Original Research Article

IN VITRO ANTIPLASMODIAL ACTIVITIES OF CRUDE EXTRACTS AND COMPOUNDS DERIVED FROM SELECTED INDIGENOUS MEDICINAL PLANTS USED TRADITIONALLY TO TREAT MALARIA IN KENYA

ABSTRACT

Aims: To identify viable phytomedicines traditionally employed for the treatment of malaria in Kenya that could be developed into antimalarial agents.

Study design: Quantitative analysis of antiplasmodial activities and brine shrimp bioassays were carried out using standard procedures.The experiment was set in duplicate for each concentration of the drug and average IC50 determined.

Place and Duration of Study: Seven indigenous plants:*Achyranthesaspera, Heinsiacrinita, Brideliacathartica, Citrus limon, Microglossapyrifolia, Vernoniaglabra* and *Carissa edulis* obtained from Kilifi and Homa-Bay counties in Kenya were evaluated for their anti-*Plasmodium falciparum*potential.Collection of samples and analysis took about three months.

Methodology: Both Chemical and aqueous crude extraction methods were carried out to identify the most active extracts against *P. Falciparum* and thenisolatepureactive phytochemicals. Pure compounds were subjected to Nuclear Magnetic Resonance (NMR), Infra-Red (IR) and Mass Spectroscopy (MS) analyses for structure elucidation.

Results: Four extracts of hexane, dichloromethane (DCM), methanol and water of seven different species of plants were analyzed for their anti-plasmodial activities.W2 and D6 strains of Falciparum were tested. However, the three most active extracts were from *Citrus lemon* roots (DCM) with IC_{50} value of 7.017 µg/mL, *C. edulis* root (aqueous) with IC_{50} value of 8.054 µg/Ml and *B. Cathertica* leaves (methanol) with IC_{50} value of 15.647 µg/Ml. However, three pure compounds were obtained; suberosinIC_{50}26.7 (Strain W2), 53.1 (Strain D6) and xanthyletin IC_{50}1580 (Strain W2) from *C. Limon* (DCM) and spinasterolIC_{50}43.2 (Strain W2) from*M. pyrifolia* (hexane).

CONCLUSION: The three different species of plants with most active compounds have demonstrated their potentiality in treatment for *falciparum* malaria. Structures of the isolated three compounds can be modelled to synthesise anti- plasmodial drugs as they are active *in vitro.*

Key words:*Phytomedicines, Antimalarials, chloroquine resistance, bioactivity-guided fractionation,coumarins.*

1.0 INTRODUCTION

Malaria is present in more than 107 tropical countries with more than 3.2 billion people comprising, 40% of the world's population [26, 28]. In Africa including Kenya, malaria caused by *Plasmodium falciparum* is one of biggest obstacles to socio-economic development [15].It`s control and prevention is based on the use of prophylactic and treatment drugs, reduction of mosquito vector population via use of insecticides and prevention of mosquito bites in endemic areas [5]. The success of these initiatives has been prohibited by parasite and vector resistance to conventional drugs (CQ, Amodiaquine, Fansidar, Mefloquine, Primaquine, Quinine & Artemisinin, etc) and insecticides (DDT) respectively [29, 42, 47], hence the need for drugs.Besides development of drug resistance, some of the antimalarial drugs are faced with challenges of toxicity that inhibit their use [29]. However, despite the use of ACT as the preferred first-line drug, there have been reports of *P. falciparum* resistance in various countries; [13, 14, 23]. The use of non-chloroquine drugs and insecticide treated nets is limited by their high cost. This have impoverish the local communities who are already experiencing high poverty levels causing them to turn to natural tradition remedies [18]), occasioning the need for research for new anti-malarial drugs [37].

The resistance of *P. falciparum* to antimalarials and mosquitoes to insecticides, has necessitated search for new compounds against malaria making use of leads from ethnopharmacology studies. To those populace relying on medicinal plants against malaria, it is vitally important that the safety and efficacy of such medicines be determined, reproducible dosage forms be developed and made available for use and their active components determined [40, 45]. It is against this strong back ground that this project was undertaken. The main objective of this study was to determine the antiplasmodial activity and safety of extracts and isolated compounds of *Achyranthesaspera*, *Heinsiacrinita, Brideliacathartica, Citrus limon, Microglossapyrifolia, Vernonia glabra* and*Carissa edulis*.

2. MATERIAL AND METHODS

Medicinal plants (Test articles)

*Achyranthes aspera*L., (ii) *Heinsia crinita*(Afz.)G,Tayl, (iii)*Bridelia catharticа*Bertol.f., (iv) *Citrus limon*L., (v) *Microglossa pyrifolia*(Lam.) O. Kutze, (vi) *Vernonia glabra*(Steetz) Vatke and(vii) *Carissa edulis* (Old name) changed to Carissa spinarum L.

Table 1.**:List of plants date, time and where collected and their characteristics**

Plant Botanical Name	Date,Time,Voucher No.&location	Common/Vernacular Name
*Achyranthes aspera*L.	22/4/2018:10.00am SG2OO1/01 Kilifi	Tama Tama (Swahili), Prickly Chaff flower, Devil`s horsewhip,Sanskrit, Apamarga ama Tama (Swahili), Prickly Chaff flower, Devil`s horsewhip,Sanskrit, Apamarga
Heinsia crinita(Afz.)G,Tayl,	22/4/2018:10.20am SG2OO1/02 Kilifi	Mfyofyo (Swahili), Mshosho (Giriama), Mushoka (Duruma) and Dewakiri (Nanya), Bush apple, Jasmine-gardenia, Small false gardenia

Bridelia catharticaBertol.f.	22/4/2018:11.00am SG2OO1/03 Kilifi	Mnembe Nembe (Swahili), Blue sweetberry
*Citrus limon*L	23/4/2018:10.00 SG2OO1/04 Homa-Bay	Malimau (Swahili) Machunga Mar Ndim (Luo), Lemon
MICROGLOSSA PYRIFOLIA(LAM.) O. KUTZE	23/4/2018:11.30am SG2OO1/05 Homa-Bay	Nyabung` Odide (Luo)
Vernonia glabra(Steetz) Vatke	23/4/2018:12.00noon SG2OO1/06 Homa-Bay	Akech Madongo (Luo), Cornflower vernonia
Carissa edulis(Old name)changed to*Carissa spinarum* L.	23/4/2018:12.40pm SG2OO1/07 HOMA-BAY	Ochuoga (Luo) Mtanda-Mboo (Swahili) Fonkole,Dagams (Boran), Mutimuli (Bonjun), Molowe, Mulolwe (Duruma), Dagamsa (Gabora), Mokalakalo,Kaka-mchangani (lwana/Malakote), Mukawa (kamba), Mukawa (Kikuyu), Olamuriaki (Maasai), Legatetwo (Marakwet/Tugen), Legetetwa, Legetetwet (Nandi, Kipsigis, Tugen), Lokotetwo (Pokot), Lmuria, Lmiriel (Samburu), Gururа, (Sanya), Kirumba (Taita) and Ekamuria (Turkana), Egyptian Carissa , Carandas plum, Karaunda (India)

2.1 Preparation of collected plant samples

The collected plants samples (Table 1) were chopped, dried at room temperature at (about 30 ^{0}C) in the presence of air for two weeks for adequate drying of roots. The dried samples were then pulverized to powder thereafter stored at room temperature. Each sample pulverized of 3000 g, was divided into two portions, a small one of 100 g and a larger portion of 2900 g. The larger (2900 g) portion was used to prepare organic extracts by cold percolation sequentially using the following solvents: n-hexane, followed by dichloromethane and finally by methanol. The crude extracts from each plant sample and each extraction solvent were concentrated *in vacuo*. The smaller (100 g) portion of pulverized plant material was used to prepare an aqueous extract by boiling in water for 2 hours. The filtrate product from extraction by water was concentrated to powder and stored at 4 ^{0}C.

2.2 Preparation of the plant extracts for the tests

The stock solution of the drug containing 1 mg/50µl DMSO was further diluted ten folds to a concentration of 2 mg/ml with medium and was purified by filtering through a 0.22 µm filter. Twenty five microlitres (25µl) of the working solution was dispensed in duplicates in row B of the test plate and diluted with an equal amount of CMS containing *P. falciparum* at 1% parasitaemia. A multichannel pippete was used to make two-fold dilutions from one row to the next such that the highest concentration of a drug in row B wasx64 that in the last row H. The final concentration after adding 200 µl of parasites into the wells was such that row B had a drug concentration of 111.1 µg /ml while in row H it was 1.74 µg /ml.

2.3 Preparation of *Plasmodium falciparum*test samples

All the strains of falciparum respond differently to variety of drugs. The laboratory-adapted multidrug resistant V1/S, a multidrug resistant strain,(ii) W2-multidrug resistant strain and (iii) D6, CQ sensitive strain were used in the laboratory tests. The parasites obtained from the stabilate were assessed for viability via a microscopial examination under a light microscope followed by culture of viable parasites.

2.3.1 Preparation of parasite culture system

Antiplasmodial test was carried out as previously described [12] on 50% non-infected human O-positive red blood cells (RBC) were prepared as per the guidelines of previous work [46]. These O-positive RBCs kept for 12 days were infected with the Plasmodium species and then cultured. The O-positives in plasma was collected into 20 ml vacutainers containing citrate phosphate dextrose (CPD)-adenine buffer and was stored at 4^0C for 24 hours; in these conditions, they were active to a time limit of three weeks. Erythrocytes were prepared for use by washing three times in WM (RPMI 1640 containing HEPES (5.94g/L), and sodium bicarbonate (7.5%, 31ml/L)). The supernatant and the buffy coat containing WBC were removed after each wash. After the final wash the RBCs were suspended in WM 50% (v/v) which would also be used in parasite cultures. Parasites stored under liquid nitrogen were rapidly thawed at 37 ^{0}C and the isotonicity reconstituted as per previous work [46]. One ml of complete medium with serum (CMS) containing ten percent normal human serum which has been pooled and heat inactivated, Rhesus +ve, (NHS) in RPM1 1640 containing HEPES buffer 25mmol/µl and sodium bicarbonate 25 mmol/l was added to the culture, homogenized spun and the supernatant removed.

Fifty percent erythrocytes and CMs were added to the cells and homogenised to produce 6 % haematocrit. A mixture of three percent carbon dioxide, five percent oxygen and ninety-five percent nitrogen gas were used to flush the parasites for 2 minutes which were then incubated at 37 °C. The supernatant in each flask was renewed after every 24 hours and the cultures mixed by gently rotating the flask on a level surface before re-gassing and re-incubating. Parasitaemia was assessed after every three days on Giemsa-stained thin films by counting the parasitised RBC among 10,000 RBC. When the parasitaemia exceeded 2% the culture was diluted to a desired level by adding fresh 50% RBC and CMS, but maintaining the 6 % haematocrit. The growth rate (GR) monitored for 48 hours was calculated from the formula GR= (Pf/Pi) 2/n where Pf = final parasitaemia, and Pi = initial parasitaemia n = number of days in the culture [9]. The parasites were considered adapted to the *in vitro* culture and ready for drug test when they achieved a growth rate of 3-fold or greater in 48 hours.

2.3.2 Harvesting the malaria parasites

The malaria parasite cultures were incubated for 24 hours and labelled by adding radiolabelled [^{3}H]-hypoxanthine solution per well and plates re-incubated further for 24 hours. The [^{3}H]-hypoxanthine incorporation was measured by liquid scintillation on a Beta counter after drying the filter papers at 60°C for 30minutes.The set up was that each drug concentration

was tested in duplicate. The parasites were harvested using a Mesh II harvester on mini mash glass filter (Wittaker M A products) with plenty of distilled water after the second incubation period. The incorporation of [^{3}H]-hypoxanthine was determined by liquid scintillation counting on a scintillaton counter. The % inhibition was calculated using the formula: [mean NTPE-mean DTPE cpm/ mean NTPE-mean NPE] x 100. Where: cpm = count per minute. Mean NTPE = mean cpm for non-treated parasitized erythrocytes, mean NPE = mean cpm for non-parasitized erythrocytes and mean DTPE = mean cpm for drug treated parasitized erythrocytes.

2.4 Isolation and characterization of active chemicals/compounds from plants

2.4.1Isolation of compounds

Slurry was prepared by mixing a known amount of silica gel with a solvent. The column (80 cm long & 5.5 cm diameter) was filled about half-full with solvent and the stopcock was opened to allow solvent to drain slowly into a large beaker. The column was packed with silica gel slurry 60 (0.063-0.2mm/70 - 230 mesh ASTM for column chromatography-Macherey Nagel-Germany) to a height of 70 cm. The void volume of the column was calculated as follows: the radius of the column (27.5 mm) squared multiplied by pi (3.1416) multiplied by the column length (800 mm), and the resulting volume was divided by 1000 [corrected formula for units]. This afforded the 1900.668 mL.The bed volume (L) was calculated as follows: bed height (70 cm) x columncross-sectional area ($\pi r^2 h$) (cm^2) / 1000 = 3.1416 x 10^2 x 700/1000 = 1663.0845 cm^2. Semi-purified biologically active extracts, obtained from solvent fractionation were dissolved in a minimum solvent and added to the top of the column to form a layer on top of the adsorbent. Care was taken not to exceed the recommended solute loading capacity for silica gel. The sample was drained into the adsorbent until the top surface just begun to dry. Solvent elution was carried out starting with the solvent in which the sample was extracted until the first fractions were obtained then polarily of the solvent was increased with the addition of the more polar solvents. Different components of the sample charge passed through the column at different rates depending on their individual adsorption coefficients. These fractions eluted from the column were collected and concentrated.

Twenty grams of silica gel powder 60 PF_{254} was transferred into 500 ml conical flask and then thoroughly mixed with 50 ml distilled water to make homogeneous slurry. The slurry was carefully poured on a scrupulously clean 20x20cm glass plate and then spread evenly to cover the whole plate. The plate was left to dry at room temperature in a dust free environment overnight. The plate was then reactivated at 110^0C for 45 minutes in an Oven. Twenty to 100 mg of sample dissolved in appropriate solvent was carefully streaked 1 cm from the end of the plate, it was left to dry and then visualized using ultraviolet (UV) light source 254nm and 366nm CAMAG limited. The plate was developed, dried and then viewed under UV light for UV active compounds. These were marked on the plate. The bands with codes such as A, B, C and D starting from the top of the plate were scrapped off and kept separately. The samples were separated from the gel using filter paper, Buchner funnel and solvent.

Any pure compound which gave a weight of 5 mg and above was subjected to ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and Mass Spectra (MS) structure elucidation (Tables 8 and9). Fractions from PTLC with the highest activity were subjected to NMR analysis.

2.4.2 Brine Shrimp Safety Screening Bioassay

Since most bioactive plant constituents are toxic at higher doses, a possible approach to developing a useful general bioassay is to screen for plant extracts that are toxic to zoologic systems. For this purpose, the brine shrimp (*Artemiasalina*) lethality test was originally proposed [31]. It represents an easy way to detect general bioactivity in plant extracts and is again a handy procedure for tracking the isolation of bioactive constituents. A rectangular plastic double-chambered box with dividing wall and which had 2.3 mm holes (Encia-Italy) was used to hatch brine shrimp eggs from Lake Urmia-Iran. Artificial sea salt water made by dissolving 16g of sea salt in five hundred millilitre of distilled water was used to fill the chamber. Dry yeast (3mg) was added to serve as food for the larvae. The eggs were sprinkled carefully in the dark compartment while the other compartment was illuminated by natural light through a hole in the lid of the box. After 48 hours the larvae were collected by using a pipette from the illuminated side to which they moved on hatching due to their phototropism behaviour. They were separated from their shells by the divider wall [43]. Dimethylsulphoxide (DMSO) was used as a solvent to dissolve the plant extracts and the drug solution was then diluted with artificial sea salt water so that the DMSO content did not exceed 0.05%. Ten brine shrimps (Lake Urmia-Iran) were transferred to 1 ml of each plant sample vial containing 125, 250, 500, and 1000 µg/ml of plant extract using a Pasteur pipette. The experiment was set in duplicate for each concentration of the drug. The control tube had only sea water and DMSO. Brine shrimp survivors were enumerated after twenty four hours and the lethality fifties (LD_{50} values) were determined by taking average of five assays using a Finney Probit analysis program on an IBM computer [30]or theED_{50} values (µg/ml) calculated using Probit, a computer program [43].

2.5 Bioactivity guided isolation of compounds from various plant extracts

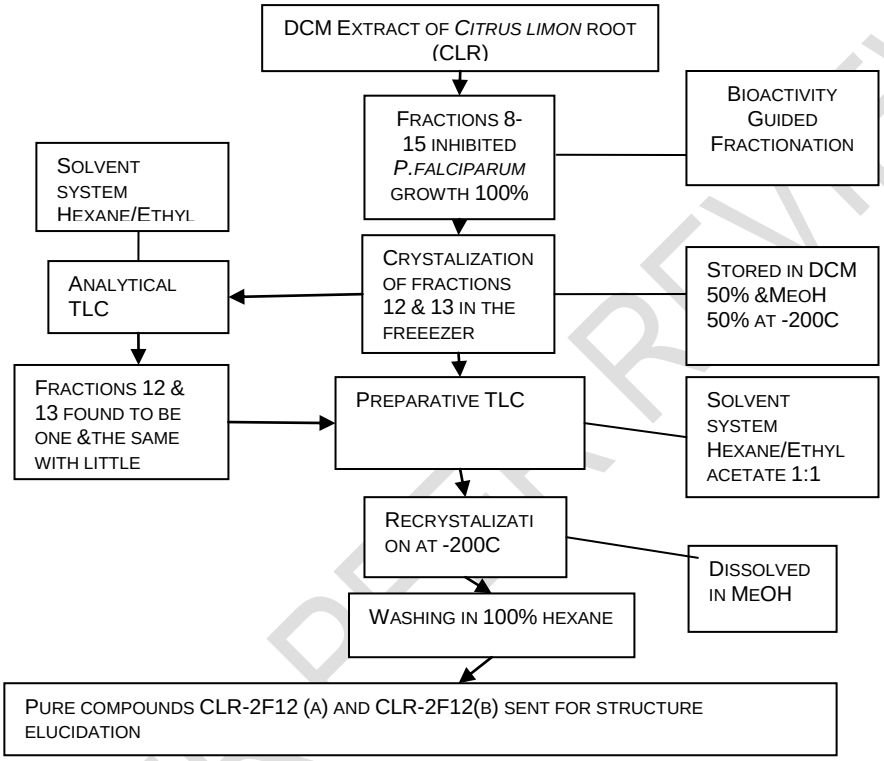
2.5.1 Isolation of compounds from DCM root extract of *C. Limon* (CLR 2)

Crude DCM root extract of *Citrus limon* (CLR-2) showed highest activities against both *P. falciparum* and brine shrimp and therefore was subjected to bioactivity-guided fractionation. The DCM extract (30 g) was packed in hexane, adsorbed on 25 g Silica gel using DCM and then extracted sequentially through a silica gel column with hexane, hexane/DCM mixtures, DCM, methanol, ethylacetate and finally acetic acid**.** Elution profile of plant *C. limon* root extract using hexane and DCM.The extract obtained from hexane/DCM mixtures yielded fractions 15 fractions from which fractions F8-15 were further purified as illustrated in scheme 3 below as up to 100 % inhibition of growth in *P. falciparum* was seen with these fractions. The column was eluted with increasing percentage of DCM in hexane and ethylacetate. Heavy

crystals that settled without centrifugation were obtained from fractions 12 and 13 as these samples were stored at -20 ^{0}C. The two fractions (12 & 13) after crystalizing out at -20 ^{0}C therefore apart from being most active against *Plasmodium* and Brine Shrimp became easy targets for isolation of compounds on crystallizing out. They gave single spots on analytical TLCs after further purification. The fractions 12 and 13 were further purified according to scheme 2.

2.5.2 Further purification of CLR-2 fractions 12 and 13 on preparative TLC

i. **Figure 1:** Further purification of CLR-2 compounds on preparative TLC



Fractions F12 and F13 were the most active against *P. falciparum* and therefore preparative TLC was performed on them. Solvent and development system containing50% Ethyl acetate in hexane gave the best separation of the two fractions on analytical TLC plates (Scheme 2). Sixty milligrams of fractions F12 and F13 were subjected to preparative TLC using hexane 1:1 ethylacetate as solvent system. Both F12 and F13 yielded two major compounds named CLR-2 F12 (a) and CLR-2 F12 (b) though with traces of contaminants. Both were recrystalised but now in methanol at -20 ^{0}C then washed several times with cold hexane in which the compound did not dissolve in except the contaminants. This resulted in two pure compounds as seen on analytical TLC plate. The structures of the compounds were determined using the following methods: ^{1}H, ^{13}C NMR and MS and the respective spectra were compared with what were available in the literature

2.5.3 Isolation of compounds from *Brideliacathartica*methanolic leaf extract (BCL-3)

The methanolic crude leaf extract of *Brideliacathartica*, which displayed good *in vitro*antiplasmodial activity (15 µg/ml), was adsorbed on silica gel in methanol and

eluted with increasing concentrations of DCM on a gel filtration column. The samples were pooled according to their analytical TLC profiles and then dried. Four major fractions were obtained which were screened for antiplasmodial activity. Individual fractions were subjected to antiplasmodial testing and the results are as shown in Table 7. BCL-3 F9 (IC_{50} 13.474 µg/ml) was the most active fraction (Table 7) and therefore was subjected to further purification which did not give a pure compound.

2.5.4 Isolation of compounds from *M. pyrifolia* hexane extract using adsorption chromatography

Microglossapyrifolia's 1,1241g of dried leaves were crushed into powder and then extracted four times with pure hexane. The extract (MPL-1) was tested for *in vitro*antiplasmodial activity to give a mean IC_{50} of 21.376 µg/ml. MPL-1 ranked 7th overall in terms of activity against *P. falciparum.* It was subjected to isolation of pure compounds as follows: the weight of the hexane extract was 23.0591g from which 20g was weighed out. This was dissolved in hexane, adsorbed onto 20g of silica and then dried. The sample was loaded on a gel filtration column (80 cm long, 5.5 cm diameter), packed with silica gel, eluted using hexane containing increasing amounts of DCM and 100ml fractions were collected (Table 7). Fractions 14 to 19 and 39 were oily. Clear white crystals were obtained as fraction 36 (solvent system: 10% MeOH in DCM) was being concentrated (BUCHI 110). Fraction 38 also gave white crystals which did not dissolve in methanol. Fraction 1 was the heaviest (2130.1mg) while fraction 40 was the lightest (10.4 mg) of them all. Fractions F28-F38 had similar analytical TLC profiles and therefore were pooled together and then coded MPL-1F37. This fraction had highest activity against falciparum. The tested combined column effluent coded MPL-1F37 was subjected to further isolation of compounds as shown in the next section.

2.5.5 Isolation of compound MPL-1F37 (a) (Spinasterol) from hexane leaf extract of*Microglossapyrifolia* by crystallization

MPL-1 F37 was then concentrated to 5ml and then kept at -20^{0}C overnight for selective crystallization. White crystals formed at the bottom of the glass tube which was spun at 4400 rpm for 5 minutes. The crystals that formed were found to dissolve in DCM and methanol at room temperature. The crystals were dried at 50^{0}C. This sample was coded MPL-1F37 (a). Analytical TLC profile of MPL-1F37 (a) developed with 2.5% MeOH in DCM showed that it was a pure compound which gave a single band. This compound was later subjected to antiplasmodial activity and NMR analysis and was proposed to be spinasterol (Figure 6).

2.6 Characterization of isolated compounds

This was executed on a HP Model 6890A gas chromatograph provided with a Model 5973 mass selective detector, a split capillary inlet system (split ratio = 1/30), a Model 6890 auto-sampler. The injection (2 µl) was made at a temperature of 250^{o}C. See Tables 2 and 3 for the compatibility models for compounds CLR 2F12 (a) and CLR 2F12 (b) as given by the MS machine**.**

Table 2: Compatibility model CLR 2F12 (a)

Name=	C:\gcms\1\data\FI2AS SUP CIR2.D
1=	PBM Apex minus start of peak
	[PBM Apex minus start of peak]
Time=	i Sep 12 13:05:44 2014

Header=	PK	RT	Area Pct	Library/ID	Ref	CAS	Qual
1=	1	9.4253	0.1429	2-Butenal, 3-methyl-	1388	000107-86-8	53
2=	2	20.9151	0.4953	36.12 Bisabolol<epi-alpha->	296	023178-88-3	93
3=	3	25.8648	1.0171	4-Nitro-1-naphthol	49193	000605-62-9	72
4=	4	26.3576	95.9367	2H-1-Benzopyran-2-one, 7-methoxy-6-(3-methyl-2-butenyl)-	87992	000581-31-7	94
5=	5	26.9175	0.2552	Benzoyl chloride, 4-hexyl-	74156	050606-95-6	59
6=	6	27.2086	2.1528	4,4'-Dimethoxy-2,2'-dimethylbiphenyl	86806	046873-19-2	58

Table 3: Compatibility model CLR 2F12 (b)

				count=1			
Name=				C:\gcms\1\data\1.D			
1=				PBM Apex minus start of peak			
				[PBM Apex minus start of peak]			
Time=				Fri Sep 12 13:08:15 2014			
Header=	PK	RT	Area Pct	Library/ID	Ref	CAS	Qual
1=	1	20.915	0.7125	36.17 Bisabolol<alpha->	302	023089-26-1	91
2=	2	23.849	0.1816	Cyclodecasiloxane, eicosamethyl-	190220	018772-36-6	91
3=	3	25.1705	98.6696	47.27 Xanthyletin	1406	000523-59-1	50
4=	4	28.1269	0.4362	Benzonitrile, m-phenethyl-	62228	034176-91-5	25

2.6.1 Determination of melting points

Melting points were measured on a Gallen Kamp® SANYO MPD 350 BM3.5 UK capillary melting point apparatus at the Chemistry Department Kenyatta University.

3. RESULTS AND DISCUSSION

3.1 Yield of plant extracts

The seven plants in this study were extracted with aqueous and organic solvents. Table 4 gives weights of ground material, weights of their respective organic and aqueous extracts and the percentage yields per plant. Aqueous extracts had the highest percentage yield in each plant. The highest aqueous extracts percentage yield was obtained from the leaf of *M. pyrifolia* (39.74 g) followed by *V. glabra* (25.66 g). The least aqueous extracts percentage yield was obtained from *C. edulis* root (8.175). Among the organic extracts the highest percentage yield was seen in the methanolic leaf extract of *V. glabra* (12.203 g) followed by another methanolic leaf extract of *M. pyrifolia* (10.43 g). Hexane extracts had the least percentage yields followed by DCM extracts (Table 4).

Table 4: Yield of the plants extracts

Plant material	Powder (g)	Solvent	Extract weight (g)	% yield
Achyranthes aspera leaves (AAL)	116	Hexane	0.3	0.259
		DCM	0.8	0.69
		Methanol	4.3	3.707
		Water	1.387	1.196

Bridelia cathartica leaves (BCL)	152.3	Hexane	4.6	3.020
		DCM	2.7	1.773
		Methanol	10.7	0.657
		Water	0.89	0.584
Hensia crinita Leaves (HCL)	92.2	Hexane	0.9	0.976
		DCM	0.7	0.759
		Methanol	2.7	2.928
		Water	1.119	1.214
Citrus limon roots (CLR)	31	Hexane	0.3	0.968
		DCM	0.9	2.903
		Methanol	1.0	3.225
		Water	0.764	2.465
Microglossapyrifolia leaves (MPL)	16.3	Hexane	23.059	2.089
		DCM	18.225	1.651
		Methanol	1.7	10.43
		Water	3.974	39.74
Vernoniaglabra leaves (VGL)	69	Hexane	0.6	0.870
		DCM	1.3	1.884
		Methanol	7.2	10.435
		Water	2.567	3.720
Carrisa edulis root (CER)	159.5	Hexane	1.1	0.690
		DCM	0.6	0.376
		Methanol	4.5	2.821
		Water	1.635	1.025

3.2 Results of the Brine shrimp lethality test

The results of the brine shrimp lethality test are displayed in Table 5. The DCM extract of *Achyranthes aspera* leaves was most active against brine shrimps with an LC_{50} of 0.460 µg/ml. *Bridelia cathartica*leaf DCM and methanolic extracts had LD_{50s} of 6.163 µg/mL and 6.197 µg/mL, respectively, against the brine shrimbs. Both *Citrus Limon* (CL) hexane (> 0.00) and DCM (> 0.00) root extracts were too active at the concentrations used against the brine shrimbs and the two killed all the Shrimps depicted by the values. *Citrus limon*methanolic root extract had LC_{50} of 2.195 µg/ml. *Microglossapyrifolia* (MP)hexane leaf extract had LC_{50} of 3.389 µg/ml while its DCM leaf extract had LD_{50} of 3.260 µg/ml. *Vernoniaglabra* (VG) hexane leaf extract had LC_{50} of 6.087 µg/ml, while its DCM leaf extract was more active with an LC_{50} of 2.449 µg/ml; its methanolic leaf extract was the most active against brine shrimp in this plant with an LD_{50} of 0.106 µg/ml second to *Citrus limon* root hexane and DCM extracts. *Achyranthes aspera* (AA) hexane and methanolic leaf extracts were not active against brine shrimp larva. *Bridelia cathartica* (BC) hexane leaf extract was inactive against the brine shrimbs. Methanolic extracts of MPL did not show any activity against brine shrimps (Table 5).

Table 5: LC_{50}s (µg/ml) of crude plant extracts against brine shrimps calculated at 95% confidence interval using probit

Drug	Hexane(1)	DCM (2)	Methanol (3)
*Achyranthesaspera*leaves (AAL)	> 1000	0.460	> 500
Bridelia cathartica leaves (BCL)	> 500	6.163	6.197
Citrus limon roots (CLR)	< 0.00	< 0.00	2.195
Microglossapyrifolia leaves (MPL)	3.389	3.260	> 500
Vernonia glabra leaves (VGL)	6.087	2.449	0.106

Three plants including *B. cathartica, C. limon* and *M. pyrifolia* extracted with DCM and methanol were further processed to obtain pure compounds.

3.3 *In vitro*antiplasmodial activity of the plant extracts

The *in-vitro*antiplasmodial activities of the extracts against V1/S, multidrug resistant strain of *P. falciparum*were as indicated in Table 9. Results indicate that the most active crude extract against *P. falciparum* was that of DCM root extract of *C. limon* with an IC_{50} of 7.017 µg/mL. The second crude extract in in terms of antiplasmodial activity was an aqueous extract of *C. edulis* roots with an IC_{50} of 8 µg/mL. The leaves of *B. cathartica* DCM extract was the third most active crude extract against *P. falciparum*with an IC_{50} of 11.537 µg/mL. The statistical differences between mean IC_{50} values were examined by the student's t-test (Table 6).

Table 6: *In vitro* antiplasmodial activity of plant extracts against V1/S Strain

Plant material	Solvent	IC_{50s} (µg/ml)
Achyranthes aspera leaves (AAL)	Hexane	18.087
	DCM	86.501
	Methanol	111.127
	Water	38.990
Bridelia cathartica leaves (BCL)	Hexane	32.908
	DCM	11.537
	Methanol	15.647
	Water	25.985
Hensiacrinita Leaves (HCL)	Hexane	34.223
	DCM	13.336
	Methanol	24.805
	Water	47.203
Citrus limon roots (CLR)	Hexane	30.092
	DCM	7.017
	Methanol	916.997
	Water	96.860
Microglossapyrifolia leaves (MPL)	Hexane	21.376
	DCM	34.88
	Methanol	313.647
	Water	203.457
Vernoniaglabra leaves (VGL)	Hexane	427.40
	DCM	53.62

Carrisa edulis root (CER)	Methanol	112.495
	Water	-
	Hexane	193.599
	DCM	30.074
	Methanol	69.969
	Water	8.054
Chloroquine (CQ)		49.915 ng/ml

Resistance to chloroquine is stated as an IC_{50} less than 100nM (approximately 0.052 µg/mL) [3]. However, K39 and V1 strains of *falciparum*fell far below this cut-off concentration in this study for reasons which could not be explained (IC_{50} of CQ was 0.040 µg/mL for W2 & 0.011 µg/mL for D6, IC50 of Mefloquine was 0.012 µg/mL for W2 and 0.040 µg/mL for D6 and IC50 of Quinine was 0.103 µg/mL for W2 & 0.031 µg/mL for D6). Most researchers consider IC_{50} values above 100 µg/mL to be inactive and that values ranging between20-100 µg/mLas moderate activity[3].Researchers have grouped plants with the following activities against malaria parasites as follows;GroupA (greater than 1µg/mL), B (1 to 5µg/mL) and C (6 to 10µg/mL) [3].

Most of the plant extracts in this study except DCM root extract of *C. limon* with an IC_{50} of 7.017 µg/mL, aqueous extract of *C. edulis* with an IC_{50} of 8.054 µg/mL and DCM and methanolic extract of *B. cathartica* with an IC_{50} of 11.537 µg/mL and 15.647 µg/mL, respectively, DCM extract of *H. crinita* with an IC_{50} of 13.336 µg/mL and hexane extract of *A. aspera* with an IC_{50} of 18.087 µg/mL are considered to be within the mild or moderate activity range. Values less than 20 µg/mL are considered to be in the high activity range for crude plants extracts.

Citrus aurantiifolia is frequently used against malaria in Brazil [33] and also *M. pyrifolia*, also studied here, has been used in Ghana against malaria. The present study also established the presence of some very popular herbal antimalarial plant species in Nyanza and the Coastal region that may not be very popular in other regions. However, though *B. carthatica* has been used in Zimbabwe against malaria, its crude extracts did not exhibit significant antiplasmodial activity in this study probably because of geographical varieties. The parts utilized by the traditional healers may also not necessarily contain the most active compounds for the choice may depend on the convenience of preparation. Most of the antimalarial concoctions are obtained from roots, leaves and at times the entire plant [35].

None of the crude extracts fell in the 1st or 2nd groups. The DCM extract of *C. limon*roots with an IC_{50} of 7.017 µg/mL, and an aqueous extract of *C. edulis* roots with an IC_{50} of 8.054 µg/mL were the only crude extracts that fell within group C. The rest had lower activities with IC_{50s} greater than 11 µg/mL; for example, DCM extract of BCL had an IC_{50} of 11.537 µg/mL, DCM extract of HCL had an IC_{50} of 13.336 µg/mL, methanolic extract of BCL had an IC_{50} of 15.647 µg/mL and hexane extract of AAL had an IC_{50} of 18.087 µg/mL. The remaining extracts had antimalarial activities above 20 µg/mL and up to 916.997 µg/mL.Contrary to work by researchers [21] who demonstrated that unprocessed aqueous and ethanolic extracts of the root and the

ethanolic stem extract of *B. cathartica* resulted in a 50% growth inhibition of *P. falciparum* when kept at 0.05 µg/mL, the present study showed that the extracts from this plant were generally active. The difference could have been due to the different localities and therefore different soil textures and climatic conditions. Out of the 28 crude extracts tested, only five had IC_{50}s greater than 100 µg/mL and thus 82% were active. This can reflect some accuracy in the part played by the herbalist and the authenticating authority at the University of Nairobi.

The following ranges of IC_{50}s were observed per plant regardless of the chemical used for extraction: AAL with an IC_{50} of 18.087-111.127 µg/mL, HCL with an IC_{50} of 13.336-47.203 µg/mL, CLR with an IC_{50} of 7.017-916.997 µg/mL, MPL with an IC_{50} of 21.376-313.647 µg/mL, VGL with an IC_{50} of 53.62-427.40 µg/mL, CER with an IC_{50} of 8.054-193.599 µg/mL and BCL with an IC_{50} of 11.537-32.908 µg/mL. The same plants that showed high activities with IC_{50s} of 7.017-11.537 µg/mL in category C against *P. falciparum*, had also significant bioactivity against brine shrimp, *Artemiasalina*. It therefore shows that these plant extracts were generally active. Some researchers [2] found a low antimalarial activity of an aqueous extract of *C. sinensis.* Various workers have claimed *C. limon* to have the following attributes: antiperiodic, astringent, antibacterial, antiscorbutic, carminative, refrigerant, stimulant, miscellany, rubifacient and stomachic. Lemons being the source of the most active crude extract with an IC_{50} of 7.017 µg/mL is an extremely good prophylactic medicine for most ailments and has many uses at home.Vitamin C in which the fruit is rich in aids the body in the fight against infections and again protects or treats scurvy infections[8,16]; it has also been employed as a replacement for quinine against malaria and other fevers [16].

3.4 Results for isolation of compounds

3.4.1 Compounds isolated from active crude extracts

The most active fractions were crude DCM root extract of *C. limon* (CLR-2), crude methanolic leaf extract of *Bridelia cathartica* (BCL-3) and the mildly active crude hexane leaf extract *M. pyrifolia* (MPL-1) were subjected to fractionation on column chromatography using solvents of increasing polarity (hexane, hexane/DCM mixtures, DCM, DCM/methanol mixtures and methanol) on the plant extracts.

3.4.2 Isolation of compounds from the DCM root of *C.limon* (CLR 2)

Sixteen fractions were obtained and fractions 12 and 13 were active against *P. falciparum.* These fractions 12 & 13 were each subjected to preparative TLC using 50:50 hexane: ethylacetate developer.

The two fractions gave single spots on analytical TLCs after further purification by preparative TLC and recrystalization in methanol. This yielded two pure coumarin compounds; (Figures 3 and 5).

3.4.3 Compounds isolated from *Brideliacathartica*methanolic leaf extract (BCL-3)

The NMR spectra for compoundsfrom this plant was not clear for structural elucidation. Fraction BCL-3F9 was the most active against falciparum (13.5µg/ml) (Table 7).

Table 7: Antimalarial test results for fractions of *Bridelia cathartica* leaf methanolic extract

Fraction	IC_{50}µg/ml
BCL-3F7	29.1
BCL-3F8	19.4
BCL-3F9	13.5
BCL-3F11	29.9

3.4.4 Compounds isolated from *M. pyrifolia* hexane leaf extract: Adsorption chromatography

One hundred ml fractions were collected (Table 8) and a total of 40 fractions of this capacity were obtained. Fractions 14 to 19 and 39 were oily.

Clear white crystals were obtained as fraction 36 (solvent system: 10% MeOH in DCM) was being concentrated (Table 8).

Table 8: Weights of fractions of *Microglossa pyrifolia* hexane leaf extract (MPL-1)

Fraction	Weight(mg)	Fraction	Weight(mg)
MPL-1F1	2130.1	MPL-1F21	288.2
MPL-1F2	1028.4	MPL-1F22	443.7
MPL-1F3	997.1	MPL-1F23	372.2
MPL-1F4	702.9	MPL-1F24	333.3
MPL-1F5	524.4	MPL-1F25	304.1
MPL-1F6	97.3	MPL-1F26	109.4
MPL-IF7	71.4	MPL-1F27	412.5
MPL-1F8	88.0	MPL-1F28	348.5
MPL-1F9	67.6	MPL-1F29	349.1
MPL-1F10	44.5	MPL-1F30	308.2
MPL-1F11	154.2	MPL-1F31	193.3
MPL-1F12	120.1	MPL-1F32	178.3
MPL-1F13	131.0	MPL-1F33	152.1
MPL-1F14	247.6	MPL-1F34	152.3
MPL-1F15	957.1	MPL-1F35	151.8
MPL-1F16	280.9	MPL-1F36	152.2
MPL-1F17	155.0	MPL-1F37	230.0
MPL-1F18	161.7	MPL-1F38	120.0
MPL-1F19	415.3	MPL-1F39	50.1
MPL-1F20		MPL-1F40	10.4

Fraction1 was the heaviest (2130.1mg) while fraction 40 was the lightest (10.4 mg) of them all. Fractions F28- F38 had similar analytical TLC profiles and therefore were pooled together and then coded

MPL-1F37. The combined column effluent coded MPL-1F37 was subjected to further isolation of compounds. Spinasterol was obtained (Table 8 and Figure 6).

3.5 Structures of isolated pure compounds

The pure compounds that were isolated from the plants were the following; (1) CLR-2 F12 (a), (2) CLR-2 F12 (b) from DCM root extract of *C. Limon* and (3) MPL -1F37 (a) from hexane leaf extract of *M. pyrifolia*(Figures 3, 5 and 6). The first twocompoundswere very closely related as they were moving together as one and the same on analytical TLC with most solvent developers except when developed with hexane-ethylacetate 1:1 mixture which separated them as two distinct compounds. The structures of the compounds were arrived at after comparing their NMR, IR data with data available in literature and confirmed by MS analysis which gave their molecular weights (Figures 3 and 5) while structural elucidation for Figure 6 was proposed by NMR analysis only.

3.5.1 Suberosin

NMR, IR and MS Results for Compound CLR 2F12

The structure proposal of HSCCC peak fractions was carried out by ^{1}H-NMR and 13C-NMR (University of Nairobi, Department of Chemistry) and IR (Jomo Kenyatta University of Agriculture & Technology, Department of Chemistry). NMR spectra were run on RKCM.07.27.06 360 (^{1}H: 360 MHz; ^{13}C: 212 MHz) spectrometer in $CDCl_3$ using TMS as internal standard or by reference to the solvent signal (CHC_3 at δ_H 7.25. EIMS were obtained at 70 eVona Shimadzu QP-2000 spectrometer. Its IR spectrum exhibited absorptions typical for 7-oxygenated coumarins. The ^{1}H NMR spectrum showed a pair of doublets at d 7.57 and 6.20 (J ¼ 9.5 Hz), characteristic of H-4 and H-3 in a coumarin nucleus. The pair of doublets at d 5.70 and 6.86 (J ¼ 10 Hz), beside the singlet at d 1.45 (6 H, s) are typical for the dimethylchromene ring. From the IR and UV it was deduced that 2 is a 7-oxygenated coumarin. The 1 H NMR spectrum showed two doublets at d 6.20 and 7.59 (J ¼ 9.5 Hz), and two singlets at d 6.75 (H-8) and 7.15 (H-5) corresponding to 6,7-disubstituted coumarinthe presence of a doublet at d 3.28 (2 H, d, J ¼ 7.5, H-9) coupled with a multiplet at d 5.26 (1 H, m, H-10) and two methyl signals at d 1.68 and 1.74 indicated a prenyl function. The singlet at d 3.87 was attributed to the methoxyl group. The MS showed [Mþ] at m/z 244, a base peak at 229 and a fragmentation pattern similar to that of suberosin. By comparison of the obtained data with those reported for suberosin, compound 2 was identified as suberosin. The ^{13}C NMRspectral data showed 20 carbon signals confirming structure 2. Their assignments were addressed herein for the first time on the basis of several NMR experiments (DEPT, COSY and HETCOR). Compound 3 was identified as xanthyletin by comparison with an authentic sample (.m.p., co-TLC and IR) and with literature data (m.p., IR, UV, MS, ^{1} H and ^{13}C NMR). The above data therefore prompted proposal of compound CLR 2F12 (a) to be suberosin. Compound CLR 2F12 (b) and xanthyletin had similar infrared spectra and therefore they were identical. For ^{1}H NMR, ^{13}C NMR (Table 8). The data in Table 8

prompted proposal of compound CLR 2F12 (a) to be suberosin as they compared well with literature information.

Physical and spectral data of compound CLR 2 F12

Compound CLR2F12 (a) was isolated as the major compound of DCM root extract of *C. limon* with dazling bluish- violet-whitish appearance under UV light. The compound appeared as colourless crystals with a melting point of 119 ^{0}C and Rf equivalent to 0.8 (50 % hexane in ethyl acetate). ^{1}H NMR δH7.61 (d, j= 9. 5Hz, 1H, H-4), 7. 58 (d, 1H, H-5), 6. 25 (s, 1H, H-8), 6. 16 (d, j=9. 5Hz, 1H, H-3,), 6. 76 (t, 1H, H-2'), 3.88 (s, 3H, OCH3), 3. 31 (m. 2H, H- 1'), 1.76 (s, 3H, H-3 '-CH3), 1. 69(s, 3H, H-3 'CH3). ^{13}C NMR (100MHz; CDCl3; ppm) δc 161.39 (C-2), 160. 57 (C- 9), 154. 40 (C- 7), 143. 50 (C-4), 133. 53 (C-3'), 127. 40 (C- 10), 127. 32 (C-5), 121. 28 (C-2'), 112. 68 (C-3), 111. 82 (C- 6), 98. 42 (C-8), 55. 76 (C-7 OCH3), 27. 69 C-1'), 25. 70 (C- 3' –CH3), 17.65 (C-3'- CH3) (Table 9).

Table 9:13C (75 MHz) 1H (360 MHz) data of Suberosin ($CDCL_3$, CD3OD, δ in ppm) in Hz

Atom Number	Carbon -13	1H
1	-	-
1'	27.69	3.31 (m,2H)
2	161.39	-
2'	121.28	6.76.28 (t, 1H)
3	112.68	6.16 (d, 9.5Hz, 1H)
3'	133.53	-
4	143.50	7.61 (d, 9.5Hz, 1H)
5	127.32	7.58 (s, 1H)
6	111.82	-
7	154.40	-
8	98.42	6.25 (s, 1H)
9	160.57	-
10	127.40	-
C3'- CH3	17.65	1.76 (s, 3H)
C3' – CH3	25.70	1.69 (s, 3H)
C-7 – OCH3	55.76	3.88 (s, 3H)

IR spectra were recorded in KBr on a Shimadzu FTIR-8201PC IR spectrometer. The IR spectra of compound CLR 2F12 (a) corresponded to the IR spectra of suberosin, the IR spectrum of which had a frequency at KBr disk) v = 1693 (c = 0) cm^{-1}. These were consistent with what is reported in literature on suberosin.

MS retention time of compound CLR 2F12 (a) (Suberosin)

A single peak was obtained confirming the purity of the compound (Figure 2).

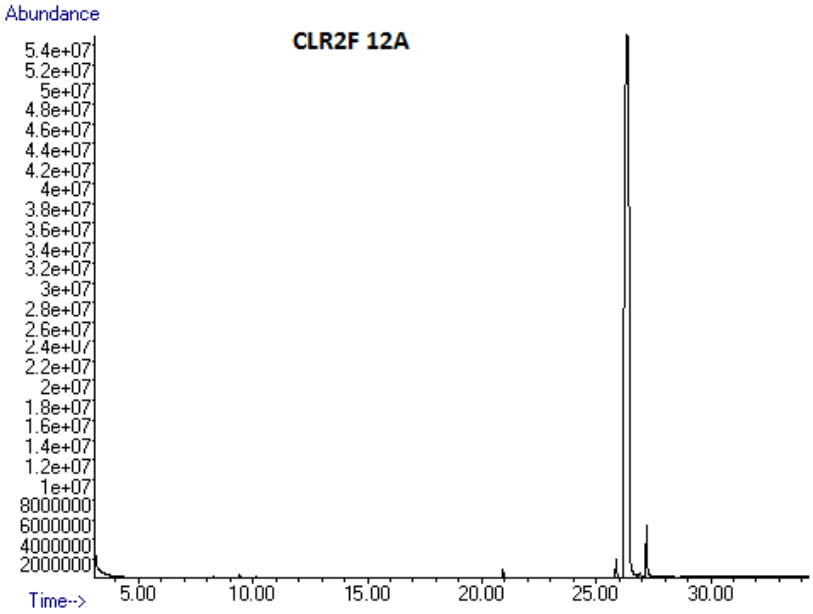


Figure 2: MS retention time of compound CLR- 2F12 (a) (Suberosin)

MS Spectra and structure of compound CLR 2F12

The above data were in agreement with those for suberosin (Figure 3). The molecular formula of compound CLR 2 F12 (a) was $C_{15}H_{16}O_3$ and therefore its molecular weight was 245. The NMR and IR data, the structure and the molecular weight (245.1) as given by the MS suited that of a coumarin known by the name suberosin. The purity of suberosin was estimated at 98 % with Gas Chromatography Analysis instrument.

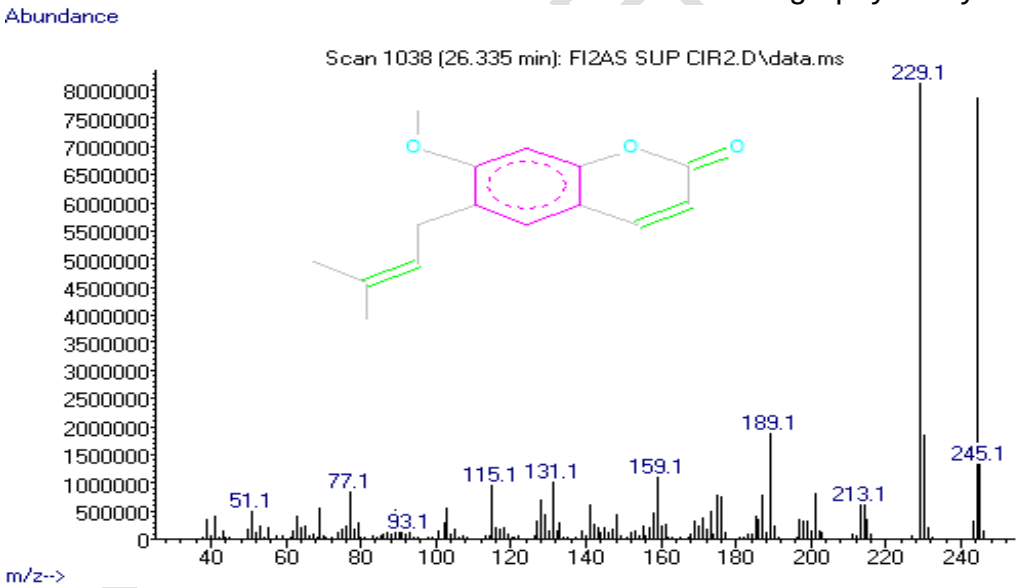


Figure 3: MS spectra, structure and molecular weight of compound CLR 2F12 (a) (Suberosin)

3.5.2 Xanthyletin

NMR, IR and MS results for compound CLR 2F12

Structural analysis was rooted on NMR data. The ^{1}H NMR spectrum demonstrated two doublet signals at δH 7.57 (1H; J = 9.5 Hz) and 6.20 (1H; J = 9.5 Hz). These signals were due to the presence of hydrogen atoms of conjugated double bond with carbonyl group. Two doublet signals at δH 6.33 (1H; J = 9.9 Hz) and 5.68 (1H; J = 9.9 Hz) were also due to alkenyl hydrogen

atoms on vicinal carbon atoms. The singlet signals at δH 7.02 (1H) and 6.70 were assigned to aromatic hydrogen atoms distant from others. The singlet signal at δH 1.45 (6H) was assigned to hydrogen atoms of two methyl groups. ^{13}C NMR spectrum showed signals at δ C 156.76, 155.38, 118.41, 112.95, and 77.63 which equated to non-hydrogenated carbon atoms. The signals at δ C 143.19, 131.12, 124.65, 120.69, 113.41, and 104.31 equated to single-hydrogenated carbon atoms. The signal at δ C 28.25 was assigned to two carbon atoms of the methyl groups. The 1D NMR spectra are typical of pyranocoumarin construction. The two hydrogen signal at 7.02 (H-5) was linked to the carbon signals at δ C 156.76 (C-7), 155.38 (C-9), 143.19 (C-4), and 120.69 (C-6). The hydrogen signal at δH 6.70 (H-8) was linked to the carbon signals at δC 156.76 (C-7), 155.38 (C-9), and 118.41 (C-6). The hydrogen signal at δ H 6.34 (H-4') was linked to carbon signals at δ C 156.76 (C-7), 124.66 (C-5), and 77.63 (C-2'). The hydrogen signal at δH 6.20 (H-3) was linked to the carbon signals at δC 161.2 (C-2) and 112.95 (C-10). The hydrogen signal at δH 5.68 (H-3´) was linked to the carbon signals at δ C 118.41 (C-6), 77.63 (C-2'), and 28.25 (C-1"/2"). The hydrogen signal at δH 1.45 (H-1"/2") was linked to the carbon signals at δC 131.12 (C-3') and 77.63 (C-2'). These suited xanthyletin [25], a coumarin hitherto separated from *Brosimumgaudichaudii*[39]. The NMR spectrum of xanthyletin in CdCl3 at 360 MHz. (CLR F12 (b) showed clearly the presence of two methyl groups at = 1,45 pmm(singlet), two signals corresponding to two protons which can be attributed to a double bond conjugated to a carbonyl group.Two further doublets (j = 10 Hz) as well as two singlets of one proton each at 6,70 and 7,02 pmm suggested the presence of a dimethyl chromene unit on an aromatic ring possessing two protons in paraposition. The above data strongly favoured as structure coumarin with an annelated dimethyl chromene ring. NMR spectra were run on RKCM.07.26.06 360(1H: 360 MHz; 13C: 212 MHz) spectrometer in CDCl3 employing TMS as internal standard or by remission to the solvent signal (CHC3 at δH 7.25 (Table 9). IR spectra were secured using KBr disks on a Shimadzu FTIR 8000, [default] FTIR 8400 Japan. The IR spectrum of 2 displayed peaks for an α, β-unsaturated carbonyl group that was reaffirmed and by comparing its physical properties with spectroscopic data (IR, 1H NMR), the substance xanthyletin is reported here. .

Physical and spectral data of compound CLR 2 F12

Compound CLR2F12 (b) was isolated as the major compound of DCM root extract of *C. limon* with dazzling bluish- violet-whitish appearance under UV light. The compound was isolated as yellow white crystals, melting point: 122-124.^{0}C, Rf = 0.6 (hexane-ethyl acetate (1:1),230Rf = 0.51 (hexane-ethyl acetate (2:1)). It was soluble in ethyl acetate, chloroform, ethanol, and methanol but not in water. ^{1}H NMR δH (7.57, d, J=9.5Hz, 1H), 7.02 (s, 1H, H-5), 6. 72 (s, 1H, H-8), 6. 70 (d, 9.9Hz, 2H-4'), 6. 33 (d,J= 9. 5Hz, 1H, H-3) 5. 68 (d, J= 9. 9Hz, 1H, H-3'), 1.45 (s, 6H, H-1'). ^{13}C NMR (100MHz; CDCl3; ppm), 156.76 (C-7), 155.38 2(C-9), 143. 19 (C-4), 131. 12 (C- 3'), 124. 66 (C-5), 120. 69 (C-4'), 118. 41 (C-6), 112.95 (C-3), 112. 64 (C-10), 104. 31 (C-8), 77. 28 (C-2), 28.25(C-3' -CH3). Molecular Formula is$C_{14}H_{12}O_3$and itschemical name is8, 8 - dimethyl pyro (3, 2 - g) chromen-2-one.

Table 10: 1H (360 MHz) and 13C (360 MHz) data of Xanthyletin ($CDCl_2$, δ in ppm) in Hz. The structure of xanthyletin was ascertained by comparison of its physical data (mp, ^{1}H- and ^{13}C- NMR) (Table 10) with reported values.

Atom Number	Carbon -13	1H
1	-	-
2	77.28	--
2’	77.63	-
3	112.95	6.33 (d, 9.5Hz, 1H)
3’	131.12	5.68 (d, 9.9Hz, 1H)
4	143.19	7.57 (d, 9.5Hz, 1H)
4’	120.69	6.70 (d, 9.9Hz, 1H)
5	124.66	7.02(s,1H)
6	118.41	-
7	156.76	-
8	104.31	6.72 (s, 1H)
9	155.38	-
10	112.64	-
CH3/CH3	28.25	1.45 (s, 6H)

MS retention time of compound CLR 2F12 (b) (Xanthyletin)

Only one peak was obtained showing that the compound was pure (Figure 4).

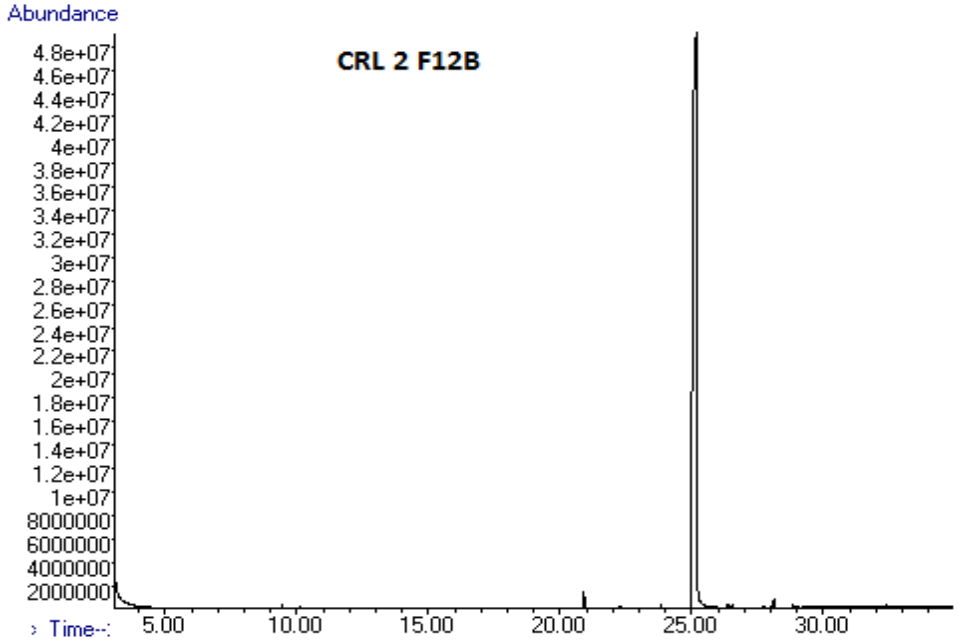
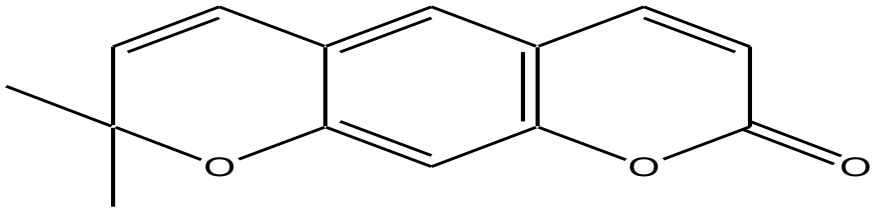


Figure 4: MS retention time of compound CLR 2F12 (b)

Chemical structure of compound CLR 2F12

The NMR and IR data, the structure and the molecular weight (228.1) suited that of a coumarin derivative known by the name xanthyletin. The molecular formula was found to be $C_{14}H_{12}O_3$ and therefore formula weight was 228.1 (Figure 5).



ii.

Figure 5: Chemical structure of compound CLR 2F12 (b) (xanthyletin)

3.5.3 Spinasterol

NMR data for compound MPL-1F37

The ^{1}H-NMR spectrum of compound MPL-1F37 (a) specified vibrational harmony for free olefinic proton at α 5.16 (dd, J=8, 8,) 15.2 Hz), δ 5.15 (br s), and δ 5.02 (dd, J=8.4, 15.2 Hz); a carbinyl proton at δ 3.59; and six methyl protons at δ 1.03 (d, J=6.8 Hz), 0.85 (d, J=6.4 Hz), 0.84 (d, J=6.0 Hz), 0.81 (t, J=7.2 Hz), 0.80 (s), and 0.55 (s). The J-mod 13C-NMR spectral data of MPL-1F37 (a) 9 indicated same vibrational quality for twenty-nine carbons with the following functionalities: four olefinic carbons, seven methane carbons, nine methylene carbons, a carbonyl carbon, two quaternary carbons, and six methyl carbons. These are characteristic resonances of a sterol with an alcohol and two olefinic bonds. NMR Spinasterol: Semisolid, Identity confirmed by ^{1}H NMR, ^{13}C NMR and co-TLC. Spinasterol eluates when freed of the solvent provided 3, identified by co-TLC, 1H NMR (Figure 6).

Physical and spectral data of compound MPL-1F37

Compound MPL-1F37(a) had the following physical properties; white needle-like crystals. This pure compound was found to be a phytosteroid. ^{1}H NMR (CDCI3, (ppm), (400 MHz) δ 5.22 (1H, d, j = 7. 2 Hz), δ 3.52 (m), δ 1.03 (3H, s), δ 0.94 (3H, d, j =8. 4 Hz), δ 0.86 (9H, m), δ 0.70 (3H, s); ^{13}C NMR (CDCI3, (ppm), 100 MHz). 7δ 11.8 (C-29), 12.0 (C-18), 18.7 (C-26), 19.0 (C-19), 19.4 (C-21), 19.7 (C-27), 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.2 (C-16), 29.2 (C-25), 31.7 (C-7), 31.9 (C-2), 31.9 (C- 22), 34.0 (C-8), 36.1 (C-10), 36.5 (C-20), 37.3 (C-1), 39.8 (C-12), 42.3 (C-4), 42.3 (C-13), 45.8 (C-24), 50.1 (C-9), 56.1 (C-17), 56.8 (C-14), 71.8 (C-3), 121.7 (C-6), 140.8 (C-5) (Figure 6).

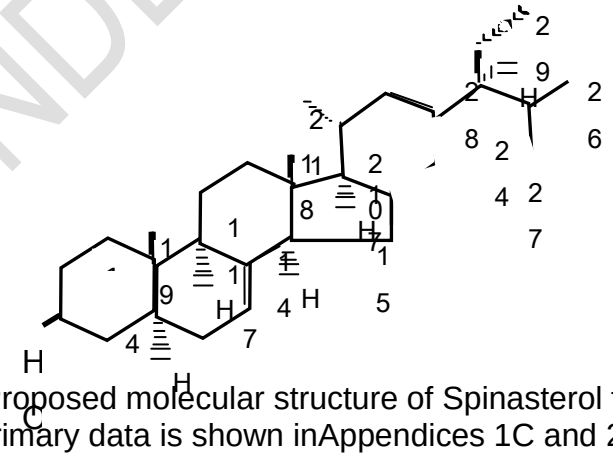


Figure 6: Proposed molecular structure of Spinasterol from NMR data (Detailed primary data is shown inAppendices 1C and 2I-L).

3.6 Antiplamodial activity of the isolated compounds

Table 11 below summarises the IC_{50}s for the activity of the isolated compounds against falciparum strains. The standard drugs CQ, Mefloquine and Quinine were all more active than isolated compounds. Although DCM extract of *V. glabra* leaves was found to be more active with an LC_{50} of 2.499 µg/ml and its MLC was the most active against brime shrimp with LD_{50} of 0.106 µg/ml, the isolated compounds from the extract were all less than 5mg and therefore could not be subjected to further analysis. Hexane extract of M. pyrifolia leaves was analyzed with inconclusive antiplasmodial results due to sample contamination.

Table 11: Pharmacological and chemical data of pure compounds and reference drugs

Compound	F Formula	IC_{50}µg/mL against falciparum strain	
		W2	D6
1.CLR-2F12(a) Suberosin	$C_{15}H_{16}O_3$	26.7	53.1
2.CLR-2F12(b) Xanthyletin	$C_{14}H_{12}O_3$	1580.0	ND
3.MPL-1F37(a) Spinasterol		ND	43.2
4. STDS (a) CQ	$C_6H_{13}C_{l2}NO$	0.040	0.011
b) Mefloquine	$C_{17}H_{16}F_6N_2O$	0.012	0.040
© Quinine	$C_{20}H_{24}N_2O_2$	0.103	0.031

Key: CQ-Chloroquine, ND-Not done, W2-multidrug resistant strain of *P. falciparum*; D6 was CQ sensitive strain of *P.falciparum*

Discussion: This study has demonstrated two coumarins namely : suberosin with an IC50 of 26.7 µg/mL for W2 and 53.1 µg/mL for D6 strains as the most active compound and xanthyletin with an IC50 of 1580 µg/mL for W2 strain both from *C. limon* DCM root extract and spinasterol with an IC50 of 43.2 µg/mL for D6 from *M. pyrifolia* leaves with amoderate activity. Work done previously [34]) showed that lemon flavonoids or eriocitrin and heparidin taken in the diet are effective antioxidant *in-vivo*.Suberosin, a simple coumarin isolated from *C. limon* was the most active antiplasmodial(26.7 µg/ml α W2 and 53.1 µg/ml α D6 strains) whereas xanthyletin (1580 µg/ml α W2 strain) a pyranocoumarin also from the same plant had no activity.Spinasterol from *M. pyrifolia* leaves with a moderate activity (43.200 µg/ml α D6). A researcher [22] while working on *M. Pyrifolia* isolated sinapyldiangelate and acetyl-6E-geranylgeraniol-19-oic acid as new compounds. The most active components in their test system were two diterpenes acetyl-6E geranylgeraniol-19-oic [IC_{50}, 12.9 µmol/L (PoW), 15.6 µmol/L (Dd2)] and E-phytol [IC_{50} 8.5 µmol/L (PoW), 11.5 µmol/L (Dd2)]. Other compounds that have been isolated from *C. Limon* are bergapten, bergamottin, yakangelicin,citropten, imperatorin, isoimperatorin, isopimpinellin, phellopterin, prangol, scoparon, scopoletin, umbelliferone, umbelliprenin and xanthyletin[19]. Suberosin is structurally related to drugs such as propranolol, osthol, quinine, chloroquine and primaquine, and it inhibits anti-inflammatory activity and prevents growth of human peripheral blood mononuclear cells by means of modulating the transcription factors NF-Kb and NF-AT [6]. It is forms yellowish crystals with melting point of 88-89^{o}C [36] as established in this present study. It has earlier been

also isolated from the root bark of *C. nobilis* var. Sunki[44], the roots of *Citrus sinensis* (Rutaceae)[4], *Citropsis articulate* [24], *Citrusgrandis*[7] and from the bark of *Xanthoxylumsuberosum*[17]. Inhibition of aggregation and ATP release of rabbit platelets induced by arachidonic acid collagen, ADP, platelet activating factor (PAF) or U46619 (athromboxane A analog) was characteristic of all the coumarins except xanthyletin [7].

Xanthyletin is structurally related to a drug named spectinomycin, and was also found to inhibit (100%) fungal growth and was isolated from the DCM extract of a plant known as *Pilocarpusriedelianus,* two shrubs found in North America, India and Bhutan; *Zanthoxylumalatum* and *Zanthoxylumamericanum, Stauranthusperforatus* root, *S. perforatus* roots [32] and also from the bark of *Xanthoxylumamericanum*, the roots of *X. ailanthoides*, the fruit of *Luvungascandens*, the wood of *Chloroxylonswietenia*, and *Citrus aurentifolia* (all Rutaceae). Also, it is found in the wood of Brosimum spp. (Moraceae). A closely related compound; xanthoxyletin with 100% inhibition on fungal growth has also been isolated from a methanolic extract of *C. limon*. It has antitumour and antibacterial activities and an efficient inhibitor of *Phytophthoracitroohthorain vitro* and also its synergistic effect was observed with other phenolics of Citrus [1]. As per work done before [38], existence of coumarins in the rootsmay protect the plant against microbial intrusion. Seselin, suberosin and xanthoxyletin have been characterized by 1HNMR, 13CNMR, IR and UV spectra as standard methods [11].

Spinasterol, a phytosteroid, isolated from *Microglossapyrifolia* leaves for the first time in this study, was the only active principle from this plant (MPL-1F37 (a) had an IC50 of 43.169 µg/ml). It was found found to structurally resemble adrenocortical antagonists; hydrocortisone, prenisolone, betamethasone, triamcinolone, 7-dehydrocholesterol and gonadal hormones. It has also been isolated from the stems of the flowers of *Cucurbita maxima* Duch [10]. *Microglassapyrifolia* was documented for use as antiplasmodial remedy by Cameroon's traditionalmedicine users. Studies elsewhere [41] on *Microglossapyrifolia* and described new dihydrobenzofurans and triterpenoids from roots but not spinasterol. Work by [20] indicated that the content of *M. pyrifolia* leaf oil was predominantly gemacrene D (17.4%), careen (15.3%) or (E)-B-ocimene (13.4%), α-humulene (27.1-36.4%) and α-piriene (18.7%) as opposed to the findings in this study.

Spinasterylglucocide and spinasterol have been separated as the main sterols from cell suspension cultures of *Saponaria officinalis* and determined by 1HNMR, ^{13}C-NMR, MS spectral data. Phytosterol are soluble in most organic solvents and contain alcohol functional group but are insoluble in water.

ABBREVIATIONS AND ACRONYMS

AAL	*Achyranthes aspera* leaves
ABO	blood groups
ACT	artemisinin base combination therapy
ACTs	artemisinin-based combination therapies
ANOVA	analysis of variance
BCL	*Bridelia cathartica* leaves
BCL-1,2,3& 4	*Bridelia cathartica* leaf hexane, DCM, methanolic &aqueous extracts
13C	carbon 13
CAMAG	the world leader in instrumental Thin-Layer Chromatography
CDCl2	dichloromethane
CER	*Carissa edulis* roots
CHCl3/MeOH	chloroform/methanol solvent system
CLR	*Citrus lemon* root extract
CLR-1,CLR-2,CLR-3,CLR-4	hexane, DCM, methanolic & aqueous extracts
CMS	complete medium with serum
CO2	carbon dioxide
COSSY	a useful method for determining which signals arise from neighbouring protons
CPD	citrate phosphate dextrose
CQ	chloroquine
D6 CQ	sensitive strain of *P.faciparum*
DCM	dichloromethane
DMSO	dimethylsulphoxide
DTPE	Department of Training Plans and Evaluation
ED50	effective dose for 50 percent of the group tested
EDTA	ethylene diamine tetraacetic acid
EI	electron impulse
FY	fiscal year
F1, F2-F100	fractions 1,2-100
FTIR	Fourier transform infrared spectroscopy
g	gram
GHz	gigahertz
GoK	Government of Kenya
GR	growth rate
GRAFIT	data analysis and scientific graphing package for Windows
[3H]	labeled proton
1H	proton
HCL	*Hensia crinita* leaves
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETCOR	heteronulear correlation
HRP2	histidine-rich protein 2
Hz	hertz (cycles per second)
IC50	half maximal inhibitory concentration
IR	infra red
KALRO	Kenya Agricultural and Livestock Research Organisation
Kbr	potassium bromide
kHz	kilohertz
LD50	median lethal concentration
LH-20	a type of sephadex for gel filtration chromatography
MeOH	methanol
MHz	megahertz
MPL 1F37	fraction 37 of hexane extract from a column
MPL	*Microglossa pyrifolia* leaves
MS	mass spectrometry
NHS	normal human serum
NMR	nuclear magnetic resonance
60 PF254	silica gel powder to be visualized under UV254
RBC,WBC	red and white blood cells
RPMI 1640	medium for culturing plasmodium

TLC	thin layer chromatography
UV	ultra violet
VGL	*Vernonia glabra* leaves
VI/S	an international multidrug resistant strain of *P.falciparum* originally from a patient in Vietnam
W2	chloroquine-resistant strain of *P.falciparum*
WHO	world health organisation

4. CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

The conclusions associated with this study include:

(i) Hexane extracts of *Citrus limon* roots (CLR), *Microglossapyrifolia* leaves (MPL), and *Vernoniaglabra* leaves (VGL), dichloromethane (DCM) extracts of *Citrus limon* roots (CLR), *Achyranthesaspera* leaves (AAL), *Brideliacathartica* leaves (BCL), *Microglossapyrifolia* leaves (PML), and *Vernoniaglabra* leaves (VGL), and methanolic extracts of *Brideliacathartica* leaves (BCL), *Citrus limon* roots (CLR), and *Vernoniaglabra* leaves (VGL) were active against the brine shrimp, *Artemiasalina*. Dichloromethane (DCM) root extract of *Citrus limon* roots (CLR) showed the highest activity *in vitro* against brine shrimp, *Artemiasalina* based on IC_{50}.

(ii) Dichloromethane (DCM) extract of *Citrus limon* roots (CLR) (7.017 µg/mL), aqueous extract of*Carrisa edulis* roots (CER) (8.054 µg/mL), DCM extract of *Brideliacathartica* leaves (BCL) (11.537µg/mL), DCM extract of *Hensiacrinita* leaves (HCL) (13.336 µg/mL), methanolic extract of BCL (15.647 µg/mL), and hexane extract of *Achyranthesaspera* leaves (AAL) (18.087 µg/mL) demonstrated high antiplasmodial activity based on IC_{50}. Hexane extract of *Microglossapyrifolia* leaves (MPL) (21.376 µg/mL), methanolic extract of *Hensiacrinita* leaves (HCL) (24.805 µg/mL), aqueous extracts of *Brideliacathartica* leaves (BCL) (25.985 µg/mL), DCM extract of *Carrisa edulis* roots (CER) (30.074 µg/mL), hexane extract of *Citrus limon* roots (CLR) (30.092 µg/mL), hexane extract of *Brideliacathartica* leaves (BCL) (32.908 µg/mL), hexane extract of*Hensiacrinita* leaves (HCL) (34.223 µg/mL), DCM extract of *Microglossapyrifolia* leaves (MPL) (34.88 µg/mL), aqueous extract of *Achyranthesaspera* leaves (AAL) (38.99 µg/mL), aqueous extract of *Hensiacrinita* leaves (HCL) (47.203 µg/mL), DCM extract of *Vernoniaglabra* leaves (VGL) (53.62 µg/mL), methanolic extract of *Carrisa edulis* roots (CER) (69.969 µg/mL), DCM extract of *Achyranthesaspera* leaves (AAL) (86.501 µg/mL), and aqueous extract of *Citrus limon* roots (CLR) (96.86 µg/mL) demonstrated moderate antiplasmodial activity based on IC_{50}. DCM extract of *Citrus limon* roots (CLR) (7.017 µg/mL) demonstrated the highest antiplasmodial activity based on IC_{50}.

(iii)Dichloromethane (DCM) extract of *Citrus limon* roots (CLR) owes is antiplasmodial activity to the presence of suberosin which together with other compounds synergistically works against *Plasmodium falciparum*. Further, Xanthyletin a compound without demonstratableantiplasmodial activity was also isolated from DCM extracts of *Citrus limon* roots. In addition, Spinasterol, a compound without demonstratableantiplasmodial activity was isolated from the *Microglossapyrifolia* leaves (MPL).

4.2 Recommendations

The observation that all the seven studied plants parts extracts including *Achyranthesaspera* leaves, *Brideliacathartica*leaves,*Microglossapyrifolia*leaves,*Vernoniaglabra* leaves, *Hensiacrinita* leaves, *Carrisa edulis* roots, and *Citrus limon* roots demonstrated high to moderate antiplasmodial activity, supports their continued use as antimalarial drugs in Kilifi and Homa-bay.

Recommendations for further studies

Further, the compounds contributing to the antiplasmodial activity in these

aqueous and organic extracts can be isolated, and characterized and identified using spectroscopic techniques based on bioassays.

(ii) Suberosin, the antiplasmodial compound isolated in DCM extracts of *Citrus limon* roots in this study should be subjected to *in vivo* antimalarial activity in rodent models such as infecting mice with *Plasmodium berghei* and treating the infected mice with varying doses of suberosin to confirm its antimalarial activity. If it has moderate antimalarial activity, a drug that can cure malarial infection can be synthesized modeled upon its structure to confer it with a high *in vivo* antimalarial activity in addition to conferring it with a high *in vitro* activity against *P. falciparum*.

(iii) More work should be done on active antiplasmodial hexane, aqueous and methanolic extracts of *C. limon* roots in order to isolate, characterize and identify more compounds.

(iv) In addition, further work for example MS needs to be done on compound MPL-1 F37 (a) (Spinasterol) to confirm its identity.

DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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