

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 General Experimental Procedure, Solvents and Fine Chemicals

Melting points were determined using Gallenkamp melting point apparatus (Manchester, UK) and are uncorrected. Optical rotation was measured on a Jasco P-1020 Polarimeter (Jasco Corporation, Tokyo, Japan). UV spectra were analysed using a Shimadzu UV-2401 A spectrophotometer (Shimadzu corporation, Kyoto, Japan). IR data were recorded on a Bruker Tensor 27 FTIR spectrophotometer (Bruker Corporation, Bremen, Germany) as KBr pellets. NMR data were measured in CDCl₃, CD₃OD and DMSO-d₆ on a JOEL NMR instrument operating 600 and 150 MHz, respectively. Some NMR data were done using Bruker AM 300 spectrometer operating at 400, 300 and 125, 75 MHz, respectively. Chemical shifts are expressed in ppm with tetramethylsilane (TMS) used as internal standard. The mass spectral data were obtained using a Varian MAT 8200 A instrument. Column chromatography was performed using silica gel 60 (0.063 - 0.200 mm, Merck-Germany) while thin layer chromatography (TLC) was performed using silica gel 60 Å F₂₅₄ (Merck) pre-coated plates. Paper chromatography was done on standard Whatman No. 1 chromatography paper. Shift reagents were prepared according to Mabry *et al* (1970). All solvents used were of analytical grade.

3.2 Plant materials, collection and identification

Lonchocarpus eriocalyx (Harms) (Reference No.: LE/58/2013) was collected from Embu-Mbeere (Lat: 0.5833° S and Long: 37.6333° E) while *Alysicarpus ovalifolius* (Schumach) (Reference No.: FAB/AO/2012) was obtained from Shimba Hills (Lat: 4° 19' 39" S and Long: 39° 21' 39" E) where the plants grow naturally. The leaves of *Erythrina abyssinica* (DC) (Reference No.: MU/EA/76/2013) were collected from Rawalo Hills (Lat: 5° and Long: 34° 30'

E) which is in Central Gem, Siaya County. The plant materials were authenticated at the herbarium of the National Museums of Kenya where voucher specimens are preserved.

3.3 Extraction of plant materials

3.3.1 Extraction of the stem bark of *Lonchocarpus eriocalyx*

The air dried and pulverized stem bark (2 kg) was soaked sequentially in *n*-hexane (3 x 3L), CH₂Cl₂ (3 x 3L) and MeOH (3 x 3L), each lasting four days at room temperature. The extracts were separately filtered and evaporated under reduced pressure to afford yellow (5 g), yellowish-brown (25 g) and reddish-brown (106 g) extracts, respectively.

3.3.2 Thin layer chromatography (TLC) analysis of *n*-hexane and CH₂Cl₂ extracts

TLC analysis of *n*-hexane extract revealed two components with R_f values 0.82 and 0.63 [*n*-hexane-CH₂Cl₂ (2:3)] which turned greenish-purple with anisaldehyde spraying reagent. On the other hand, TLC analysis of CH₂Cl₂ extract using *n*-hexane-CH₂Cl₂ (2:3) afforded five spots of R_f values 0.82, 0.63, 0.48, 0.32 and 0.18 which turned bluish-purple using anisaldehyde spraying reagent.

3.3.3 Fractionation of *n*-hexane extract

A portion of the *n*-hexane extract (4 g) was mixed with 4 g of silica gel in 20 mL of CH₂Cl₂. The solvent was removed using a rotary evaporator and the free flowing solid chromatographed over silica gel packed column (2.0 x 60 cm, 120 g) using *n*-hexane with increasing amount of CH₂Cl₂ up to 100% of the latter. This afforded 60 fractions, each 20 mL which were collected and their homogeneity monitored by TLC (solvent systems: *n*-hexane EtOAc, 9:1 and 4:1). The eluants were grouped into three major pools (**I-III**) depending on TLC profiles. Fractions 1-15 constituted pool **I**, which upon evaporation of the solvent afforded a yellow oily material that lost color with time and was not followed further. Pool **II** (fractions 20-40, 1.5 g) showed a single

spot of R_f 0.82 (solvent system: *n*-hexane-EtOAc, 4:1) which on further recrystallization gave **133** (55 mg) as white amorphous powder. Pool **III** (fractions 41-57) showed a single spot of R_f 0.63 (eluant, *n*-hexane-CH₂Cl₂, 2:3) which crystallized in *n*-hexane CH₂Cl₂ mixture to give compound **134** as white needle-like crystals (100 mg).

3.3.4 Physical and spectroscopic data of compounds from *n*-hexane extract

3.3.4.1 Compound 133

White needle-like crystals, R_f , 0.82 [*n*-hexane-CH₂Cl₂, (2:3)], m.p. 254-256°C (Lit. 260-262°C; Majidul *et al.*, 2015); ¹H and ¹³C NMR data: (See **Table 10, page 73, Appendix 4**); **EI-MS**: m/z (rel int.): 426 [M⁺] (25), 408 (10), 343 (18), 331 (20), 316 (9), 154 (10), 127 (17), 97 (30), 73 (100).

3.3.4.2 Compound 134

White needle-like crystals R_f , 0.63 [*n*-hexane-CH₂Cl₂, (2:3)], m.p. 132-134°C (Lit. 136-138°C; Orabi, 2011); IR ν_{max} , (KBr) cm⁻¹: 3470.3 (OH), 2933. 2859.3 1693.5 (C=C), 1457.0, 1270.4, 995.7, 926.4. ¹H and ¹³C NMR data: (See **Table 11, page 76, Appendix 5**); **EI-MS**: m/z (rel int.): 414 [M]⁺ (100), 396 (70), 381 (40), 329 (50), 303 (50).

3.3.5 Fractionation of CH₂Cl₂ extract

A portion of the CH₂Cl₂ extract (20 g) was adsorbed onto 20 g silica gel and then subjected to column chromatography (3.0 x 60 cm, SiO₂, 200 g, and pressure≈1 bar) using *n*-hexane, *n*-hexane-CH₂Cl₂ gradient (increment 10%) up to 100% CH₂Cl₂) and elution concluded with 100% ethyl acetate. A total of 200 fractions, each 20 ml were collected. This process afforded sub-fractions (**I-IV**) as determined by TLC profiles [solvent systems: *n*-hexane-CH₂Cl₂ (4:1, 1:3, 2:3) and CH₂Cl₂-MeOH (99:1)]. Sub-fraction **I** (fractions 1-30) mainly eluted with *n*-hexane did not show clear spots on TLC and was not followed further. Sub-fraction **II** (fractions 35-90) afforded

yellow oil which became colourless and was not followed further. Sub-fraction **III** (fractions 92-141) showed two spots of R_f values of 0.82 and 0.63 (eluant: *n*-hexane- CH_2Cl_2 , 2:3) which on evaporation of the solvent followed by recrystallization in *n*-hexane- CH_2Cl_2 mixture afforded a further amount of **133** (R_f , 0.82, 45 mg). The mother liquor of this sub-fraction was further purified using medium pressure liquid chromatography eluting with *n*-hexane- CH_2Cl_2 (4:1) as the mobile phase to give a further amounts of **133** (15 mg) and compound **135** (R_f , 0.63, 40 mg). Lastly, sub-fraction **IV** (fractions 143-200), 6 g upon repeated chromatographic separation afforded a further amounts of **134** (R_f , 0.63, 15 mg), **135** as white powder (R_f , 0.48, 40 mg) and **136** as amorphous white powder (R_f , 0.32, 70 mg) and **27** (R_f , 0.18, 300 mg) [TLC solvent system; *n*-hexane- CH_2Cl_2 (2:3)].

3.3.6 Physical and Spectroscopic data of compounds from CH_2Cl_2 extract

3.3.6.1 Compound 27

White needle-like crystals, R_f , 0.18 [*n*-hexane- CH_2Cl_2 , (2:3)], m.p. 214-216°C (Lit. 215-216°C; Saratha *et al.*, 2011); IR ν_{max} (KBr) cm^{-1} : 3315, 2900, 1650, 1462, 1190, 1037, 997, 681; ^1H and ^{13}C NMR data: (See **Table 7, page 66, Appendix 1**); EIMS: m/z (rel int.): 426 $[\text{M}]^+$ (12), 238 (60), 180 (22).

3.3.6.2 Compound 135

White powder, R_f , 0.48 [*n*-hexane- CH_2Cl_2 , (2:3)], m.p. 254-256°C (Lit. 260-262°C, Aher *et al.*, 2010) ^1H and ^{13}C NMR data δ ppm: (See **Table 12, page 79, Appendix 6**); EI-MS: m/z (rel int.): 424 $[\text{M}]^+$ (30), 318 (27), 206 (75), 189 (35), 109 (60).

3.3.6.3 Compound 136

Amorphous white powder R_f , 0.32 [*n*-hexane- CH_2Cl_2 , (2:3)], m.p. 270-272 °C (Lit. 272-274 °C; Mahbuba *et al.*, 2012); ^1H and ^{13}C NMR data: (See **Table 13**, **page 82**, **Appendix 7**); EI-MS: m/z (rel int): 576 $[\text{M}+2]^+$ (7), 574 $[\text{M}]^+$ (6), 559 (1.5), 531.

3.3.7 Fractionation of MeOH extract

A portion of the powdered extract (100 g) was mixed with 20 g of silica gel and put in a desiccator with a drying agent to remove any traces of water. The sample was then subjected to column chromatography on oxalic acid deactivated silica gel (5.0 x 60 cm, 500g, pressure \approx 1 bar), starting with CH_2Cl_2 followed by $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient with increasing concentration of the polar solvent (increment 3%) and elution concluded until 100% MeOH was used. A total of 300 fractions each 20 mL were collected and their homogeneity determined by TLC (eluent: $\text{CH}_2\text{Cl}_2/\text{MeOH}$, 99:1, 98:2, 97:3, 95:5, 4:1 and 4:1; *n*-BuOH/HOAc/ H_2O , 4:5:1) and those exhibiting similar profiles were combined into five major pools (**1-VI**). Pool **I** (fractions 1-30, 4 g) eluted using CH_2Cl_2 afforded a mixture of compounds with R_f values of 0.63, 0.18 and 0.32 and were resolved into individual components using medium pressure chromatography as already described in subsections **3.3.5** to give a further amount of **27** (15 mg), **134** (20 mg) and **135** (30 mg). Fractions 32-80 (6 g) constituted pool **II** and was similarly chromatographed as described above using CH_2Cl_2 -MeOH (99:1) followed by the same solvent system in the ratio 98:2 to give **137** (R_f 0.42, 30 mg) and **68** (R_f 0.39, 31 mg, (CH_2Cl_2 -MeOH, 98:2). Pool **III** (fractions 83-108) showed one major spot of R_f value of 0.39 (solvent system CH_2Cl_2 -MeOH, 98:2) and was purified by crystallization from MeOH- H_2O mixture and yielded more amount of **68** (R_f 0.39, 33 mg). Pool **IV** (fractions (110-153, 11 g) showed two spots of R_f values 0.5 and 0.36 (CH_2Cl_2 -MeOH (97:3) and were separated using the same solvent system into compounds

65 (R_f 0.5, 90 mg) and **138** (R_f 0.36, 25 mg). Fractions 157-230 (10 g) constituted pool **V** which upon repeated medium pressure chromatographic separation using CH_2Cl_2 -MeOH (97:3) followed by the same solvent system in the ratio 96:4 and 96:4 gave a further amount of **65** (R_f 0.5, 15 mg), **139** (R_f 0.21, 25 mg) and **140** (R_f 0.17, 45 mg). Pool **VI** (5.5 g) similarly on repeated flash chromatography using CH_2Cl_2 -MeOH (96:4 and 95:5) afforded two compounds which were crystallized from MeOH- H_2O mixture to give a further amount of **139** (R_f 0.21, 68 mg) and **141** (R_f 0.30, 45 mg).

3.3.8 Physical and spectroscopic data for compounds isolated from MeOH extract

of *Lonchocarpus eriocalyx*

3.3.8.1 Compound 65

Pale yellow amorphous powder R_f 0.5 [CH_2Cl_2 -MeOH, (98:2)], m.p. 314-316°C (Lit. 316-318°C; Esra, *et al.*, 2015); UV λ_{max} , (MeOH): 358, 298, 258 nm; IR ν_{max} (KBr): 3500-2500 (OH), 1610 (conjugated C=O), 1450, 1340, 1250, 930 cm^{-1} ; ^1H and ^{13}C NMR data: (See **Table 8, page 68, Appendix 2**); ESI-MS: m/z (rel int.): 302 $[\text{M}]^+$ (100), 274 (08), 153 (11), 137 (20), 69 (10).

3.3.8.2 Compound 68

Yellow amorphous powder, R_f 0.39 [CH_2Cl_2 -MeOH, (98:2)], m.p. 346-348°C (Lit. 345-350°C; Chaturvedula and Prakash, 2013); UV λ_{max} , (MeOH): 268 and 337 nm; ^1H and ^{13}C NMR data: (See **Table 9, page 71, Appendix 3**); ESI-MS: m/z (rel int.) 270 $[\text{M}+1]^+$ (100), 178 (10), 153 (15), 121 (5).

3.3.8.3 Compound 137

Yellow powder R_f 0.42 [CH_2Cl_2 -MeOH, (98:2)], m.p. 291-292°C (Lit. 285-286°C; Miyaichi *et al.*, 2006); ^1H and ^{13}C NMR data: (See **Table 14 and 15, page 85, Appendix 8**); ESI-MS: m/z (rel int.): 255, $[\text{M}+1]^+$ (62), 210 (47), 186 (32), 145(75), 110 (52), 104 (100).

3.3.8.4 Compound 138

Light yellow powder, R_f 0.36 [CH_2Cl_2 -MeOH, (98:2)], m.p. 301-303°C (Lit. 300-301°C; Liu *et al.*, 2012; Younghee, 2012); UV λ_{max} (MeOH): 254, 324 and 370 nm; IR ν_{max} (KBr) 1 : 3400 (OH), 1650 (α,β -unsaturated C=O), 1600, 1450 cm^{-1} ; ^1H and ^{13}C NMR data: (See **Table 14** and **15**, **page 85**, **Appendix 9**); **ESI-MS**: m/z (rel int.) 332 (20), 289 (100), 233(70), 177 (36), 109 (17).

3.3.8.5 Compound 139

Yellow powder, R_f 0.21 [CH_2Cl_2 -MeOH, (96:4)], m.p. 227-230°C (Lit. 225-227°C; Ahmad *et al.*, 2012; Ana *et al.*, 2009); ^1H and ^{13}C NMR (400 and 100 MHz, DMSO- d_6) data: (See **Table 16**, **page 88**, **Appendix 10**); **ESI-MS**: m/z (rel int.): 463 $[\text{M}-1]^+$ (20), 423 (7), 306 (4), 342 (7), 301 (100).

3.3.8.6 Compound 140

Red amorphous powder, R_f [CH_2Cl_2 -MeOH (95:5)], 0.17, m.p. 220-222°C (Lit. 223-226°C; Liu *et al.*, 2015); ^1H and ^{13}C NMR data: (See **Table 17**, **page 90**, **Appendix 11**); **ESI-MS**: m/z (rel int.): 390 $[\text{M}]^+$ (100).

3.3.8.7 Compound 141

Greenish-yellow crystalline solid R_f 0.3 [CH_2Cl_2 -MeOH (95:5)], m.p. 234-236 °C (Lit. 238-240 °C; Okoth, 2013; Okoth *et al.*, 2013); ^1H NMR and ^{13}C NMR data data: (See **Table 18**, **page 93**, **Appendix 12**); **ESI-MS**: m/z (rel int.): 633 $[\text{M} + \text{Na}]^+$ (70), 611 (30), 465 (25), 273 (10), 215 (20).

3.3.9 Extraction, isolation and identification of the pure isolates from the root bark of *Alysicarpus ovalifolius*

3.4.0 TLC analysis of *n*-hexane of extract

Only the root bark of *A. ovalifolius* showed positive results upon preliminary bioactivity analysis hence the aerial part was not investigated. The air dried and pulverized root bark (1 kg) of the plant was soaked sequentially in *n*-hexane (3 x 3 L), CH₂Cl₂ (3 x 3L) and MeOH (3 x 3 L), each lasting four days at ambient temperature. The extracts were separately filtered and evaporated under reduced pressure to obtain yellow (7 g), brown (25 g) and reddish-brown (176 g) extracts respectively.

TLC analysis of the extract using *n*-hexane-CH₂Cl₂ (2:3) showed two spots of R_f values 0.63 and 0.67. The spot with R_f value of 0.63 appeared reddish-purple upon spraying with anisaldehyde reagent and heating while the second spot gave purple colour on exposure to concentrated ammonia suggesting the presence of a sterol/and/or a terpenoid and a quinone derivatives, respectively.

3.4.1 Fractionation of the *n*-hexane extract

The *n*-hexane extract (5 g) was adsorbed onto same amount of silica in 30 mL of CH₂Cl₂, solvent removed under reduced pressure and chromatographed over silica gel packed column (2.0 x 60 cm, 120 g) using *n*-hexane containing increasing amounts of CH₂Cl₂ (up to 100%). A total of 120 fractions, each 20 mL were collected and their homogeneity monitored by TLC (solvent systems: *n*-hexane-EtOAc, 9:1, 4:1 and 2:3). The eluants were grouped into three pools (**I-III**) depending on TLC profiles. Fractions 1-25 constituted pool **I**, which upon evaporation of solvent afforded a yellow oily compound that lost colour with time and was not followed further. Pool **II** (fractions 30-55, 1.5 g) showed a single yellow spot of R_f 0.63 (solvent system: *n*-hexane-

EtOAc, 4:1) which on recrystallization gave **134** (90 mg). Pool **III** (fractions 57-85, 1.0 g) crystallized [*n*-hexane-EtOAc, 4:1] to give an orange solution which on further re-crystallization afforded **142** (R_f , 0.34, 75 mg).

3.4.2 Physical and spectroscopic data of compounds from *n*-hexane extract

3.4.2.1 Compound 142

An orange amorphous powder, R_f 0.34 [*n*-hexane-EtOAc (4:1)], , m.p. 74-76°C (Lit. 76-78°C; Sing *et al.*, 2012; Tangmouo *et al.*, 2005); ^1H NMR and ^{13}C NMR data: (See **Table 19** on **page 95, Appendix 13**); ESI-MS: m/z (rel int.) 188 $[\text{M}]^+$ (100).

3.4.3 Fractionation of the CH_2Cl_2 extract

The CH_2Cl_2 extract (22 g) was adsorbed onto same amount of silica gel and then subjected to column chromatography (3.0 x 60 cm, 240 g, pressure \approx 1 bar) using *n*-hexane- CH_2Cl_2 gradient (increment 10%) up to 100% CH_2Cl_2 and elution concluded with ethyl acetate, collecting 20 mL each. The process afforded various sub-fractions (**I-VI**) as determined by TLC [solvent systems: *n*-hexane- CH_2Cl_2 (1:2, 2:3) and CH_2Cl_2 -MeOH, 99:1)]. The sub-fraction **I** (fractions 1-8) did not show any detectable spot and was not followed further. Sub-fraction **II** (fractions 9-15) showed a single spot R_f 0.60 (eluent: *n*-hexane- CH_2Cl_2 , 1:1), which upon crystallization in *n*-hexane- CH_2Cl_2 mixture gave **143** (23 mg). Sub-fraction **III** (fractions (20-60) also showed a single spot R_f 0.43 (eluent: *n*-hexane - CH_2Cl_2 , 1:1), that was Dragendorff's reagent positive was subjected to repeated column chromatographic separation followed by evaporation of the solvent and afforded compound **144** (R_f 45 mg). Sub-fraction **IV** (fractions 63-86, 3.7 g) showed two spots of R_f 0.30 and 0.38 (eluent: *n*-hexane- CH_2Cl_2 , 1:1) and upon repeated chromatography gave a further **144** (15 mg) and **145** (20 mg). Sub-fraction **V** (fractions 93-130, 3.4 g) showed one major spot of R_f 0.40 and upon repeated chromatography gave **146** (165 mg). Fractions 134-180

constituted sub-fraction **VI** (5 g) was further purified by medium pressure chromatography (2.5 x 50 cm, 150 g, pressure \approx 1 bar) to give a further amounts of compound **145** (50 mg) and **146** (70 mg) (eluent: *n*-hexane-CH₂Cl₂, 1:1).

3.4.4 Physical and Spectroscopic data of compounds from CH₂Cl₂ extract

3.4.4.1 Compound 143

Yellow powder R_f 0.60 [*n*-hexane-CH₂Cl₂, (1:1)], m.p. 255–257°C (Lit. 260-262°C; Wen *et al.*, 2007); ¹H NMR and ¹³C NMR data: (See **Table 20, page 98, Appendix 14**); ESI-MS: *m/z* (rel int.) 449 [M⁺+1] (5), 432 (21), 431 (100), 414 (34), 384 (30), 354 (10), 330 (20).

3.4.4.2 Compound 144

Colourless powder R_f 0.43 60 [*n*-hexane-CH₂Cl₂, (1:1)], m.p. 92-94°C (Lit. 88-90°C; Abu Bakar *et al.*, 2007; Mohammad *et al.*, 2013); ¹H and ¹³C NMR data: (See **Table 21 and 22 on page 101, 102, Appendix 15**); ESI-MS: *m/z* (rel int.): 332 [M]⁺ (100), 331 (10), 276 (7), 250 (30), 248 (10), 210 (10).

3.4.4.3 Compound 145

Colourless crystals, R_f 0.38 60 [*n*-hexane-CH₂Cl₂, 1:1)], m.p. 196-197°C (Lit. 194-195°C; Nayak *et al.*, 2010); ¹H and ¹³C NMR data: (See **Table 21 and 22, page 101, 102, Appendix 15**); ESI-MS: *m/z* (rel int.): 294 [M]⁺ (100), 296 (10), 293 (24).

3.4.4.4 Compound 146

Pale yellow crystals R_f, 0.40, m.p. 225-226°C (Lit. 224-225°C; Mohammad *et al.*, 2013); ¹H and ¹³C NMR data: (See **Table 21 and 22, page 101, 102, Appendix 15**); ESI-MS: *m/z* (rel int.) 324 [M]⁺ (100), 323 (50), 308 (5), 282 (5).

3.4.5 Fractionation of MeOH extract

Medium pressure chromatographic separation of the MeOH extract (150 g) over 2% oxalic acid solution-deactivated silica gel column using a mixture of CH₂Cl₂-MeOH (5% -100% MeOH) gave a total of 150 fractions of 50 mL each. Fractions exhibiting similar TLC profiles were pooled together (Pools **I-III**). Pool **I** (fractions 7-20, 5 g) showed one major spot R_f 0.50 (eluant: CH₂Cl₂-MeOH, 97:3) and was further purified by crystallization gave **65** (180 mg). Fractions 25-45 (pool **II**, 7 g) was repeatedly fractionated over 2% oxalic acid-deactivated silica gel (SiO₂ 150 g; 3.5 x 50 cm; 2-3 % MeOH-CH₂Cl₂) affording sub-fractions which resulted in the isolation of further amounts of **65** (15 mg), **68** (35 mg), **139** (70 mg). Pool **III** (5 g) gave **136** (35 mg) under similar purification procedure.

3.4.6 Acid hydrolysis of compounds 136 and 139

Compounds **136** and **139** (each 10 mg) in a mixture of 8% HCl (2 mL) and MeOH (20 mL) were separately heated under reflux for 2 h. The reaction mixtures were dried under reduced pressure to, dissolved in H₂O (3 mL) and neutralized with NaOH. The neutralized products were then subjected to TLC (eluent: EtOAc–MeOH–H₂O–HOAc, 6:2:1:1) and paper chromatography (PC) (eluent: n-BuOH–HOAc–H₂O, 4:1:5). The sugar chromatograms were sprayed with aniline hydrogen phthalate followed by heating at 100 °C and the spots were identified after comparison with authentic samples. Similarly, the aglycones were confirmed upon exposure to conc. ammonia solution (Maciej, 2000).

3.5 Extraction of *Erythrina abyssinica* leaves

The air dried and pulverized leaves (≈ 2 Kg) was soaked sequentially in *n*-hexane (3 x 3L), CH₂Cl₂ (3 x 3L) and MeOH (3 x 3L), each lasting four days at room temperature with occasional

shaking using orbital shaker. The extracts were filtered and the filtrate evaporated to give 16 g, 27g and 86 g of dark green materials, respectively.

3.5.1 TLC analysis of *n*-hexane and CH₂Cl₂ extracts

Thin layer chromatographic analysis of *n*-hexane extract (eluent: *n*-hexane-CH₂Cl₂, 2:3) showed a spot of R_f 0.82 which turned bluish-purple with anisaldehyde spraying reagent after heating at 100 °C for 1 minute. On the other hand, TLC analysis of CH₂Cl₂ extracts using *n*-hexane-CH₂Cl₂ (2:3) followed using same solvent in the ratio 1:3 showed spots of R_f 0.82, 0.69 and 0.52 respectively. The spot with R_f 0.82 gave bluish-purple colour on spraying with anisaldehyde reagent followed by heating at 100 °C for a minute. The spot with R_f 0.52 gave an orange coloration on spraying with the same reagent.

3.5.2 Chromatographic separation of the *n*-hexane extract

Approximately 10 g of the *n*-hexane extract was dissolved in CH₂Cl₂, adsorbed on an equivalent amount of silica gel in CH₂Cl₂ and then dried under *vacuo*. The free flowing material was then subjected to column chromatography (3 cm diameter by 60 cm long) glass column packed with silica gel (≈ 150 g) using *n*-hexane, *n*-hexane-CH₂Cl₂ mixtures and finally with CH₂Cl₂ as the elution solvent. A total of 130 fractions, each 20 mL were collected and their composition monitored using analytical TLC with solvent system *n*-hexane- CH₂Cl₂ (4:1, 2:3). Fractions with similar profiles were combined into three pools (**I-III**). Pool **I** (fractions 1-30) did not show any spot on TLC and was not followed further. Fractions 33-40 were combined to give pool **II** which gave an orange oily substance which faded in colour after some time and was not followed further. Pool **III** (fractions 50-120, 1.5 g) showed a single spot of R_f 0.82 (eluent- *n*-hexane-CH₂Cl₂, 2:3) which upon crystallization in CH₂Cl₂-MeOH mixture gave compound **133** (70 mg).

3.5.3 Chromatographic separation of the CH₂Cl₂ of extract

A portion of the CH₂Cl₂ (25 g) was adsorbed onto silica gel and then subjected to column chromatography (3.0 x 60 cm, silica gel 200 g, and pressure ≈ 1 bar) using *n*-hexane-CH₂Cl₂ gradient (increment 10%) up to 100% CH₂Cl₂ and finally eluted with 100% ethyl acetate. This process afforded sub-fractions (I-IV) as determined by the TLC profiles [solvent system (*n*-hexane-CH₂Cl₂, 4:1, 2:3) and CH₂Cl₂-MeOH (99:1)]. Sub-fraction I (Fractions 1-30) did not show any spot and was not followed further. Sub-fraction II (Fractions 35-90, 1.5 g) showed a single spot of R_f value 0.63 (solvent: *n*-hexane-CH₂Cl₂ (solvent: *n*-hexane-CH₂Cl₂, 2:3) and upon evaporation of solvent followed by crystallization in *n*-hexane-CH₂Cl₂ mixture afforded **134** (50 mg). Sub-fraction III (Fractions 92-104, 2.5 g) showed a single spot of R_f 0.52 (eluent: *n*-hexane-CH₂Cl₂, 2:3) which upon crystallization in *n*-hexane-CH₂Cl₂, gave compound **147** (55 mg). Sub-fraction IV (Fractions 140-186, 5 g) showed two spots of R_f values 0.52 and 0.30 (eluent: *n*-hexane-CH₂Cl₂, 2:3) which on repeated chromatography afforded a further **147** in 15 mg and compound **148** (45 mg).

3.5.4 Fractionation of the MeOH extract

A portion of the extract (40 g) was adsorbed on silica gel and then subjected to column chromatography with CH₂Cl₂ containing increasing amounts of MeOH (gradient elution with increasing concentration of the polar solvent (increment 3%) and elution concluded with 100% MeOH. A total of 50 fractions were sampled and their composition analysed by TLC (eluent: CH₂Cl₂/MeOH, 98:2, 97:3, 19:1, 9:1 and 4:1) and those exhibiting similar TLC profiles were combined. This process afforded two sub-fractions (I and II) as determined by the TLC profiles [solvent system (*n*-hexane-CH₂Cl₂, 3:1, 1:2) and CH₂Cl₂-MeOH (97:3)]. Sub-fraction I (Fractions 10-26, 3 g) contained one major spot of R_f value 0.30 (eluent: *n*-hexane-CH₂Cl₂, 2:3)

and was similarly fractionated as in **3.5.3** above to give compound **147** (15 mg). Pool **II** (Fractions 32-105, 6 g) was similarly resolved into individual components using CH₂Cl₂-MeOH (98:2) followed by the same solvent in the ratio 98:3 to give **148** (21 mg).

3.5.5 Physical and spectroscopic data of compounds isolated from *Erythrina abyssinica*

3.5.5.1 Compound 147

Grenish-yellow powder, R_f 0.30, [*n*-hexane-CH₂Cl₂, (2:3)], m.p. 194-196°C (Lit. 192-193°C; Yenesew *et al.*, 2009); ¹H and ¹³C NMR data: (See **Table 23, page 105, Appendix 16**); LCMS : *m/z* (rel int.) 336 [M]⁺ (80), 321 (21), 279 (25), 149 (100), 137(79), 115 (25), 108 (20), 69 (15), 55 (14), 41 (25).

3.5.5.2 Compound 148

White powder, R_f 0.45 [CH₂Cl₂-MeOH (98:2)], m.p. 112-114°C (Lit. 114-116°C; Nkengfack, *et al.*, 1997; Yenesew *et al.*, 1997); ¹H and ¹³C NMR data: (See **Table 24, page 107, Appendix 17**); LCMS : *m/z* (ret int.): 588 [M]⁺ (100), 573 (30), 560 (40), 559 (98), 554 (15), 561 (10).

3.6 Biological activity studies

3.6.1 *In vitro* antiplasmodial assay

An *in vitro* antiplasmodial activity was carried out using the *P. falciparum* multi-drug resistant Indochicha I (W2) and chloroquine-sensitive Sierra Leone I (D6) strains according to procedures of Desjardins *et al.*, (1979) and Chulay *et al.*, (1983) to determine the IC₅₀. The parasites were grown in a continuous culture supplemented with mixed gas (90% nitrogen, 5% oxygen), 10% human serum and 6% haematocrit a, plus red blood cell (Trager and Jensen, 1976). When the cultures had reached a parasitemia of 3% with at least a 70% ring developmental stage present, parasites were transferred to a 96 well microtitre plate precoated with the extracts/isolates dissolved in DMSO. The samples were diluted across the plate to provide a range of concentrations to determine IC₅₀ values. The plate was then incubated in a mixed gas incubator for 24 h. Thereafter 3H-hypoxanthine was added and the parasite allowed to grow for 18 h. Cells were then processed with a plate harvester (TomTec) on a filter paper and washed to eliminate unincorporated radioisotope. Mefloquine (1 µg/mL) was used as a standard drug. The experiments were done in triplicate-(MEMRI-Kenya).

3.6.2 Larvicidal and mosquitocidal tests

1 mg of crude extracts and isolates were weighed and dissolved in 1 mL acetone (analytical grade, Lobarchemi) to give a stock solution of 1000 µg/mL. The larvae were exposed to a wide range of test concentrations and a control to find out the activity range of the crude samples and isolates. After determining the mortality of larvae in this wide range of concentrations, a narrow range of five concentrations (10, 100, 250, 500 µg/mL) yielding up to 100% mortality in 24 h was used to determine LC₅₀ values. Temperatures were maintained at 26±3°C while humidity

was kept at 70-75%. Batches of 20 3rd instar *Anopheles gambiae* larvae were transferred by means of droppers into a small disposable test cup each containing 50 mL of the extract and isolates at concentrations of 10, 100, 250, 500 µg/mL dissolved in acetone and fed on larval food (Globade *et al.*, 2002). Temephos (3 µg/mL) was used as positive control, while 1% acetone was used as negative control. After 24 hours, larval mortality was recorded whereby moribund larvae were counted and added to the dead ones for calculating % mortality. Dead larvae were considered as those which could not be induced to move when they were probed with a needle in the siphon region. For mosquitocidal assays, sample solutions of 10, 100, 250, 500 µg/mL dissolved in acetone were impregnated on Whatman filter paper No. 1 measuring 140 x 100 mm. The bioassay was conducted in an experimental kit consisting of two cylindrical plastic tubes, both measuring 125 x 44 mm (Globade, *et al.*, 2002). One tube served to expose the mosquitoes to the extracts and isolates while another tube was used to hold the mosquitoes before and after the exposure periods. The impregnated papers were rolled and placed in the exposure tube. Each tube was closed at one end with a 16 mesh size wire screen. Sucrose-fed and blood starved female mosquitoes (20) were released into the tube, and the mortality effects of the extracts and isolates were observed every 10 minutes for 3 hours exposure period. At the end of 1, 2, and 3 hrs exposure periods, the mosquitoes were placed in the holding tube. Cotton pads soaked in 10% sugar solution with vitamin-B complex were placed in the tube during the holding period of 24 hours and mortality rate was recorded (Globade, *et al.*, 2002). Any mosquito was considered to be dead if did not move when prodded repeatedly with a soft brush. The above procedure was carried out in triplicate for each concentration. The mortality of the mosquitoes was monitored and toxicity levels of the test samples evaluated graphically to give LC₅₀ values.

Lambdacyhalothrin (1 µg/mL) was used as a positive control while 1% acetone was employed as a negative control. The tests were done in triplicate.

3.6.3 Antifungal and antibacterial tests

The disc diffusion assay method was applied (Singh *et al.*, 2002) using clinical isolates of *Candida albicans* (HG 392), *Aspergillus fumigatus* (HG 420) and *Aspergillus niger* (ATCC 90028) as the representative fungi. *Staphylococcus aureus* (ATCC 25922), *Streptococcus faecalis* (ATCC 25925) and *Bacillus anthracis* (QST 713) were used as the representative Gram positive bacteria while *Klebsiella pneumoniae* (ATCC 90028), *Salmonella typhimurium* (ATCC 25927), *Pseudomonas aeruginosa* (ATCC 25923) and *E. coli* (K 12) were representatives of Gram negative bacteria. Crude samples were tested *in vitro* at sample concentration of 1000 µg/mL dissolved in dimethylsulfoxide (DMSO), while pure isolates were tested at 100 µg/mL. Mueller Hinton agar was aseptically aliquoted at volumes of 25 mL into petri dishes and left to congeal. 250 µL of fungi/bacteria cell suspension (at a concentration of 10^8 CFU/mL) was spread onto the surface of agar medium in petri dish (Singh *et al.*, 2002). Sterile paper discs (Schleicher and Schuell type 602 H, Germany, 5 mm diameter) previously impregnated with 10 µL of test samples were placed approximately equidistant into the seeded agar using a sterile forceps. This was done in triplicate. Disc containing 10µL of the 20 µg/mL solution of the standard drug (fluconazole and amoxyllin, respectively) was used as the positive control. The agar plates were incubated at 37°C for 24 h after which the inhibition zones were measured in millimeters (McChesney *et al.*, 1991).

3.7 Data analysis

3.7.1 Antiplasmodial activity

The filters were measured for activity in a microtitre plate scintillation counter (Wallace). Data from the counter were imported into a Microsoft Excel Spreadsheet, which was then imported into an Oracle Database Program to determine IC₅₀ values (Trager and Jensen 1976).

3.7.2 Larvicidal and mosquitocidal Activity

The activity of the extracts and the isolates were evaluated according % mortality scale; (>75%: highly active; 50-74%: moderate; 25-49%: weak; <25%: inactive (Globade, *et al.*, 2002). The results are also reported as lethal concentration; LC₅₀ for extracts and each compound and the minimum concentration that gives 50% larvicidal activity was determined in µg/mL. Average 24 hour knock down (or mortality) data were compared with both negative and positive control (Globade *et al.*, 2002).

3.7.3 Antifungal and antibacterial activity

The results are reported as zones of inhibition measured in mm. The size of the zone of inhibited growth (in mm) indicated the degree of antimicrobial susceptibility according to the scale Activity scale: (> 17: Highly active; 11-16: intermediate; 7-10: weak; <6: resistant) (McChesney *et al.* 1991).