s, 3H), 2.44 (s, 3H), 2.56 (t, J = 6.4 Hz, 2H), 3.71 (s, 3H), 3.99 (t, J = 8.4 Hz, 2H), 6.22 (s, 1H), 6.35 (s, 1H), 6.59 (s, 1H), 6.64 (s, 1H); ^{13}C NMR (CD_3OD) δ 18.6, 19.3, 22.8, 29.1, 46.6, 50.9, 95.7, 104.9, 106.9, 107.7, 115.1, 135.4, 140.1, 146.8, 155.8, 166.0, 173.5, 177.7; ESI-MS *m/z* 314.1 ([M+H]$^+$); HR-ESI-MS obsd 314.1387 ([M+H]$^+$), calcd 314.1387 ([M+H]$^+$), 313.1314 (M^+; M = $C_{18}H_{19}NO_4$).

3.2.7 General Procedure for Synthesis of *N*-Substituted Cassiarin A Chloride 28a–31a

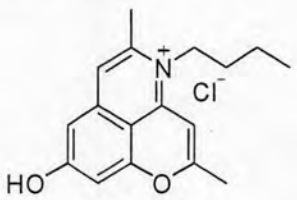


3

28a, R = butyl
29a, R = cyclohexyl
30a, R = phenyl
31a, R = benzyl

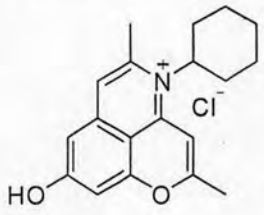
A mixture of **3** (0.125 g, 0.501 mmol) and amine (1.00 mmol) in methanol (2.0 mL) was stirred at room temperature for 2 h. The resulting mixture was further reacted with concentrated hydrochloric acid (0.5 mL) and the stirring was continued at room temperature for 12 h. Methanol was removed under reduced pressure, and the residual solid was treated with THF (10.0 mL). The resulting solid was collected by filtration and washed again with THF to afford a light yellow solid of *N*-substituted cassiarins A **28a–31a**.

3.2.7.1 *N*-Butyl Cassiarin A Chloride (28a):



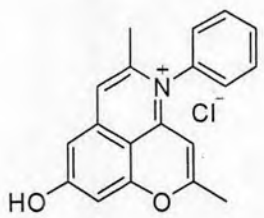
(0.135 g, 88%). m.p. 245 °C (dec.); ^{1}H NMR δ 1.04 (t, J = 7.4 Hz, 3H), 1.53–1.44 (m, 2H), 1.75–1.67 (m, 2H), 2.53 (s, 3H), 2.62 (s, 3H), 4.27 (t, J = 8.2 Hz, 2H), 6.84 (s, 1H), 6.89 (s, 1H), 6.90 (s, 1H), 7.21 (s, 1H); ^{13}C NMR δ 12.6, 18.9, 19.2, 19.5, 29.8, 48.5, 97.4, 101.4, 104.2, 110.7, 117.2, 136.4, 142.7, 149.2, 155.8, 165.4, 168.8; ESI-MS *m/z* 270.1 ($[M-Cl]^+$); HR-ESI-MS obsd 270.1531 ($[M-Cl]^+$), calcd 270.1489 ($[M-Cl]^+$), 305.1183 (M^+; M = $C_{17}H_{20}ClNO_2$).

3.2.7.2 *N*-Cyclohexyl Cassiarin A Chloride (29a):



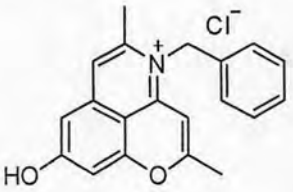
(0.145 g, 87%). m.p. 338 °C (dec.); ^{1}H NMR ($CD_3OD/CDCl_3$ (1:4)) δ 1.41–1.35 (m, 1H), 1.56–1.46 (m, 2H), 1.82 (d, J = 13.2 Hz, 1H), 1.96 (d, J = 11.6 Hz, 2H), 2.05 (d, J = 13.2 Hz, 2H), 2.41–2.33 (m, 2H), 2.54 (s, 3H), 2.63 (s, 3H), 4.61 (t, J = 12.0 Hz, 1H), 6.83 (s, 1H), 6.87 (s, 1H), 6.93 (s, 1H), 7.14 (s, 1H); ^{13}C NMR ($CD_3OD/CDCl_3$ (1:4)) δ 20.9, 21.5, 24.4, 25.98, 25.98, 28.71, 28.71, 63.3, 99.3, 102.6, 105.0, 111.9, 118.9, 136.0, 142.2, 149.4, 155.7, 165.9, 166.9; ESI-MS *m/z* 296.2 ($[M-Cl]^+$); HR-ESI-MS obsd 296.1674 ($[M-Cl]^+$), calcd 296.1645 ($[M-Cl]^+$), 331.1339 (M^+; M = $C_{19}H_{22}ClNO_2$).

3.2.7.3 *N*-Phenyl Cassiarin A Chloride (30a):



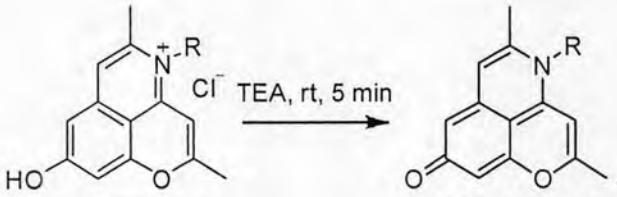
(0.123 g, 76%). m.p. 258 °C (dec.); ^{1}H NMR δ 2.15 (s, 3H), 2.35 (s, 3H), 5.81 (s, 1H), 6.99 (d, J = 1.6 Hz, 1H,), 7.01 (d, J = 1.6 Hz, 1H), 7.33 (s, 1H), 7.51–7.50 (m, 2H), 7.77–7.75 (m, 3H); ^{13}C NMR δ 19.3, 19.7, 98.3, 101.5, 104.8, 110.0, 116.0, 127.10, 127.10, 130.97, 130.97, 130.97, 136.8, 136.9, 142.8, 150.5, 156.4, 166.3, 169.1; ESI-MS *m/z* 290.1 ($[M-Cl]^+$); HR-ESI-MS obsd 290.1110 ($[M-Cl]^+$), calcd 290.1176 ($[M-Cl]^+$), 325.0870 (M^+; M = $C_{19}H_{16}ClNO_2$).

3.2.7.4 *N*-Benzyl Cassiarin A Chloride (31a):



(0.152 g, 89%). m.p. 291 °C (dec.); ^{1}H NMR δ 2.43 (s, 3H), 2.54 (s, 3H), 5.64 (s, 2H), 6.80 (s, 1H), 6.96 (d, J = 1.6 Hz, 1H), 7.00 (d, J = 2.0 Hz, 1H), 7.15 (d, J = 7.2 Hz, 2H), 7.32 (s, 1H), 7.36 (d, J = 7.2 Hz, 1H), 7.43–7.39 (m, 2H); ^{13}C NMR δ 18.9, 19.4, 51.4, 97.6, 101.6, 104.6, 110.7, 117.2, 125.26, 125.26, 128.0, 129.13, 129.13, 133.3, 136.7, 143.1, 150.5, 156.2, 166.0, 169.4; ESI-MS *m/z* 304.1 ($[M-Cl]^+$); HR-ESI-MS obsd 304.1365 ($[M-Cl]^+$), calcd 304.1332 ($[M-Cl]^+$), 339.1026 (M^+; M = $C_{20}H_{18}ClNO_2$).

3.2.8 General Procedure for Synthesis of *N*-Substituted Cassiarin B 28b–31b

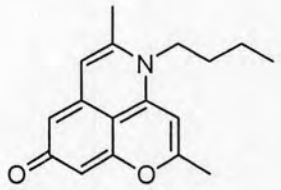


28a, R = butyl
29a, R = cyclohexyl
30a, R = phenyl
31a, R = benzyl

28b, R = butyl
29b, R = cyclohexyl
30b, R = phenyl
31b, R = benzyl

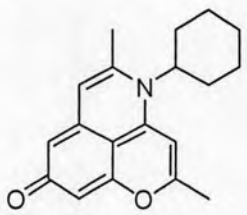
To a solution of *N*-substituted cassiarin A chloride **28a–31a** (0.30 mmol) in water (5.0 mL), was added triethylamine (0.061 g, 0.60 mmol). The mixture was stirred at room temperature for 5 min. The resulting solution was extracted with chloroform (15 mL × 4) or, in the other case of **31b**, the resulting solid filter off. For **28b–30b**, the combined chloroform extracts were concentrated under reduced pressure until the volume of organic extract was reduced to 1.0 mL. The crude extract was kept in the room temperature until the yellow precipitate was obtained. The precipitate was dried in vacuum to give the *N*-substituted cassiarin B derivatives **28b–31b**.

3.2.8.1 *N*-Butyl Cassiarin B (28b):



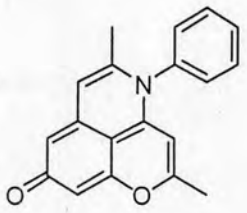
(0.054 g, 67%). m.p. 167 °C (dec.); ^{1}H NMR δ 1.02 (t, J = 7.4 Hz, 3H), 1.53–1.43 (m, 2H), 1.68–1.61 (m, 2H), 2.35 (s, 2H), 2.41 (s, 3H), 3.96 (t, J = 8.2 Hz, 2H), 6.21 (s, 1H), 6.33 (s, 1H), 6.34 (s, 1H), 6.63 (s, 1H); ^{13}C NMR δ 12.6, 18.7, 19.2, 19.2, 30.2, 47.2, 95.6, 104.9, 106.9, 107.7, 115.2, 135.4, 140.1, 146.6, 155.7, 165.9, 177.7; ESI-MS *m/z* 270.2 ($[M+H]^+$); HR-ESI-MS obsd 270.1538 ($[M+H]^+$), calcd 270.1489 ($[M+H]^+$), 269.1416 (M^+; M = $C_{17}H_{19}NO_2$).

3.2.8.2 *N*-Cyclohexyl Cassiarin B (29b):



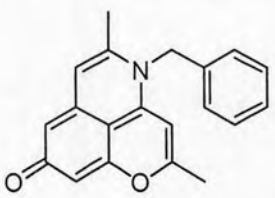
(0.059 g, 67%). m.p. 136 °C (dec.); ^{1}H NMR ($CD_3OD/CDCl_3$ (1:4)) δ 1.31–1.21 (m, 1H), 1.48–1.39 (m, 2H), 1.80 (d, J = 12.8 Hz, 1H), 1.90 (d, J = 12.4 Hz, 2H), 2.00 (d, J = 13.2 Hz, 2H), 2.26–2.22 (m, 2H), 2.43 (d, J = 5.6 Hz, 3H), 2.43 (s, 3H), 4.39–4.32 (m, 1H), 6.32 (s, 1H), 6.39 (s, 1H), 6.49 (s, 1H), 6.63 (s, 1H); ^{13}C NMR ($CD_3OD/CDCl_3$ (1:4)) δ 20.8, 21.4, 24.7, 26.14, 26.14, 29.34, 29.34, 61.5, 97.7, 106.2, 108.5, 108.7, 116.9, 135.0, 139.4, 146.9, 155.9, 163.6, 178.4; ESI-MS *m/z* 296.2 ($[M+H]^+$); HR-ESI-MS obsd 296.1676 ($[M+H]^+$), calcd 296.1645 ($[M+H]^+$), 295.1572 (M^+; M = $C_{19}H_{21}NO_2$).

3.2.8.3 *N*-Phenyl Cassiarin B (30b):



(0.056 g, 65%). m.p. 132 °C (dec.); ^{1}H NMR δ 2.01 (s, 3H), 2.21 (s, 3H), 5.45 (s, 1H), 6.43 (d, J = 1.2 Hz, 1H), 6.49 (d, J = 1.2 Hz 1H), 6.86 (s, 1H), 7.47–7.45 (m, 2H), 7.72–7.68 (m, 3H); ^{13}C NMR δ 19.1, 19.5, 96.6, 104.9, 106.3, 108.6, 114.1, 127.74, 127.74, 130.4, 130.62, 130.62, 135.8, 137.3, 140.3, 147.8, 156.5, 166.2, 178.4; ESI-MS *m/z* 290.1 ([M+H]$^{+}$); HR-ESI-MS obsd 290.1137 ([M+H]$^{+}$), calcd 290.1176 ([M+H]$^{+}$), 289.1103 (M^{+}; M = $C_{19}H_{15}NO_2$).

3.2.8.4 *N*-Benzyl Cassiarin B (31b):



(0.079 g, 87%). m.p. 192 °C (dec.); ^{1}H NMR δ 2.27 (s, 3H), 2.37 (s, 3H), 5.38 (s, 2H), 6.31 (s, 1H), 6.38 (d, J = 2.0 Hz, 1H), 6.47 (d, J = 1.6 Hz, 1H), 6.82 (s, 1H), 7.10 (d, J = 7.6 Hz, 2H), 7.33–7.30 (m, 1H), 7.40–7.36 (m, 2H); ^{13}C NMR δ 18.7, 19.2, 50.3, 95.9, 105.1, 106.9, 108.3, 115.2, 125.14, 125.14, 127.7, 128.99, 128.99, 134.3, 135.6, 140.7, 147.8, 156.1, 166.4, 178.3; ESI-MS *m/z* 304.1 ([M+H]$^{+}$); HR-ESI-MS obsd 304.1365 ([M+H]$^{+}$), calcd 304.1332 ([M+H]$^{+}$), 303.1259 (M^{+}; M = $C_{20}H_{17}NO_2$).

3.3 Biological Activities

3.3.1 Antiplasmodial Activity

Determination of antiplasmodial activity was performed by National Center for Genetic Engineering and Biotechnology (BIOTEC). Firstly, *Plasmodial falciparum* (K1, multi drug resistant strain) was cultivated *in vitro* according to Trager and Jensen procedure [21] in RPMI 1640 medium containing 20 mM *N*-2-hydroxyethypiperazine-*N'*-2-ethanesulfonic acid (HEPES), 32 mM $NaHCO_3$ and 10% heat activated human serum with 3% erythocytes and incubated at 37 °C in an incubator with 3% CO_2. Cultures were diluted with fresh medium and erythocytes everyday according to cell growth. Quantitative assessment of antiplasmodial activity i*n vitro* was determined by microculture radioisotope techniques based upon the methods described by Desjardins and co-workers [22]. Briefly, a mixture of 200 µL of 1.5% erytrocytes with parasitemia at the early ring stage was pre-exposed to 25 µL of the medium containing at test sample dissolved in 1% DMSO (0.1% final concentration) for 24 h employing the incubation conditions described above. Subsequently, 25 µL of [^{3}H]-hypoxanthine in culture medium (0.5 µCi) was added to each well and plates were incubated for an additional 24 h. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the TopCount microplate scintillation counter. Inhibition concentration (IC_{50}) represents the concentration which indicates 50% reduction in parasite growth. The standard sample was Dihydroartemisinine (DHA).

3.3.2 Cytotoxic Activity

Determination of cytotoxicity test was performed by Institute of Biotechnology and Genetic Engineering, which using the following protocol. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method [23, 24]. In principle, viable cell number/well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

The human cell line was harvested from exponential-phase maintenance cultures (T-25 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100 μL volumes using a repeating pipette. Following a 24 h incubation at 37 °C, 5% CO_2, 100% relative humidity, 100 μL of culture medium, culture medium containing sample was dispensed within appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium/tetrazolium reagent blank (N = 6) "background" determinations. Culture plates were then incubated for 4 days prior to the additions of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/mL PBS was sterile and filtered with 0.45 μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μL) was added to each culture well resulting in 50 μg MTT/250 μL total medium volume and cultures were incubated at 37 °C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically: culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10−20 μL of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μL of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a mocroculture plate reader at 540 nm (single wavelength, calibration factor = 1.00)

Cell line growth and growth inhibition were expressed in terms of mean (± 1 SD) absorbance units and/or percentage of control absorbance (± 1 SD%) following subtraction of mean "background" absorbance.

Sample were also tested for cytotoxic activity towards 6 cell lines, which contain SW620 (colon), BT474 (breast), KATO-III (gastric), Hep-G2 (hepatoma), Chago (lung) and CH-Liver (liver) following the experimental method of bioassay of cytotoxic activity.