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Original Research Article

Isolation, characterization and biological activities of phytoconstituents from *Lonchocarpus eriocalyx* Harms leaves

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ABSTRACT

The plant species *Lonchocarpus eriocalyx* Harms (Fabaceae) is a deciduous slender tree or shrub which grows up to 15 m tall. It is an important medicinal plant in Kenya used to treat a wide range of ailments including eye infection, wound and ulcer. However, little is known about its chemical constituents. The present study aims to isolate secondary metabolites from the plant leaves along with their various biological activities. Eight compounds, namely friedelin (**1**), lupenone (**2**), lupeol (**3**), stigmasterol (**4**), chrysin (**5**), stigmasterol 3-*O*-β-glucoside (**6**), apigenin (**7**) and 3,5,7,2′,4′-pentahydroxyflavonol (**8**) were isolated from the CH_2Cl_2 and MeOH extracts of *Lonchocarpus eriocalyx* Harms leaves. Their structures were determined by using physical and spectroscopic methods as well as comparison with literature data. The CH_2Cl_2 and MeOH extracts together with the isolated compounds were subjected to an array of biological tests including anti-plasmodial, larvicidal and mosquitocidal, antimicrobial and anti-inflammatory assays. The compounds **1-4** and **6** together with their anti-inflammatory activities are reported from this plant for the first time.

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1. Introduction

The plant *Lonchocarpus eriocalyx* Harms (Fabaceae) is a small deciduous generally slender tree or shrub, 3-12 m tall, with rounded crown; bark pale greyish, smooth to reticulately fissured; slash pinkish-white, producing a red resinous exudate from the inner side (Bentjee, 1994; Jong et al., 2012). In Kenya, an infusion of the plant bark is used as an effective remedy for fever, headache, diarrhea and also as an insecticide agent (Ceres et al., 1981; Kokwaro, 2009; Adem et al., 2018). Previous phytochemical study on the plant yielded lupeol triterpene which attracted interest on account of its antiplasmodial activity against *Plasmodium ovale* (Tuwei, 2006). In the present report, phytochemical analysis of CH_2Cl_2 and MeOH extracts of the plant leaves has resulted in the isolation of eight compounds (**1-8**) (Fig. 1) which showed an array of biological activities.

2. Experimental

2.1. Equipment used, solvents and fine chemicals

Melting points were determined using Gallenkamp melting point apparatus (Manchester, UK). UV spectra were measured 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 $CDCl_3$ and DMSO-d_6 on a JOEL NMR instrument operating at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR, respectively. Chemical shifts were 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

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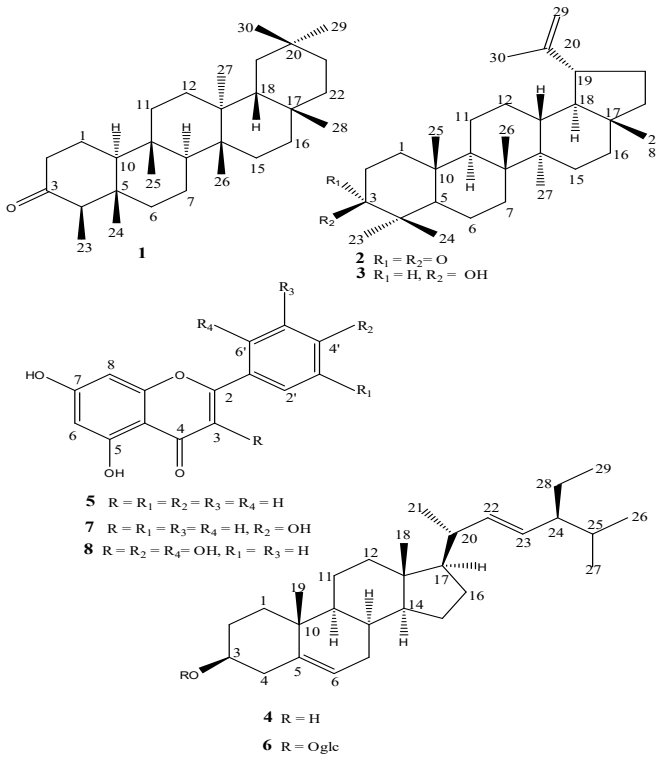

Fig. 1. Compounds **1-8** isolated from *L. eriocalyx* Harms leaves.

(TLC) was performed using silica gel 60 A° F_{254} (Merck, Germany) pre-coated plates. Paper chromatography was done on standard Whatman No 1 chromatography paper (Kobian Kenya LTD, Kenya).

2.2. Plant materials collection and identification

The plant *L. eriocalyx* Harms was collected from Embu-Mbeere (Lat: 0.5833° S and Long: 37.6333° E) where it naturally grows. The plant materials were authenticated at the herbarium section of the National Museums of Kenya by Mr. Mutiso where voucher specimens were (No. 2013/58) deposited.

2.3. Extraction and isolation of secondary metabolites

The air-dried and pulverized leaves (1.5 kg) of the plant were soaked sequentially using CH_2Cl_2 (3x3 L) and MeOH (3x3 L) solvents, each lasting four days at room temperature. The extracts were separately filtered and the solvents evaporated under reduced pressure to afford dark green (16 g, CH_2Cl_2 extract) and greenish-brown (36 g, MeOH extract) materials, respectively.

2.4. Fractionation of CH_2Cl_2 extract

A portion of the CH_2Cl_2 extract (12 g) was pre-adsorbed onto silica gel and then subjected to column chromatography (3.5x60 cm; SiO_2 240 g; pressure≈1 bar) using *n*-hexane-CH_2Cl_2 mixture (increment 10%) up to 100% CH_2Cl_2 and elution concluded with a liter of CH_2Cl_2-MeOH (99:1) mixture. A total of 330 fractions were collected (each 20 mL). The process afforded sub-fractions (I-VI) as was determined by TLC profiles [solvent systems: *n*-hexane-CH_2Cl_2 (1:3, 1:2) and a liter of CH_2Cl_2-MeOH (99:1 and 98:2)]. Sub-fraction I (fractions 1-30) showed no spot. Sub-fraction II (fractions 35-80, 3.5 g) produced a yellowish oil which lost color with time and was discarded. Sub-fraction III (fractions 85-170) showed a single spot R_f 0.63 (eluent: *n*-hexane-CH_2Cl_2, 1:3) which upon evaporation of solvent followed by crystallization in CH_2Cl_2-MeOH mixture afforded compound **1** as white crystals (75.1 mg). Sub-fraction IV (fractions 174-230, 5 g) showed two spots of R_f values 0.63 and 0.56 (eluent: *n*-hexane-CH_2Cl_2, 1:3) which upon repeated chromatographic separation (2.5x60 cm, 140 g, pressure≈1 bar) afforded a further **1** (45.3 mg) and **2** (90.0 mg). Sub-fraction V (fractions 243-270, 2.4 g) showed one major spot R_f value 0.46 (solvent: *n*-hexane-CH_2Cl_2, 1:2) and was further purified by crystallization (MeOH) to give compound **3** as white crystals (105.4 mg). Fractions 274-310 which constituted sub-fraction VI (5 g) showed two major spots R_f 0.46 and 0.34 (eluent: *n*-hexane-CH_2Cl_2, 1:2) and on repeated chromatographic separation (2.5x50 cm, SiO_2 150 g, pressure≈1 bar), followed by crystallization (*n*-hexane-CH_2Cl_2, 4:1) gave compounds **3** and **4** in the yields of 64.2 and 55.0 mg, respectively.

Friedelin (**1**). White crystals with m.p. 254-256 ºC; IR ν_{max} (KBr) cm^{-1}: 2930, 2862, 1711, 1626, 1460, 1380, 1176, 1112, 1071, 1004, 931. 786; ^{1}H-NMR and ^{13}C-NMR data ($CDCl_3$) δ: see Table 1 and Table 2; EI-MS (rel. int): *m/z* (%) 426 $[M]^+$ (30), 408 (9), 343 (11), 313 (32), 316 (17), 205 (6), 154 (35), 127 (21), 97 (37), 73 (100).

Table 1

^{1}H-NMR of compounds **1-8** (*J* in Hz).

H	1	2	3	4	5	6	7	8
1	1.76 m	1.69 m	1.68 m	1.73 m		1.99 m		
2	1.62 m	1.64 m	1.64 m	1.65 m		1.72 m		
3			3.20 (dd, *J*=11.4, 4.8)	3.52 m	6.73 s	3.63 m	6.60 s	
4	2.27(q, *J*=5.4)					2.35 m		
5	1.57 m	1.61 s	1.56 m					
6	1.50	1.49 m	1.50 m	5.35 (t, *J*=5.2)	6.23 (d, *J*=1.5)	5.28 (t, *J*=5.4)	6.26 (d, *J*=2.2)	6.17 (d, *J*=1.5)
7	1.31 m	1.32 m	1.35 m		6.48 (d, *J*=1.5)			
8	1.39 m						6.40 (d, *J*=1.8)	6.43 (d, *J*=1.5)
9		1.41 m	1.43 m		6.73 s			
10	1.40 m							
11	1.55 m	1.49 m	1.52 m					
12	1.54 m	1.47 m	1.46 m					
13		1.41 m	1.40 m					
14								
15	1.33 m	1.39 m	1.36 m					
16	1.30 m	1.45 m	1.43 m					
17								
18	1.41 m	2.62 m	2.49 m	1.03 s		1.07 s		
19	1.44 m	1.61 m	1.62 m	0.95s		0.94 s		
20		1.44 m	1.47 m	1.33 m		1.36 m		
21	1.36 m	1.43 m	1.40 m	1.12 (d, *J*=6.5)		1.30 (d, *J*=6.6)		
22	1.37 m			4.88 m		4.95 m		
23	0.94(d, *J*=6.5)	1.23 s	1.20 s	5.20 m		5.10 m		
24	1.02 s	1.08 s	1.03 s	1.26 m		5.1 m		
25	0.74 s	1.67 s	1.73 s	1.67 m		1.70 m		
26	0.91 s	0.92 s	0.94 s	0.84 (d, *J*=6.8)		0.90 (d, *J*=6.5)		
27	1.20 s	0.78 s	0.76 s	0.83 (d, *J*=6.8)		0.88 (d, *J*=6.7)		
28	1.07 s	1.1 s	0.96 s	1.20 m		1.19 m		
29	0.88 s	4.57(d, *J*=0.5) 4.68 (d, *J*=0.5)	4.55 (d, *J*=0.6) 4.65 (d, *J*=0.6)	0.86 (t, *J*=7.1)		1.03 (t, *J*=7.2)		
30	0.89 s	0.83 s	0.83 s					
1′						4.44 (d, *J*=7.7)		
2′					7.99 (d, *J*=6.5)	3.58 m	7.86 (d, *J*=9.0)	
3′					7.58 m	3.37 m	6.94 (d, *J*=9.0)	6.33 (d, *J*=4.0)
4′					7.58 m	3.46 m		
5′					7.58 m	2.89 m	6.94 (d, *J*=9.0)	6.50 (dd, *J*=4.0, 3.0)
6′					7.99 (d, *J*=6.5)	3.88 m 3.72 m	7.86 (d, *J*=9.0)	7.43 (d, *J*=7.0)

Lupenone (**2**). White crystals, m.p. 166-168 ºC; ^{1}H and ^{13}C-NMR data ($CDCl_3$) δ: See Table 1 and Table 2; EI-MS: *m/z* (%): 424 (9), 368 (11), 342 (5), 313 (26), 297 (4), 273 (5), 267 (7), 231 (24), 206 (100), 189 (35), 161 (25), 109 (80), 81 (70), 65 (50).

Lupeol (**3**). White crystals with m.p. 216-218 ºC; IR ν_{max} (KBr) cm^{-1}: 3315, 2920, 2860, 1650, 1462, 1190, 1037, 997, 681; ^{1}H-NMR and ^{13}C-NMR ($CDCl_3$) δ: see Table 1 and Table 2; EI-MS (rel. int): *m/z* 426 $[M]^+$ (8), 411 (11), 385 (8), 355 (20), 220 (31), 218 (45), 207 (33), 189 (100).

Stigmasterol (**4**). White crystals, m.p. 163-166 ºC; IR ν_{max} (KBr) cm^{-1}: 3470, 2968, 2939, 2859, 1633, 1457, 1387, 1072; ^{1}H-NMR and ^{13}C-NMR ($CDCl_3$) δ: see Table 1 and Table 2; EI-MS (rel. int): *m/z* 412 (16), 397 (10), 369 (7), 327 (8), 300 (11), 281 (28), 271 (65), 255 (23), 207 (50), 191 (13), 161 (15), 147 (25), 95 (30), 81 (60), 55 (100), 43 (89).

2.5. Fractionation of MeOH extract

A portion of the extract (30 g) was mixed with 20 g of silica gel, dried and then subjected to column chromatography (4.0x60 cm; SiO_2 300 g; pressure≈1 bar), starting with CH_2Cl_2 followed by CH_2Cl_2-MeOH mixture with increasing concentration of the more polar solvent (increment 10%) and elution concluded with MeOH (100%). A total of 150 fractions, each 50 mL were sampled and their homogeneity determined by TLC (eluent: CH_2Cl_2-MeOH, 99:1, 98:2, 97:3, 95:5, 4:1 and 1:1; *n*-BuOH-HOAc-H_2O, 4:1:5) and those exhibiting similar profiles were combined into five major pools (VII-X). Pool VII (fractions 15-30, 4 g) eluted using CH_2Cl_2 afforded a mixture of compounds with R_f values of 0.46 and 0.34, and were resolved into individual components using medium pressure chromatography (2.5x60 cm, SiO_2 120 g, Pressure≈1 bar) to give further **3** (15 mg) and **4** (20 mg). Fractions 32-90 (6 g) eluted using CH_2Cl_2-MeOH (99:1) followed by the same solvent in the ratio 98:2 constituted pool VIII and was further chromatographically purified using CH_2Cl_2-MeOH (99:1) to give **5** (R_f=0.42, 30.0 mg) and **6** (R_f=0.39, 41.0 mg). Pool IX (fractions 92-117) showed one major spot of R_f value of 0.37 (solvent system CH_2Cl_2-MeOH, 98:2) and was purified by crystallization in MeOH-H_2O (99:1) mixture to give **7** (63.1 mg). Pool X (fractions (119-150, 8 g) showed two spots of R_f values 0.37 and 0.28 and were

Table 2
^{13}C-NMR of compounds **1-8**.

C#	1	2	3	4	5	6	7	8
1	22.5	39.9	38.7	37.3		36.7		
2	41.7	27.5	27.2	31.7	163.3	32.0	164.0	162.8
3	213.4	216.7	74.7	71.8	106.1	78.2	103.9	133.4
4	58.5	42.3	38.7	39.8	183.9	40.0	180.7	177.7
5	42.4	56.6	54.0	140.8	165.7	142.5	164.4	162.5
6	41.5	17.7	18.6	121.7	105.6	122.3	104.8	99.3
7	36.3	34.5	34.3	31.9	159.6	33.40	160.0	165.6
8	53.4	42.11	41.7	31.7	95.2	31.8	95.1	94.7
9	37.7	50.7	49.2	50.1	165.6	50.6	160.7	149.9
10	59.8	36.8	37.5	36.5	106.1	36.7	109.5	105.4
11	33.0	21.3	21.3	21.1		21.5		
12	30.8	29.2	37.9	37.2		39.9		
13	41.8	37.6	38.0	42.3		42.6		
14	41.7	40.0	41.6	56.8		56.6		
15	30.3	26.2	28.6	24.3		23.2		
16	35.9	35.5	35.9	28.3		28.4		
17	30.8	42.2	42.9	56.1		56.4		
18	43.1	51.4	49.2	12.0		13.2		
19	35.7	42.2	48.0	19.4		19.3		
20	29.9	150.8	149.7	11.7		39.7		
21	33.1	29.8	30.0	19.3		22.0		
22	39.6	40.0	39.6	138.88		137.5		
23	7.1	32.4	27.3	130.1		124.3		
24	14.9	33.3	32.8	45.9		12.6		
25	20.5	19.3	15.9	23.1		30.0		
26	18.9	18.0	16.0	19.0		19.4		
27	18.5	14.8	15.0	19.6		20.3		
28	20.3	21.2	18.0	23.1		26.1		
29	32.4	109.5	108.1	11.9		12.6		
30	32.7	21.6	19.5					
1′					127.5	101.7	123.2	109.4
2′					126.4	74.0	129.2	157.6
3′					130.4	76.8	117.1	104.6
4′					132.0	71.0	162.5	159.0
5′					130.4	76.2	116.0	111.6
6′					126.4	63.1	129.5	132.7

separated using CH_2Cl_2-MeOH (98:2) into compounds **7** (15.0 mg) and **8** (90.0 mg).

Chrysin (**5**). Yellow powder, m.p. 285-287 ºC; ^{1}H and ^{13}C-NMR (DMSO-d_6) δ: (See Table 1 and Table 2); ESI-MS (%): *m/z* 255, $[M+H]^+$, (70), 210 (47), 186 (32), 153 (70), 143 (75), 110 (52), 104 (100).

Stigmasterol 3-*O*-β-glucoside (**6**). White powder, m.p. 289-290 ºC; IR ν_{max} (KBr) cm^{-1}: 3390, 2931, 2868, 1644, 1432, 1369, 1256, 1164, 1061, 1017, 931; ^{1}H-NMR and ^{13}C-NMR (DMSO-d_6) δ: see Table 1 and Table 2; EI-MS (%): *m/z* 576 $[M+2]^+$ (2), 574 $[M]^+$ (3), 412 (10), 397 (2), 393 (11), 383 (22), 271 (100), 162 (3).

Apigenin (**7**). Yellow powder, m.p. 346-348 ºC; UV λ_{max}, (MeOH): 268 and 337 nm; ^{1}H and ^{13}C-NMR (DMSO-d_6) δ: see Table 1 and Table 2; ESI-MS: *m/z* 271 $[M+H]^+$.

Compound **8**. Pale yellow powder, m.p. 299-300 ºC; 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}; ^{1}H and ^{13}C-NMR (DMSO-d_6) δ: See Table 1 and Table 2; ESI-MS (%): *m/z* 302 $[M]^+$ (100), 274 (8), 153 (11), 137 (20), 69 (10).

2.6. Acid hydrolysis of compound **6**

Compound **6** (10 mg) in a mixture of 2% HCl (2 mL) and MeOH (20 mL) was heated under reflux for 2 h. The reaction mixture was reduced under pressure to dryness, dissolved in H_2O (2 mL) and neutralized with NaOH. The neutralized product was then subjected to TLC analysis (eluent: EtOAc-MeOH-H_2O-HOAc, 6:2:1:1) and paper chromatography (PC) (eluent: *n*-BuOH-HOAc-H_2O, 4:1:5). The sugar chromatograms were sprayed with aniline hydrogen phthalate followed by

Table 3

In vitro antiplasmodial activity (IC_{50}) of crude extracts and isolated compounds against W2 and D6 strains of *P. falciparum*.

Test Samples	IC_{50} values (ng/mL)	
	W2 strain	D6 strain
CH_2Cl_2 extract	575.5 ± 0.5	478.8 ± 0.6
MeOH extract	423.0 ± 0.1	365.2 ± 0.3
Friedelin (**1**)	250.7 ± 0.6	231.4 ± 0.5
Lupenone (**2**)	208.1 ± 0.4	394.7 ± 0.6
Lupeol (**3**)	104.4 ± 0.4	109.9 ± 0.4
Stigmasterol (**4**)	187.6 ± 0.4	206.6 ± 0.3
Chrysin (**5**)	998.1 ± 0.5	606.1 ± 0.5
Stigmasterol-3-*O*-glucoside (**6**)	579.3 ± 0.3	559.9 ± 0.5
Apigenin (**7**)	972.1 ± 0.5	606.1 ± 0.5
Morin (**8**)	369.9 ± 0.4	481.9 ± 0.8
Mefloquine	16.1 ± 0.2	22.3 ± 0.1

Values are means ± SD of three replicates.

heating at 100 ºC and were identified after comparison with authentic samples.

2.7. *In vitro* antiplasmodial assay

An *in vitro* antiplasmodial activity was done using *Plasmodium falciparum*, the multi-drug resistant *Indochicha I* (W2) and chloroquine-sensitive *Sierra Leone I* (D6) strains to determine the concentration of inhibition where the response (or binding) is reduced by half (IC_{50}) (Desjardins et al., 1979; Chulay et al., 1983). The parasites were grown in a continuous culture supplemented with mixed gas (90% nitrogen, 5% oxygen), 10% human serum and 6% haematocrit A, and red blood cells (Trager and Jensen, 1976). When the cultures had reached a parasitemia of 3% with at least a 70% ring developmental stage present, the parasites were transferred into a 96 well microtitre plate precoated with the extracts (0.5 mg) or isolated (0.1 mg) compounds dissolved in DMSO. The samples were diluted across the plates to provide a range of concentrations to determine IC_{50} values. The plates were 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 (MQ) was used as standard drug. The filters were measured for activity in a microtitre plate scintillation counter (Wallace) and data from the counter were imported into a Microsoft excel spreadsheet, which was then imported into an Oracle Database Program to determine IC_{50} values. The experiments were done in triplicate and the obtained results tabulated in Table 3.

2.8. Larvicidal and mosquitocidal tests

For larvicidal test, batches of 20 third instar *Anopheles gambiae* larvae were transferred using a dropper into disposable test cups containing 100 mL of extracts and isolates of different concentrations (10, 100, 250, 500 and 1000 µg/mL). The experiments were done in triplicate (Gbolade et al., 2002). Temephos (3 µg L^{-1}) was used as positive control, while acetone was used as a negative control. Dead larvae were counted after 24 h. For mosquitocidal bioassay, the sample solutions dissolved in acetone were applied onto filter paper discs (5 cm diameter), then placed in perforated dishes (treated set). After drying the filter papers, ten unfed insects were introduced into each of the dishes through a hole and allowed to be in contact with the filter papers discs for 20 min, then transferred into cages containing sugar-water and observed for 24 h (Gbolade et al., 2002). The mortality of the insects was monitored and the toxicity levels of the test samples evaluated graphically to give LC_{50} values. Temephos

Table 4

Larvicidal and mosquitocidal activities of extracts and isolates as mortality% and LC_{50} values.

Test Samples	Larvicidal activity		Mosquitocidal activity	
	Mortality (%)	LC_{50} (µg/mL)	Mortality (%)	LC_{50} (µg/mL)
CH_2Cl_2 extract	53.0 ± 0.02	423.56	23.2 ± 0.02	788.59
MeOH extract	29.9 ± 0.05	654.98	59.4 ± 0.06	745.09
Friedelin (**1**)	47.6 ± 0.50	456.99	17.8 ± 0.54	891.23
Lupeol (**3**)	60.7 ± 0.50	157.88	50.3 ± 0.53	171.20
Stigmasterol (**4**)	57.6 ± 0.06	212.33	26.1 ± 0.03	587.43
Chrysin (**5**)	10.9 ± 0.12	621.90	14.1 ± 0.21	341.45
Stigmasterol-3-*O*-glucoside (**6**)	5.5 ± 0.43	998.77	15.1 ± 0.14	923.40
Apigenin (**7**)	NT		NT	
Morin (**8**)	NT		17.8 ± 0.50	991.23
1% acetone	1.0 ± 0.34			
Temephos	99.9 ± 0.01	0.5		
Lambdacyhalothrin(1µg/mL)			98.2 ± 0.1	0.01

NT=not tested

Values are means ± SD of three replicates

(>75%: highly active; 50-74%: moderate; 25-49%: weak; <25%: inactive (Globade et al., 2002)

Table 5

In vitro antimicrobial activities of extracts and isolates.

Test samples	Bacteria							Fungi		
	Diameter/zones of inhibition (mm)							Diameter/zones of inhibition (mm)		
	S. aureus	*S. faecalis*	*B. anthracis*	*E. coli*	*K. pneumoniae*	*S. typhumurium*	*P. aeuriginosa*	*C. albicans*	*A. fumigatus*	*A. niger*
CH_2Cl_2 extract	6.2 ± 0.1	6.1 ± 0.1	7.4 ± 0.2	7.9 ± 0.1	6.2 ± 0.2	3.4 ± 0.1	5.8 ± 0.1	5.0 ± 0.2	5.5 ± 0.2	2.1 ± 0.3
MeOH extract	5.6 ± 0.3	3.3 ± 0.2	1.1 ± 0.4	1.3 ± 0.4	3.1 ± 0.2	6.3 ± 0.3	8.4 ± 0.3	5.4 ± 0.2	6.3 ± 0.3	8.5 ± 0.4
Friedelin (**1**)	0.2 ± 0.2	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.2	0.7 ± 0.2	2.0 ± 0.1	0.2 ± 0.2	4.8 ± 0.3	0.2 ± 0.2	1.3 ± 0.7
Lupenone (**2**)	2.3 ± 0.2	3.1 ± 0.4	1.3 ± 0.4	3.1 ± 0.2	4.3 ± 0.3	7.4 ± 0.3	5.4 ± 0.4	5.0 ± 0.2	4.5 ± 0.2	4.1 ± 0.3
Lupeol (**3**)	5.4 ± 0.0	6.3 ± 0.1	5.1 ± 0.1	6.7 ± 0.2	2.2 ± 0.1	1.0 ± 0.2	1.6 ± 0.2	9.6 ± 0.1	4.4 ± 0.2	3.1 ± 0.2
Stigmasterol(**4**)	1.1 ± 0.1	2.1 ± 0.2	4.4 ± 0.2	3.7 ± 0.3	4.6 ± 0.3	9.9 ± 0.1	4.1 ± 0.3	3.8 ± 0.1	NT	NT
Chrysin (**5**)	7.2 ± 0.1	4.1 ± 0.1	5.4 ± 0.2	4.9 ± 0.1	5.2 ± 0.2	5.4 ± 0.1	4.8 ± 0.1	6.6 ± 0.1	4.4 ± 0.2	3.1 ± 0.2
Stigmasterol-3-*O*-glucoside (**6**)	4.4 ± 0.0	5.3 ± 0.1	2.1 ± 0.1	6.3 ± 0.1	4.3 ± 0.1	3.0 ± 0.2	3.6 ± 0.2	4.9 ± 0.2	4.1 ± 0.4	4.1 ± 0.1
Apigenin (**7**)	1.3 ± 0.2	1.3 ± 0.2	3.2 ± 0.4	2.1 ± 0.2	1.1 ± 0.2	2.3 ± 0.3	NT	7.0 ± 0.2	6.5 ± 0.2	6.1 ± 0.3
Morin (**8**)	2.3 ± 0.2	3.1 ± 0.4	1.3 ± 0.4	3.1 ± 0.2	4.3 ± 0.3	7.4 ± 0.3	5.4 ± 0.4	8.4 ± 0.2	2.3 ± 0.3	3.5 ± 0.4
Amoxyillin	19.5 ± 0.1	9.3 ± 0.3	16.7 ± 0.1	18.5 ± 0.1	19.9 ± 0.2	19.6 ± 0.0	17.7 ± 0.3			
Fluconazol								17.3 ± 0.2	19.5 ± 0.1	15.8 ± 0.3

Values are means ± SD of three determinations. Paper disc size is=5 in mm diameter (subtracted); **Activity scale**: (>17: Highly active; 11-16: intermediate; 6-10: weak; <5: resistant) (Singh et al., 2002); **Bacteria**: *S. aureus*, (ATCC 25922), *S. faecalis* (ATCC 25925), *K. pneumoniaie* (ATCC 90028), *S. typhimurium* (ATCC 25927), *E. coli* (K 12), *P. aeuruginosa* (ATCC 25923), *B. anthracis* (QST 713), Am=Amoxycillin, **NT**=Not tested; **Fungi**: *C.albicans* (HG 392), *A. fumigatus* (HG 420), *A. niger* (ATCC 90028), Flu=Fluconazol, NT=Not tested.

Table 6

Effects of crude extracts and isolated compounds of *L. eriocalyx* Harms on hot plate-induced pain and acetic acid-induced writhing in mice.

Dose Treatment (100 mg/kg)	Anti-inflammatory tests										
	Pre-treatment latency (s)	Pain threshold (time lapse after treatment)								Writhing response	
		Post treatment latency (s) and % inhibitions								Total no. of writhes	% Inb
	0 min	30 min		60 min		90 min		120 min			
			% Inb		% Inb		% Inb		% Inb		
CH_2Cl_2	3.0 ± 0.01	4.4 ± 0.04	8.23	11.5 ± 0.25	32.16	7.4 ± 0.20	25.13	6.1 ± 0.21	13.55	27.3 ± 2.51	36.05
MeOH	3.5 ± 0.12	4.8 ± 0.10	7.33	12.0 ± 0.31	33.18	12.6 ± 0.20	28.52	6.9 ± 0.25	11.98	34.7 ± 2.63	53.70
Cpd **1**	3.2 ± 0.12	6.8 ± 0.13	7.97	15.1 ± 015	38.09	16.4 ± 0.10	41.94	7.5 ± 0.13	38.31	19.7 ± 2.08	66.47
Cpd **2**	3.1 ± 0.15	6.4 ± 0.13	6.04	12.3± 0.31	35.04	11.0 ± 0.25	22.92	8.5 ± 0.20	14.33	24.3 ± 1.49	50.52
Cpd **3**	3.1 ± 0.12	6.8 ± 0.10	10.33	17.0± 0.31	53.18	20.6 ± 0.20	56.52	9.5 ± 0.25	30.98	14.7 ± 2.63	76.70
Cpd **4**	3.2 ± 0.15	4.8 ± 0.10	5.29	8.0 ± 0.10	13.5	5.8 ± 0.22	8.51	7.3 ± 0.23	31.90	21.3 ± 2.50	62.24
Cpd **6**	3.6 ± 0.11	4.1 ± 0.14	6.97	8.1 ± 015	27.31	8.4 ± 0.12	19.94	7.5 ± 0.13	22.61	35.3 ± 4.53	45.44
Cpd **5**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cpd **7**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cpd **8**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Morphine (10 mL/kg)	2.9 ± 0.15	7.2 ± 0.15	15.86	20.4 ± 0.20	64.57	23.3 ± 0.57	75.27	15.8 ± 0.35	47.6		
Acetyl- salicylic acid										10.9 ± 1.10	87.37

V values are mean ± SEM (n=6); NS=Non significant ($p<0.05$) vs. control (one-way ANOVA followed by Bonferroni posttests); NA=not active

was used as positive control and acetone as negative one. The results were reported as lethal concentration LC_{50} for extracts and compounds, and the minimum concentration that gives 100% larvicidal activity was determined in ppm (Table 4). The tests were done in triplicate.

2.9. Antimicrobial tests

The disc diffusion assay method was applied (Singh et al., 2016) using *Candida albicans* (HG 392), *Aspergillus fumigatus* (HG 420) and *Aspergillus niger* (ATCC 90028) as the representatives of fungi. *Staphylococcus aureus* (ATCC 25922), *Streptococcus faecalis* (ATCC 25925) and *Bacillus anthracis* (QST 713) were used as the representatives of gram-positive bacteria, while *Klebsiela pneumaoniaie* (ATCC 90028), *Salmonella typhimurium* (ATCC 25927), *Pseudomonas aeruginosa* (ATCC 25923) and *Escherichia coli* (K 12) were representatives of gram negative bacteria. The test organisms were obtained from the stock kept at the Microbiology Section of the Kenya Medical Research Institute, Kisumu County, Kenya. The anti-biogram pattern was studied using CH_2Cl_2 and MeOH extracts at 0.1 mg/mL (dissolving 10 mg of the extracts in 100 mL of DMSO). Sterile paper discs (6 mm of diameter) were impregnated with 20 µg/mL of each solution of the extracts, while pure isolates were tested at 30 µg/mL. Overnight cultures of bacteria and fungi species were used for inocula preparation whereby the microorganisms were grown in a sterile saline and turbidity adjusted to yield 0.5 McFarland standards [approximately 1-2x10^8 colony-forming units per milliliters (for bacteria) and approximately 1-5x10^6 colony-forming units per milliliters (for fungi)]. Petri

dishes containing Mueller-Hinton agar and Sabouraud dextrose agar were seeded with 100 µL of the prepared bacterial and fungal inocula, respectively. The discs were then incubated at 37 ºC for bacteria and at 25 ºC for fungi for 24 h. Similarly, paper discs containing standard concentrations of antibacterial (amoxycillin, 20 µg per disk) and antifungal (fluconazole, 30 µg per disk) were used as positive control. Antimicrobial activities were determined by measuring the zone of growth inhibition (mm) around the disk (Table 5). The assays were done in triplicate and analyzed statistically using MSTAT-C statistical package.

2.10. Antiinflammatory tests

2.10.1. Analgesic effect on the hot plate test

The modified method of Eddy and Leimbach (1953) was used whereby groups of mice (5 per group) of either sex (17-30 g) were used as test organisms. The mice were initially screened by placing the animals in turn on a hot plate set at 55 ± 1 ºC and those which failed to lick the hind paw or jump within 15 s were discarded. Eligible animals were divided into five groups of five each and pre-treatment reaction time for each mouse was determined before drug treatment so that each animal serves as it is own control. The time taken for the animals to lick the paws, flutter any of the paws or jump was taken as reaction time and recorded with the aid of an inbuilt stopwatch. Mice in the different groups were then treated with normal saline water [10 mL/kg, per oral (p.o)], CH_2Cl_2 and MeOH extracts (100 mg/kg, p.o), together with compounds **1**, **2**, **3**, **4** and **6** (10 mg/kg) and morphine (10 mg/kg, s.c). The latency was recorded after 30 and 60 min after oral administration of the substances. A post-treatment cut off time of 30 s was used to avoid paw tissue damage (Omisore et al., 2004).

%Inhibition=[post-treatment latency]-[pre-treatment latency]/[cut-off time-pre-treatment latency]×100 (Eqn.1)

2.10.2. Acetic acid (chemical-induced) writhing method

Abdominal writhes consist of contraction of the abdominal muscle together with a stretching of the hind limbs, induced by intra-peritoneal injection (i.p) in mice using acetic acid (0.8% solution in normal saline (0.1 mL/10 kg), the nociceptive agent (Koster et al., 1959). The CH_2Cl_2 and MeOH extracts (100 mg/kg, p.o) and compounds **1**, **2**, **3**, **4** and **6** (100 mg/kg, p.o) were administered to mice (animals fasted overnight and divided into five groups of six animals each) 60 min before intra-peritoneal injection with acetic acid (0.6%, v/v in normal saline, 10 mL/kg, i.p). Normal saline was used as the control. The number of writhes (characterized by contraction of the abdominal musculature and extension of the hind limbs) were counted for 30 min at 5 min interval of intra-peritoneal injection of acetic acid (Adeyemi et al., 2004). Statistical analysis of results obtained were expressed as mean ± standard error of mean (SEM) or standard deviation (SD) (Table 6). The data were analyzed using one-way ANOVA followed by Bonferroni posttests and Dunnett's multiple comparison tests. Values were considered significant when $p \leq 0.05$.

Inhibition(%)=number of writhes [control]−number of writhes [treatment]/number of writhes [control]×100 (Eqn. 2)

3. Results and Discussion

3.1. Structure elucidation of compounds **1-8**

Compound **1** was isolated as white powder with m.p. 254-256 ºC. It afforded positive Libermann-Burchard test suggesting that it could be a terpene or a sterol (Attarde et al., 2010). Its IR spectrum showed an intense band at 1711 cm^{-1} suggesting the presence of a carbonyl moiety in the compound and was confirmed by the presence of ^{13}C-NMR signal at δ_C 213.4 typical of a carbonyl carbon of ketone function (Haque et al., 2015). The ^{13}C-NMR spectral data (Table 2) showed the presence of 30 carbon signals resolved into eight sp^3 methyls, eleven sp^2 methylenes, four sp^3 methines, six sp^3 quartenary carbons and one sp^2 quartenary carbon as evidenced by 135 DEPT experiment, a fact that was supported by the EI-MS (70 eV) molecular ion peak at *m/z* 426 corresponding to a $C_{30}H_{50}O$ formula. In the ^{1}H-NMR spectrum (Table 1), a doublet at δ 0.94 (*J*=6.5 Hz, Me-23) integrating into three protons together with resonances for seven tertiary methyl groups observed at δ 0.74 (25-Me), 0.88 (29-Me), 0.89 (30-Me), 0.91 (26-Me), 1.02 (24-Me), 1.03 (27-Me) and 1.07 (28-Me) with corresponding ^{13}C-NMR signals at δ_C 20.5 (C-25), 32.4 (C-29), 32.7 (C-30), 18.9 (C-26), 14.9 (C-24), 18.5 (C-27), 7.1 (C-23) and 32.0 (C-28), suggested that the compound is a friedelane type triterpene (Duraipandiyan et al., 2016). The foregoing evidence was further supported by the fragmentation pattern in the EI-MS spectrum and the appearance of a quartet signal integrating for one proton at δ 2.27 which was assigned to H-4. In fact, the presence of signals due to one secondary and seven quartenary carbons in the ^{13}C-NMR spectrum together with cross peak correlation between a doublet at δ 0.94 and a quartet of methine at signal at δ 2.27 in the ^{1}H-^{1}H COSY spectrum confirmed the structure of the compound to be friedelin, a compound previously reported from *Conyza floribunda* (Opiyo et al., 2009).

Compound **1** along with compound **2** with R_f 0.56 (eluent: *n*-hexane-CH_2Cl_2, 1:3) and m.p. 166-168 ºC were isolated from another white crystal. Like compound **1**, it (compound **2**) gave a positive Liebermann-Burchand test suggesting that it could be a terpene or sterol derivative (Attarde et al., 2010). Its EI-MS showed a molecular ion at *m/z* 424 which is 2 amu less than that

of **3** corresponding to $C_{30}H_{48}O$ formula. In addition, both the ^{1}H and ^{13}C-NMR data (Table 1 and Table 2) of 2 were in agreement with those of 3 with notable difference being the absence of oxymethine proton which appeared at δ 3.20 in the latter compound. Apparently, in compound **2**, the hydroxyl group was replaced by a keto moiety as evidenced by a peak at δ_C 216.0 in the ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum displayed 7 tertiary methyl groups at δ 1.67, 1.23, 1.10, 1.08, 0.92, 0.83 and 0.78 with corresponding ^{13}C-NMR signals at δ 19.3, 32.4, 21.2, 33.3, 18.0, 21.6 and 14.8, respectively. This together with characteristic peaks resonating at δ 4.68 (d, *J*=0.5 Hz) and 4.57 (d, *J*=0.5 Hz) assignable to the exocyclic double bond further supported that compound **2** is a derivative of lupeol (**3**) in which C-3 has a keto-group (Malca-Garcia et al., 2015). The 1H-1H COSY spectrum showed significant connectivities; (H-1 and H-2, H-5 and H-6, H-15 and H-16, H-21 and H-22) leading to a conclusion that the compound was lupenone.

Compound **3** was isolated as white crystals, m.p. 216-218 ºC. It gave a positive Libermann-Burchard test indicating a triterpenoid or steroid skeleton (Attarde et al., 2010). The IR spectrum had significant peaks appearing at 3315 and 1650 cm^{-1} typical of OH and olefinic functional groups. The ^{1}H spectrum (Table 1) had signals consistent with a pentacyclic lupane-type triterpene with olefinic protons appearing at δ 4.55 and 4.65 (2H, H_2-29) (Pavia et al., 2009), confirmed by the appearance of a peak at δ 108.1 attributable to the olefinic carbon at C-29 in ^{13}C-NMR spectrum (Table 2). This evidence was further supported by the down field olefinic carbon at δ 149.7 (C-20) typical of a quaternary sp^2 carbon. Both the ^{1}H and ^{13}C-NMR spectra showed a signal signifying the presence of hydroxymethine group (H-3) at δ_H 3.20 (dd, *J*=11.4, 4.8 Hz) with corresponding carbon peak at δ_C 74.7 (C-3). The hydroxyl group at C-3 was deduced to be in equatorial orientation based on axial-axial and axial-equatorial couplings between H-3 and H-2 protons, an interpretation that was further facilitated by HMBC spectrum which exhibited cross-peaks between H-3 and C-23 (δ 29.3) and C-24 (δ 32.8) and in turn with C-5 (δ 54.0), a fact further corroborated by a 1,3-diaxial correlation between H-3 and H-5 in the NOESY spectrum. Seven singlets for tertiary methyl protons at δ_H 0.76 (Me-27), 0.83 (25-Me-25), 0.94 (Me-26), 0.96 (Me-28), 1.03 (Me-24),1.20 (Me-23) and 1.67 (25-Me-26) (integrating for 3H each) with corresponding ^{13}C-NMR signals at δ_C 15.0 (C-27), 15.9 (C-25), 16.0 (C-26), 18.0 (C-28), 32.8 (C-24), 29.3 (C-23) and 19.5 (C-30), respectively as deduced from the HSQC which were in agreement with the structure of lupeol previously isolated from *Lonchocarpus sericeus* and *Holarrhena floribunda* (Abdullahi et al., 2013). Confirmation of lupeol structure was further supported by EI-MS (70 eV) molecular ion at *m/z* 426 $[M]^+$ corresponding to a molecular formula $C_{30}H_{50}O$. Thus, on the basis of physical and spectroscopic data as well as comparison with literature data (Abdullahi et al., 2013), compound **3** was confirmed and identified as lupeol.

Compound **4** was isolated as white crystals with R_f=0.34 (eluent: *n*-hexane-CH_2Cl_2, 1:2) and m.p. 162-164 ºC. It afforded a bluish-purple colour when sprayed with anisaldehde on TLC followed by heating at 100 ºC for a minute. The EI-MS spectra of this compound gave a molecular ion peak at *m/z* 412 suggesting a formula of $C_{29}H_{50}O$. This together with the daughter ions observed at *m/z* 397 (loss of H_2O from the molecular ion), 381 (cleavage of C_{23}-C_{24} bond from M^+), 354 (M^+-isopropyl-H_2O ion), 327 (cleavage at C_{23}-C_{24} bond), (300 due to C_{24}-C_{23} bond scission) and 271 (cleavage of C_{17}-C_{20} bond) strongly suggest that the compound was a sterol derivative (Reginatto et al., 2001). The ^{1}H-NMR spectrum two tertiary methyl singlets [δ 1.03 (Me-18) and 0.95 (Me-19)], three secondary methyl doublets [δ 1.12 (*J*=6.5 Hz, Me-21), 0.83 (*J*=6.8 Hz, Me-27) and 0.84 (d, *J*=6.8, Me-26)] and a primary methyl triplet at δ 0.86 (*J*=7.1 Hz, 29-Me) together with characteristic $\Delta^{5,22}$-sterol signals at δ 5.35 (t, *J*=5.2 Hz, H-6), 4.88 (m, H-22) and 5.20 (m, H-23) substantiated with corresponding ^{13}C-NMR peaks appearing at δ_C 140.8 (C-5), 121.7 (C-6), 138.7 (C-22) and 130.0 (C-23) suggested that the compound is stigmasterol (Alam et al., 1996; Reginatto et al., 2001). The ^{13}C-NMR spectrum showed the presence of 29 carbon atoms which were resolved into six methyl, nine methylenes, eleven methines and three non-protonated carbon as evidenced by 135 DEPT spectrum, thus remaining proton was part of the hydroxyl functionality as evidenced by multiplet at δ 3.52. Thus, on the basis of spectroscopic data as well as comparison with literature, compound **4** was concluded to be stigmasterol.

Compound **5** was isolated as yellow powder with m.p. 285-287 ºC. Its ^{1}H-NMR spectrum (Table 1) showed the presence of two *meta*-coupled protons at δ 6.48 (d, *J*=1.5 Hz) and 6.23 (d, *J*=1.5 Hz) typical for 5,7-dihydroxy substituted ring A of a flavonoid derivative (Mabry et al., 1970). Another set of five aromatic signals involving *ortho*-coupled AX system doublets at δ 7.99 (d, *J*=6.5 Hz, H-2′/H-6′) and a multiplet ABX system at δ 7.58 (3H) assignable to H-3′, H-4′ and H-5′ together with a characteristic singlet resonance at δ 6.73 assigned to H-3 suggested that the compound is a flavone in which ring B is unsubstituted (Miyaichi et al., 2006). The foregoing evidence was further corroborated by the ESI-MS which exhibited a protonated molecular ion peak at *m/z* 255 $[M+H]^+$ consistent with the formula $C_{15}H_{11}O_4$. Thus, on the basis of spectroscopic data, compound **5** was confirmed to be chrysin.

Compound **6**, R_f value of 0.39 (eluent: CH_2Cl_2-MeOH; 98.2) was isolated as an amorphous white powder, m.p. 289-290 ºC. It showed a positive Liebermann-Burchard test (Attarde et al., 2010) and with acidified vanillin, it gave bluish-purple colour on TLC after heating, which signified either a sterol or a terpene derivative. The EI-MS showed a peak at *m/z* 574 $[M]^+$ corresponding

to $C_{35}H_{60}O_6$ formula. A peak appearing at *m/z* 412 indicated loss of a hexose moiety from the molecule. This was supported by acid hydrolysis (2% HCl) which yielded a free sugar identified as D-glucose after comparison with authentic samples on silica gel TLC as well as paper chromatography. The ^{1}H-NMR spectrum (Table 1) determined in DMSO confirmed the acid hydrolysis results by showing a characteristic signal for anomeric proton as a doublet at δ 4.44 (d, *J*=7.7 Hz). The coupling constant ascertained the β-configuration of the sugar residue. The ^{1}H-NMR spectrum further showed signals for two tertiary methyl singlets [δ 0.94 (Me-19) and 1.07 (Me-18)], three secondary methyl doublets [δ 1.30 (*J*=6.6 Hz, Me-21), 0.90 (d, *J*=6.5 Hz, Me-26) and 0.88 (d, *J*=6.7 Hz, Me-27)] and a triplet [δ 1.03 (t, *J*=7.2 Hz, Me-29) due to a primary methyl group] besides the characteristic of Δ^5-exocyclic double bond proton resonating as a triplet at δ 5.28 (H-6) (Ahmad et al., 2012). In addition, multiplets appearing at δ 4.95 and 5.10 were attributed to olefic protons on C-22 and C-23, respectively, thus suggesting the compound is glycosidated stigmasterol (Mahbuba et al., 2012). The attachment of the sugar residue at C-3 was confirmed by a shift of the H-3 resonance to higher value of δ 3.63 compared to 3.20 in **4**. The ^{13}C-NMR spectrum of compound **6** (Table 2) showed the presence of 35 carbon signals of which six were in the glycosidic region corresponding to a hexose moiety. The remaining 29 carbons were due to the aglycone. The olefinic carbon signals at δ 142.5 and 122.3 corresponded to the exocyclic double bond between C-5 and C-6, while those appearing at δ 124.3 and 137.5 assignable to C-22 and C-23, are characteristic of $\Delta^{5,22}$-type of sterol (Mahbuba et al., 2012). Conclusive evidence for the structure of compound **6** was further provided by the extensive interpretation of ^{1}H and ^{13}C chemical shift correlations experiment which further supported both ^{1}H and ^{13}C-NMR results (Ahmad et al., 2012). Therefore, on the basis of the above accumulated evidence, the structure of **6** was established as stigmasterol 3-*O*-β-D-glucoside.

Compound **7** was isolated as a yellow amorphous powder, m.p. 346-348 ºC. The yellow colour intensified on exposure to conc. ammonia vapor. It also turned dark-brown upon spraying with ferric chloride solution suggesting that it was a flavonoid derivative (Batterham and Highet, 1964). This was further supported by UV spectrum which gave an absorption maxima at λ_{max} 268 and 336 nm (Mabry et al., 1970). The ESI-MS showed a molecular ion peak at *m/z* 270 which is 16 amu less than that of kaempferol (Mabry et al., 1970) corresponding to $C_{15}H_{10}O_5$ formula. The ^{1}H-NMR spectral data (Table 1) of compound 7 exhibited the presence of two *meta*-coupled aromatic doublets at δ_H 6.40 (*J*=2.2 Hz) and 6.26 (*J*=2.2 Hz) corresponding to H-8 and H-6, respectively. In ring B, an AA'XX' system at δ_H 7.86 (d, *J*=9.0 Hz) and 6.94 (d, *J*=9.0 Hz) were assigned to H-2'/H-6' and H-3'/H-5', respectively, while a characteristic singlet at δ 6.60 represented H-3 proton, thus suggesting the presence of a flavone moiety. In fact, the ^{1}H-NMR spectral data were in agreement with a 5,7,4'-trisubstituted flavone (Batterham and Highet, 1964), confirmed by typical RDA fragments appearing at *m/z*; 153 and 121 in the MS. The ^{13}C-NMR data (Table 2) showed the presence of fifteen carbon signals sorted out into seven aromatic CH, CH of double bond and seven quaternary carbons including a conjugated carbonyl carbon. Thus, based on physical and spectroscopic data as well as comparison with data already in various literature, compound **7** was identified as apigenin.

Compound **8** was isolated as light yellow powder with m.p. 299-300 ºC. Its EI-MS displayed a molecular ion peak at *m/z* 302 consistent with the formula $C_{15}H_{10}O_7$, suggesting it is a derivative of quercetin (Mabry et al., 1970). As in the case for 7, its ^{1}H-NMR spectrum (Table 1) showed the presence of two *meta*-coupled protons of ring A appearing at δ 6.43 (d, *J*=1.5 Hz, H-8) and 6.17 (d, *J*=1.5 Hz, H-6), an observation that was further evidenced by the daughter ion at *m/z* 153, indicating that ring A had no other substituents other than the two hydroxyl groups at C-5 and C-7. Careful examination of the ^{1}H-NMR spectrum revealed the absence of H-3 of favones implying that C-3 was substituted, a fact further supported by the ^{13}C-NMR peak at δ 133.4. The ^{1}H-NMR also exhibited an AMX spin system in which peaks at δ 7.43 (d, *J*=7.0 Hz, H-6'), 6.50 (dd, *J*=4.0, 3.0 Hz, H-5'), 6.33 d, *J*=4.0 Hz, H-3') suggested oxygenation at C-2' in ring B instead of C-3' as in quercetin (Miyaichi et al., 2006). Changes of diagnostic values were observed in ^{13}C-NMR spectrum of **8** in comparison with quercetin whereby C-3' shifted up field at δ 104.6 due to the absence of OH group at this position, while C-2' shifted down field at δ 157.6 arising from OH substitution (Hussain et al., 2014), a fact that corroborated by HMBC correlation between H-5' (δ 6.50) and C-3' (δ 104.6). Thus, on the basis of spectroscopic data, compound **8** was elucidated as 3,5,7,2',4'-pentahydroxyflavonol (trival name morin).

3.2. *In vitro* anti-plasmodial activities of extracts and isolates

Both the extracts and all isolated compounds were tested for antiplasmodial activities and the corresponding results are summarized in Table 3. The MeOH extract was moderately more active than the CH_2Cl_2 showing activities of 423.0 ± 0.01 and 365.2 ± 0.3 ng/mL against W2 and D6 strains of *P. falciparum*, respectively. Among the isolates, lupeol (**3**) showed the highest activity with values of 104.4 ± 0.4 and 109.9 ± 0.4 ng/mL against W2 and D6 strains. Standard drug, mefloquine used as positive control gave impressive results with IC_{50} values of 16.1 ± 0.02 and 22.30 ± 0.01 against the clones, respectively. In a previous investigation, the MeOH extract of the root bark of the plant showed antiplasmodial activity against

chloroquine-sensitive W2 and chloroquine-resistant D6 strains of *P. falciparum*. Although the IC_{50} values were not reported, the compound (**3**) showed good antiplasmodial activity (Tuwei, 2006).

3.3. Larvicidal and mosquitocidal activities of crude extracts and isolates

The CH_2Cl_2 and MeOH extracts as well as the compounds **1-8** were subjected to both larvicidal and insecticidal assays and the obtained results are displayed in Table 4 in terms of %mortality and LC_{50}. In this case, the CH_2Cl_2 extract showed moderate larvicidal activity of 53.0 ± 0.02% mortality which translated into LC_{50} value of 423.56 µg/mL. The isolates lupeol (**3**) and stigmasterol (**4**) had mortality% of 60.7 ± 0.50 and 57.6 ± 0.06 with LC_{50} values of 157.88 and 212.33 µg/mL. The MeOH extract was observed to be active against adult mosquito with mortality% value of 59.4 ± 0.06% with a corresponding LC_{50} value of 745.09 µg/mL. This study concurs with a previous investigation in which the crude extracts showed moderate larvicidal and mosquitocidal activities against *Aedes aegypti* (Yenesew et al., 2003). The observed activity confirms the use of this plant by the Embu-Mbeere people as an effective mosquito repellant (Coates et al., 2002; Kareru et al., 2007).

3.4. Antimicrobial activities of extracts and isolates

Both the extracts and isolated secondary metabolites were subjected to antifungal and antibacterial assays and inhibition diameters are summarized in Table 5. The CH_2Cl_2 extract was inactive against *C. albicans*, *A. fumigatus* and *A. niger*, however the MeOH extract showed mild activity against A. niger with an inhibition zone of 8.5 ± 0.4 mm. Lupeol (**3**), apigenin (**7**) and morin (**8**) showed mild activity against *C. albicans* with inhibition zones of 9.60 ± 0.10, 7.0 ± 0.20 and 8.4 ± 0.2 mm, respectively. Fluconazole was used as the standard drug and had inhibition zones of 17.3 ± 0.2, 19.5 ± 0.1 and 15.8 ± 0.3 against *C. albicans*, *A. fumigatus* and *A. niger*, respectively. For antibacterial activity tests, stigmasterol (**4**) showed an intermediate activity against *S. typhimurium* with inhibition zone of 9.9 ± 0.1 mm. The activities of other compounds obtained from this plant were relatively quite low compared to the standard drug used. The activity of amoxycillin which was used as the standard drug was superior than all the isolates.

3.5. Anti-inflammatory assays

3.5.1. Analgesic effect on the hot plate test

The analgesic effect of the crude extracts and the isolated compounds **1**, **2**, **3**, **4** and **6** were studied in mice using hot plate-induced pain. Preliminary results (Table 5) showed that the pre-treatment latency for morphine (2.9 ± 0.15 s) was quite comparable to that of crude extracts (100 mg/Kg) at the zero minute whose values were 3.1 ± 0.15 and 3.0 ± 0.01 for CH_2Cl_2 and MeOH implying that they delayed infliction of pain more less with the same magnitude as the standard drug just as the instant time of administration. Similarly, the CH_2Cl_2 extract had a significant effect in delaying the pain within 30 minutes which was quite comparable to that of morphine (10 mg/Kg) meaning longer post treatment latency. Generally, the crude extracts significantly increased the reaction time for nociception from the beginning to 60 minutes post treatment. However, the effects of the crude extracts (100 mg/kg) were significantly ($p<0.05$) lower than those produced by morphine in the same tests. Compounds **1** and **3** delayed incubation of pain from the beginning to 60 minutes after which the effect was insignificant. The two compounds had longer latency compared to the crude extracts from the beginning to 60 minutes suggesting that purity enhanced the efficacy of the compounds.

3.5.2. Acetic acid (chemical-induced) writhing method

Acetic acid-induced writhing test in mice was also used to study the analgesic effect of the crude extracts and the isolates. After intraperitoneal injection with the crude extracts, comparatively less number of writhes was observed (contraction of abdominal muscles together with stretching of the hind limbs) implying that the extracts had significant ability to relieve pain.

4. Concluding remarks

CH_2Cl_2 and MeOH crude extracts along with compounds **1-8** were tested for their analgesic activities. The crude extracts as well as compounds **1**, **2**, **3**, **4** and **5** showed strong to moderate analgesic effects. However, the rest of the compounds did not show any activity. The result obtained confirms the folkloric information contained in literature that this plant has anti-inflammatory activity and is also used in managing fever and this authenticates its use as an herbal remedy.

Conflict of interest

The authors declare that there is no conflict of interest.

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