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2020-08-31

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Journal of Medicinal Plants Research

https://doi.org/10.5897/JMPR2020.6936

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Vol. 14(8), pp. 406-414, August, 2020
DOI: 10.5897/JMPR2020.6936
Article Number: C6BE8F764237
ISSN 1996-0875
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http://www.academicjournals.org/JMPR

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Full Length Research Paper

Antimalarial potential and phytochemical composition of fractions of *Lippia kituiensis* Vatke *(*Verbenaceae*)* growing in Northern Tanzania

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Received 26 February, 2020; Accepted 27 March, 2020

Despite past decades of steady advances in the fight against Malaria, statistics show that the disease is still a threat to human health. Previous successes in the development of antimalarial drugs from medicinal plants have fuelled research in this area. However, antimalarial studies on fractionated extracts from such plants have progressed slowly. This study reports the antimalarial potential of fractions from *Lippia kituiensis* Vatke, for the first time. Column chromatography was used during fractionation. Antiplasmodial assay against chloroquine-sensitive (D6) and resistant (W2) plasmodium strains were done using hypoxanthine incorporation assay. MTT assay was used to assess the cytotoxicity of fractions against the Vero cell line. Fractions obtained exhibited varied inhibitory concentrations (IC50); with the most efficacious being, Lk-5 (19.45 ± 6.20 µg/ml), Lk-3 (30.43 ± 0.68 µg/ml), Lk-4 (30.82 ± 18.01 µg/ml), and Lk-6 (36.53 ± 14.42 µg/ml) against D6. Generally, lower activity against W2 was obtained with the most active being LK-4 (24.18±2.50 µg/ml), and Lk-5 (24.42±5.95 µg/ml), while chloroquine (positive control) exerted IC50 of 77.86±4.09 ng/ml (W2) and 15.71±6.49 ng/ml (D6). LK-4 was the most cytotoxic showing cytotoxic concentration (CC50), 46.26 µg/ml. When tested in mice, fractions suppressed *Plasmodium berghei* significantly compared to the negative control with Lk-3 being most efficacious (80.01±1). Due to substantive efficacy, GC-MS done on Lk-3 revealed 8 compounds where three have previously been ascribed to antimalarial activity and other pharmacological effects. This study adds to present knowledge of antimalarial efficacy of *L. kituiensis* and provides the basis for further work to be done on the isolation of compounds from its extracts.

Key words: Malaria, chromatography, alkaloids, cytotoxicity, phytochemistry.

INTRODUCTION

The fight against malaria is at a critical point with an estimated 2 million increase in global cases between 2016 and 2017 alone. Africa is continuously the most affected with 92% of global cases happening in the

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continent. Among 80% of Malaria deaths in 17 countries of Sub-Sahara Africa and India, 5% happened in the United Republic of Tanzania (World Health Organization, 2018). This possible global resurgence of malaria spells a looming crisis in human health particularly in the African continent (World Malaria Day, 2019).

The disease is caused by *Plasmodium falciparum*, *Plasmodium ovale, Plasmodium vivax, Plasmodium malariae* and *Plasmodium knowlesi* all of which are transmitted by the bites of Plasmodium spp. infected female mosquitoes of the Anopheles genus (Singh et al., 2004; Van Hellemond et al., 2009). However, *P. falciparum* and *P. vivax*, for which humans are exclusive mammalian hosts, are responsible for the largest public health burden (Phillips et al., 2017). Furthermore, the recent emergence of malaria as a zoonosis due to *P. knowlesi* transmission poses obvious complications for elimination (Imwong et al., 2011). With currently no approved vaccine, chemotherapy and, prevention are predominant methods of malaria control (Chen et al., 2018).

Artemisinin has been the cornerstone of malaria chemotherapy. However, worrying reports of failure attributed to growing resistance have been published; highlighting a clear need to find alternative treatment options that are effective, well-tolerated, and safe (Landier et al., 2018; Pereira et al., 2011). Additionally, the previously cheap and effective drugs like chloroquine are no longer effective due to the rapid spread of the resistant Plasmodium strains. Even with existing reports of vaccine development, chemotherapy will still be required. More urgently, new and robust anti- malarial drugs, acting on new targets that have not been mutated into resistant forms, are urgently needed (De Beer et al., 2012).

Plants present a wealth of compounds with novel mechanisms of action (Bero et al., 2009), hence this particular study. Lippia is a genus of flowering plants in the family Verbenaceae, known for its characteristic aromaticity from its essential oil (Arthur et al., 2011). With approximately 200 species, Verbenaceae consists of herbs, shrubs and small trees. These species are mainly distributed throughout tropical African countries and South and Central American territories (Pascual et al., 2001). Several studies have reported pharmacological effects of lippia including antimalarial, antimicrobial, antifungal, analgesic, larvicidal, antispasmodic, inflammatory, and antipyretic activities (Stashenko et al., 2013). *Lippia kituiensis* is used by local communities in Tanzania to treat malaria, with only a single study reporting scientific validation of the efficacy of its total extracts. The current study reports the antimalarial efficacy of the fractions obtained from *L. kituiensis*. This is a subsequent study guided by previous report showing a significant chemo-suppression (95.19%) efficacy of total extracts of the plant against *Plasmodium berghei* (Mzena et al., 2018).

Reagents and chemicals

Silica gel powder (70-230 mesh), pre-coated Thin Layer Chromatography Plates, solvents (Hexane, Ethyl Acetate, Dichloromethane and, Methanol) and biological reagents and consumables were purchased from Sigma Aldrich. Chloroquine diphosphate and radio-labeled Hypoxanthine Monohydrate salt were generously donated by Dr. Gathirwa of the Kenya Medical Research Institute.

MATERIALS AND METHODS

Ethics statement

Approvals for the study were obtained from Kenya Medical Research Institute's Review Board which are, Scientific and Ethics Review Unit (SERU) approval number-KEMRI/SERU/CTMDR/063/3644 and the Animal Care and Use Committee (ACUC)-KEMRI/ACUC/01.02.19.

Collection and preparation of plant material

Previous studies by Mzena et al. (2018) demonstrated significant antimalarial activity of total extracts of *L. kituiensisVatke* leading to this study. Samples of fresh aerial parts of the plant were harvested sustainably from Ugweno village in Kilimanjaro region, Northern Tanzania (3° 39' 0" South and 37° 39' 0"), where they are traditionally used to treat Malaria. The samples were harvested in the morning hours of 13th March 2018 and transported to NM-AIST. Identification and authentication of the plant were done with assistance from a resident herbalists and a professional taxonomist (Mr Josephat Mboya of the Tropical Research Institutes, Arusha). A voucher specimen was taken and deposited at the Nelson Mandela African Institute of Science and Technology and assigned specimen number LKV-160-2018. Soil and other contaminants were washed off using cold water. The plant material was dried under shade till complete dryness, milled into a fine powder and packed in manila bags. The sample was then stored at room temperature. The powdered plant material was transported to the Kenya Medical Research Institute, Nairobi where subsequent studies were done.

Extraction of plant material

Organic extraction was done with brief modifications of the method described by Gebrehiwot et al. (2019). Briefly, a top pan balance (Slater model-323, China), was used to weigh 1 kg of plant powder into a 5 L flat-bottomed flask. Methanol (MeOH) and Dichloromethane (DCM) mixture in the ratio 1:1 was used to macerate the plant material. This is because methanol being a polar solvent would extract polar compounds while DCM (non-polar) would extract the non-polar secondary metabolites. The powdered material was extracted for 24 h at room temperature and this was repeated two times. The material was filtered using Whatman's filter paper (No. 1), and concentrated at reduced pressure using a rotary evaporator (BUCHI Rotavapor model No. R-114; Switzerland) giving a dark green gum of 87.8 g hence a percentage yield of 8.78%.

Thin layer chromatography

A small portion of the total extract of *L. kituiensis Vatke* was reconstituted in MeOH: Dichloromethane mixture and spotted on

cut pieces of pre-coated thin layer chromatography plates (TLC). The plates were then developed in different solvent pairs (Begum et al., 2017). Upon development, assessment of plates was done by observation on open light, under UV (254 and 365 nm), and after vanillin spray with plates heated to 110°C for 5 min. Based on retention factors (Rf) obtained from developed plates, the best solvent mixture to run the column was identified. Hexane and Ethyl acetate mixed in the ratio 4:1 gave the best separations and hence the pair was established as an ideal mobile phase in liquid column chromatography.

Column chromatography

Open column chromatography was used to separate the total extract where briefly, a glass column of diameter 10 cm was packed with a slurry of 300 g of Silica powder (70-230 mesh, using100% Hexane) (Walker et al., 2015). The slurry was immediately transferred into the column with intermittent swirling to prevent solidification. The wetness of the gel was maintained by several elutions of 100% hexane. After 24 h of undisturbed packing, Hexane was drained and 60 g of the total extract of *L. kituiensis* was mixed with 70 g of silica and 100% hexane. The mixture was swirled to homogeneity and transferred into the packed column. 500 ml of 100% hexane was then loaded into the column to begin fractionation as fractions were collected in different flasks. The polarity of the mobile phase was progressively increased until no further separations could be identified as guided by TLC profiles gathered in the process.

***In-vitro* antiplasmodial assay**

Antiplasmodial activity of fractions was done using a semi-automated microdilution technique on culture-adapted *P. falciparum* D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains as earlier described by (Desjardins et al., 1979). Briefly, culture media consisted of RPMI 1640, 25 MM HEPES at pH 7.4, supplemented with 12.5% heat-inactivated human serum (obtained from pooled blood types A, AB and, O). Haematocrit was adjusted to 6% human erythrocytes (O^+ type RBCs) of 5 ml total culture volume. Cultures were kept in sealed flasks at 37°C, supplied with a gas mixture (3% O_2, 5% CO_2 and 92% N_2). Stock solutions of drugs were prepared at 100 mg/ml in DMSO diluted by RPM1640 to attain a final 0.2% content of DMSO. Test samples were diluted in DMSO except for CQ; (constituted in water) and reduced to < 0.2% DMSO content. 25 µl of screening medium was then added to each well in a 96-well flat-bottom micro-culture plate (Costar®, Corning Incorporated, NY, U.S.A). 50 µl of constituted test samples at a starting concentration of 100 µg/ml was added to wells of row B in duplicate. Upon proper mixing, 25 µl of drug solution was transferred from wells of row B to wells of row C successively to row H. 200 µl of infected erythrocytes (1.5% hematocrit and 0.5% parasitemia) were then added to each well except for wells A9 to A12, to which, 200 µl of uninfected RBCs (diluted in screening medium to 1.5% hematocrit) were added as a negative control. The plates were incubated at 37°C in a chamber gassed by gas mixture at 95% humidity. After 48 h of incubation, 25 µl of [3H] hypoxanthine was added by pulsing to each well (0.5 µCi per well). Plates were incubated for a further 18 h after which it was frozen to negative 20°C. The plates were then thawed and the contents harvested onto glass-fiber filter mats (A 1450-421; Perkin Elmer) using a Betaplate cell harvester (1295-004 Betaplate; Wallac Perkin Elmer) The filter mats were then oven-dried and drenched in 10 ml of scintillation fluid (Beta Scintilla®, Perkin Elmer, Schwerzenbach, CH) while in a plastic foil (1450-432; Perkin Elmer). A Betaplate liquid scintillation counter (1205 Betaplate; Wallac Perkin Elmer) was then used to determine [3H] hypoxanthine incorporation which was measured in terms of counts per minute (cpm). The result of each well was recorded as counts per minute and expressed as a percentage of the untreated (positive) control (wells A1 to A8; cultures without test compound). Negative control was used for background subtraction. IC50s (drug concentration at which 50% of [3H] hypoxanthine incorporation was inhibited compared to drug-free controls) were estimated by linear interpolation.

Cell culture and cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measures the conversion of MTT into formazan crystals by living cells, hence determining mitochondrial activity was used to asses cytotoxic levels of fractions as earlier described by Kaur et al. (2018). Vero (E6) cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated Fetal bovine serum (Himedia), l-Glutamine and treated with penicillin/streptomycin (100 μgml^{-1}), gentamycin (100 μgml^{-1}). Culture flasks were incubated in a CO_2 incubator (5% CO_2; 90% Relative Humidity) at 37°C. Upon attainment of maximum confluence (90-100%), the cell monolayer was trypsinized and seeded at 2×10^5 cells/ml in each well of 96-well plates and incubated at the same conditions for 24 h. Drug concentrations were prepared (1000µg/ml), seeded at the highest concentration and serial diluted to afford different concentrations across the plate. The plates were further incubated for 48 h after which 10 µl of MTT solution (5 $mgml^{-1}$) added into each well and incubated for a further 4 h. The plates were emptied of media and the formazan product was dissolved in 100 µl of neat Dimethyl sulfoxide (DMSO) seeded per well and shaken for 5 min. Optical density was measured using a microplate reader at 550 nm using a Thermofisher, MultiscanGo Spectrophotometer model. Inhibition data were plotted as dose-response curves, from which CC_{50} (concentration required to cause alterations in 50% of intact cells) was estimated. Selectivity index ($SI=IC_{50}/CC_{50}$) a parameter that measures the clinical significance of the test samples by comparing general toxins and selective inhibitory effect on *P. falciparum* was calculated (Wright and Phillipson, 1990).

***In-vivo* antimalarial assays**

Dosages for *in-vivo* assay were prepared in a vehicle composed of 10% Tween 80, 3% DMSO and PBS. The vehicle served as the negative control in each case respectively. We adopted a model involving the use of *P. berghei* NK65 strain to study *in vivo* antimalarial activity on Swiss albino mice according to Peter's 4-day suppressive test (Peters, 1975). Upon attainment of ethical approvals, Naïve Swiss albino mice weighing 20±2 g were acclimatized for 48 h. Frozen stabilates of *P. berghei* were thawed and injected into a group of 3 animals for propagation. Assessment of parasite growth was done microscopically and upon attainment of approximately 20% parasitaemia, blood was drawn from the passage mice (parasite donors) by cardiac puncture and reconstituted to 2% in phosphate saline glucose. Each of the 40 mice was injected in the peritoneal cavity with 0.2 ml of infected blood at approximately 2×10^7 *P. berghei* on day 0. Animals were then randomized into cages of 5 each that is, positive control (chloroquine 10 mg/kg), negative control and the 6 fraction-test groups which received 1000 and 500 mg/kg of test groups. All cages were well labeled with experimental details and animals supplied with clean water and standard rodent pellets. Stainless steel, 22-gauge cannula (Harvard Apparatus; needle of length = 25.4 mm, ball diameter = 1.25 mm) was used for oral administration of drugs 2 h after infection. This continued on days 1 to 3 (24, 48 and 72 h. Drug administration was stopped after 96 h since infection. On day 4, microscopic slides were prepared by drawing

Table 1. Fractions obtained from *L. kituiensisVatke* and their properties.

Fraction code	Number of fractions combined	Weights of fractions (g)	Percentage yield of fractions	Physical appearance
Lk-1	3	3.48	3.96	Dark green gum
Lk-2	6	5.07	5.77	Dark green gum
Lk-3	3	2.05	2.33	Dark Yellow paste
Lk-4	4	3.60	4.10	Dark green paste
Lk-5	5	3.34	3.80	Dark green paste
Lk-6	11	5.91	6.73	Dark green paste

blood from tail veins. Slides were fixed in methanol, stained using 10% Giemsa for 15 min. Parasitemia in each group was assessed microscopically under high magnification and oil immersion. Percentage chemosuppression of fractions was calculated with mean parasitemia on day 4 as described by Tona et al. (2001).

Qualitative phytochemical screening

Qualitative assessment of secondary metabolites present in the fractions was done using standard chemical procedures and tests (Kasolo et al., 2011). Availability or absence of alkaloids, tannins, saponins, phenols, and glycosides was determined.

FTIR

Fraction *Lk-3* (dark yellow paste) exhibited outstanding antimalarial activity against *P. berghei* at an oral dose of 1000mg/kg. A sample from this fraction was therefore subjected to FTIR at Jomo Kenyatta University of Agriculture, Science, and Technology (JKUAT). The Shimadzu FTIR spectrophotometer (FTS- 8000, Japan) was used to analyse the functional groups as done by Memon et al. (2015). The standard KBr disc method was used, where briefly, crystal sample was ground with KBr in the ratio 1 to 10 mg respectively. The mixture was pressed into a pellet using a hydraulic press and scanned with a spectral resolution set at 4 cm^{-1} and the scanning range from 400 to 4000 cm^{-1}.

GC-MS

GC-MS at JKUAT was used to further determine compounds in fraction *Lk-3.* This was done as in earlier method (Igwe, 2015). A Shimadzu QP 2010-SE GC-MS coupled to an autosampler was used for analysis. Ultrapure Helium was used as the carrier gas at a flow rate of 1 ml / minute. A BPX5 (non-polar column, 30 m; 0.25 mm ID; 0.25 µm film thickness) was used for separation. The GC was programmed as follows: 50°C (1 min); 5°C /min to 250°C (1 min). Total runtime was 42 min. Injection temperature was set to 200°C, while the interface temperature was set at 250°C. The EI ion source was set at 2000C and mass analysis was done in full scan mode, 50-550amu. The National Institute of Advanced Industrial Science and Technology (NAIST) 2014 library of mass spectra was used to aid in the identification of compounds.

Data analysis

Data are presented as mean± standard deviation. Analsysis of *in vivo* antimalarial assay was carried using Windows SPSS Version 20.One-way analysis of variance (ANOVA) followed by Tukey's test was done to determine statistical significance for comparison of parasitaemia suppression between groups. P value at <0.05 was considered statistically significant.

RESULTS

A total of 32 fractions were obtained and concentrated as described previously. The combination of fractions was guided by TLC profile, where fractions that showed similar retention factors were pooled together. Eventually, 6 fractions were obtained as summarised in Table 1. Under 254 nm of UV, fractions *Lk-4* and largely *Lk-5* showed fluorescent spots. Preliminary *in vitro* antiplasmodial assay was conducted against culture-maintained W2 (chloroquine-resistant) and D6 (chloroquine-sensitive) strains of *P. falciparum*. Chloroquine diphosphate was used as the positive control while DMSO (0.2%) was the negative control. Results are shown in Table 2.

The fractions exhibited moderate to low activity; where efficacy was classified as high at IC_{50} ≤10 µg/ml, moderate at 10-50 µg/ml, low at 50-100 µg/ml and inactive at >100 µg/ml, (Berthi et al., 2018). Generally, fraction LK-5 showed the highest antiplasmodial activity against chloroquine-sensitive strain (D6) with IC50 of 19 µg/ml and second highest activity against chloroquine-resistant strain (W2) IC50 24.42 µg/ml respectively. Notably, efficacy increased with increasing polarity of the mobile phase except in fraction Lk-6. The judgement for cytotoxic levels was made where a fraction was considered very toxic when CC50< 10 µg/ml, moderately toxic with CC50 ranging from 11-30 µg/ml, slightly toxic with CC50, 31-50 µg/ml, and potentially non-toxic with CC50> 50 µg/ml (Berthi et al., 2018). Hence, most fractions were potentially nontoxic with majority showing CC50 values >50, except Lk-4 (46.26 µg/ml). Selectivity indices of fractions were calculated as previously done (Rocha et al., 2012) and fractions generally depicted lower selectivity indices against D6 compared to W2. However, with SI>2 against both parasite strains, Lk-5, which also exerted the best antiplasmodial efficacy showed specific activity rather than toxicity (Berthi et al., 2018). Since antimalarial drug efficacy is best assessed *in vivo* rather than *in vitro,* two dosages of each fraction

Table 2. *In vitro* antiplasmodial activity, corresponding cytotoxicity against Normal Mammalian Cell line (Vero E6) and Selectivity indices of each fraction.

Fraction	IC_{50} of anti-plasmodial assay against strains tested (M±SD)		CC_{50}(µg/ml)	*Selectivity Indices of Fractions*	
	W2 (µg/ml)	D6 (µg/ml)	Vero E6 Cell line	SI -W2	SI-D6
Lk-1	>100	79.41±16.61	71.21	0.7121	0.8967
Lk-2	68.42±5.55	78.85±5.82	55.00	0.8038	0.6975
Lk-3	45.16±9.05	30.43±0.68	64.90	1.44	2.13
Lk-4	24.18±2.50	30.82±18.01	46.26	1.91	1.50
Lk-5	24.42±5.95	19.45±6.20	67.13	2.75	3.45
Lk-6	56.75±5.12	36.53±14.42	69.96	1.23	1.92
Cq	77.86±4.09ng/ml	15.71±6.49ng/ml	n/d	n/d	n/d

IC_{50} –an effective concentration that inhibits 50% of the cultured parasite from hypoxanthine uptake, CC_{50}- Drug concentration that reduces cell viability (Vero E6 cell line) by 50%. Selectivity index- Ratio of IC50 to CC_{50}-measure of drug's ability to target parasite, Cq: Chloroquinen/d: not done.

Table 3. In vivo antimalarial activity of fractions of L. kituiensis against P. berghei (ANKA 65).

Fractions	Parasite density per dose (Mean±SD)		Chemo-suppression per dose (Mean±SD)	
	500 mg/kg	1000 mg/kg	500 mg/kg	1000 mg/kg
Lk-1	2.30±0.43	3.99±0.07	78.64±4.01[b]	73.04±044[b]
Lk-2	5.47±0.65	4.10±0.52	49.24±6.06[b]	72.27±3.51[b]
Lk-3	3.99±0.82	2.96±0.16	62.24±7.61[b]	80.01±1.07[b]
Lk-4	5.33±0.44	4.39±0.55	50.57±4.16[b]	70.35±3.72[b]
Lk-5	4.14±0.42	4.01±0.89	61.59±3.95[b]	72.91±6.06[b]
Lk-6	5.74±0.59	4.09±0.55	46.77±5.51[b]	72.37±3.71[b]
Cq(10 mg/kg)	0.56±0.37	1.10±0.31	94.72±3.43[a]	92.57±2.11[a]
Placebo	10.78±0.70	14.82±0.90	0.00[b]	0.00[b]

Cq-Chloroquine-positive control, Placebo-Negative control- (vehicle), Parasite Density-Denotes percentage of infected erythrocytes, Chemosuppression- measure of drugs' effectiveness in reducing parasite growth over the period tested, [a, b]P≤ 0.05 level of significant difference between groups against positive control and negative control respectively.

(500 and 1000 mg/kg) were screened in a mouse model following Peter's suppressive test (Peters, 1975). Results are as shown in Table 3.

Overall, all fractions exhibited significant antimalarial activity when compared to the negative control. Their efficacy was however insignificant in comparison with the positive control (chloroquine). A dose dependent activity was observed with the highest efficacy seen in animals treated with 1000 mg/kg. However, 500 mg/kg of Lk-1 was more efficacious than it was at 1000 mg/kg. The dose was repeated in a separate group of animals and almost similar results were obtained. This scenario can be linked to toxicity since mortality was seen day 2 post-infection contrary to expectation. Additionally, the fraction showed a low selectivity index in-vitro alluding to cytotoxicity.Fraction Lk-3 was found to possess the most pronounced activity during in vivo testing with percentage chemosuppression of 80.01 at 1000 mg/kg. This was consistent with *in vitro* data in Table 2, particularly against D_6 strain.

Qualitative phytochemical screening was conducted to determine classes of secondary metabolites in each fraction (Table 4). Comparatively*, Lk-3 and Lk-5* demonstrated availability of different secondary metabolites, hence the efficacy observed from the fractions. Notably, all the fractions except *Lk-6* indicated the presence of alkaloids. On account of outstanding *in vivo* efficacy, fraction Lk-3 was further analyzed for its phytochemical content using FTIR and GC-MS. Spectral data are indicated in Figure 1, Tables 5 and 6 (GC-MS) respectively.

Spectral results for Lk-3 complement the qualitative phytochemical screening depicted in Table 4. For instance, the presence of phenols during the qualitative screening was confirmed by absorbance at 1379.0 from the spectra. GC-MS method identified 8 compounds with hexatriacontane (28.75%) being the major compound identified followed by hentriacontane, 3-methyl (7.13%) and tetratetracontane (6.13%). The first compound with retention time 6.758 min needs further identification using nuclear magnetic resonance which was beyond the scope of the current study. Although the NIST library

Table 4. Qualitative phytochemical composition of fractions.

Fractions	Secondary metabolites screened				
	Alkaloids	Terpenoids	Phenols	Tannins	Glycosides
Lk-1	+	-	-	-	+
Lk-2	+	-	-	-	+
Lk-3	+	+	+	-	-
Lk-4	+	-	-	+	+
Lk-5	+	-	+	-	+
Lk-6	-	+	+	-	+

+ = Presence - = Absence.

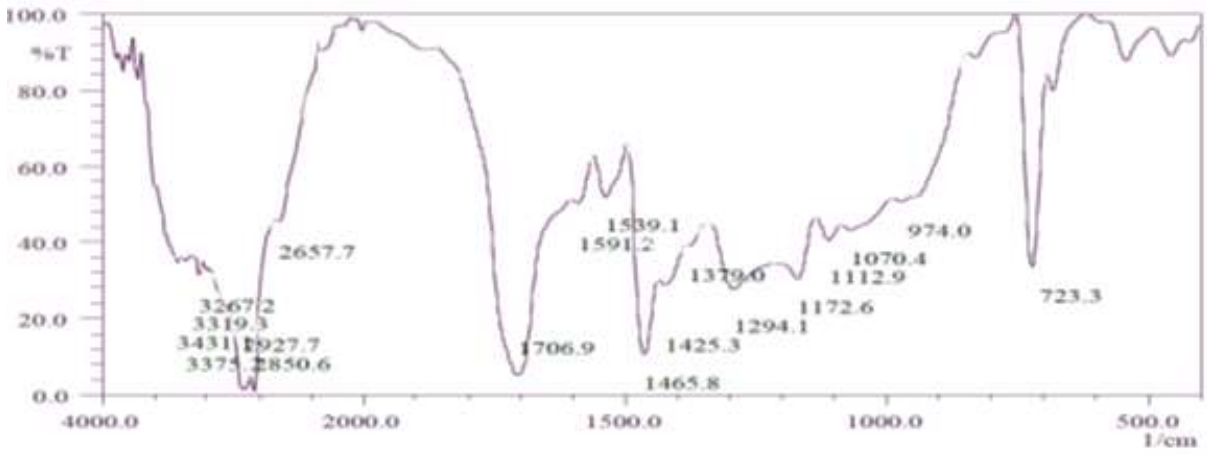


Figure 1. FTIR Spectral pattern of Lk-3.

Table 5. FTIR Spectral interpretation of Lk-3.

S/N	Frequency range	Functional Group identified
1	3329.3-2657.7 cm^{-1}	O-H carboxylic acid
2	1706.9 cm^{-1}	C=O saturated carbonyl group
3	1465.8 cm^{-1}	C-H methylene group from an alkane
4	1591.2 and 1425.3 cm^{-1}	C=C stretching in an aromatic ring
5	1172.6, 1112.9, 1070.4	C-H weak bending in the aromatic ring
6	1379.0	O-H bending due to Phenol
7	974.0, and 723	Long-chain Alkanes

suggested 1,3-Butanediol, the similarity index is so low to imply so. Three compounds identified have previously been reported to possess antimalarial activity and other pharmacological properties (structures are indicated in Table 7).

DISCUSSION

The antimalarial activity of fractionated organic extracts of *L. kituiensis Vatke* and their cytotoxic levels have been revealed in this study. Moderate to low antiplasmodial activity was seen. This can be attributed to instability of the fractionated extracts which are normally protected by chlorophyll and/or other components in their native forms (Zani et al., 1997). The concerted antimalarial effects of the total extracts of *L. kituiensis* as depicted in previous reports could have also been compromised by the semi-purification process during fractionation.

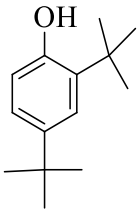
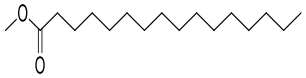
Outstanding efficacy observed during *in vivo* studies

Table 6. GC-MS spectral elucidations.

S/N	Retention time (min)	Name	Molecular formula	Molecular weight	Peak area %
1	6.758	Unknown	$C_8H_{14}O_4$ (S)	174 (S)	0.99(S)
2	10.772	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	0.98
3	13.417	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	268	2.37
4	14.02	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.91
5	14.33	l-(+)Ascorbic acid2,6 dihexadecanoate	$C_{38}H_{68}0_8$	652	5.88
6	23.055	Tetratetracontane	$C_{44}H_{90}$	618	6.31
7	26.435	Hentriacontane, 3-methyl-	$C_{32}H_{66}$	450	7.13
8	21.594	Hexatriacontane	$C_{36}H_{74}$	506	28.75

S-suggested by NIST library.

Table 7. Names and Structures of compounds with previously reported pharmacological properties identified in fraction Lk-3 by GC-MS.

Name of compound	Structure of compound
2,4-Di-tert-butylphenol	
Hexadecanoic acid, methyl ester	
Hentriacontane, 3-methyl	

rather than *in vitro* suggests immunomodulatory activity of drugs which may be due to localization of specific phytochemicals including fatty acids such as linoleic acids, squalene and Tannins which have previously been reported to possess immune-stimulating properties (Kumaradevan et al., 2015; Chakrabarti et al., 2012). These class of compounds (except Tannins) were found to be present in fraction Lk-3 as illustrated by GC-MS data in Table 5.

To the best of our knowledge, this study reports the antimalarial potential of fractionated extracts of *L. kituiensis* for the first time the. However, a number of pharmacological properties have been ascribed to the genus *Lippia*, including antimalarial, antimicrobial, antifungal, larvicidal, antispasmodic, analgesic, anti-inflammatory, and antipyretic activities (Santos et al., 2016). Numerous studies on this genus have focused on their essential oils (Stashenko et al., 2013). The promising antimalarial activity observed is consistent with other reports on the use of plants in the same family for the management of Malaria. For example among other things, *Lippia javanica* has been reported for use to cure fever or malaria (Maroyi, 2017). Ludere et al. (2013) isolated a compound (Lippialactone) with antiplasmodial effects from aerial parts of *L. javanica*. In a different study involving plants collected from Nigeria, *Lippia multiflora* was among plant samples from 11 families collected based on antimalarial ethnopharmacology (Ajaiyeoba et al., 2006).

The presence of secondary metabolites, especially alkaloids, phenolic compounds, and terpenoids could be responsible for the observed antimalarial activity. Quinine is a good example of alkaloids that have earlier been reported to possess antiplasmodial properties (Mekonnen, 2015). Most of the compounds in fraction Lk-3 identified by GC-MS have been reported previously. Although the specific antimalarial efficacy of the identified compounds has not been reported before, it is worthy stating that these compounds have been reported in plant extracts of medicinal plants that are traditionally used to manage Malaria. For instance, hexadecanoic acid, methyl ester was earlier identified in whole plant extracts of *Phyllanthus amarus*a medicinal plant used to treat malaria in Eastern Nigeria (Igwe, 2015).

Hexadecanoic acid, methyl ester, is a polyunsaturated fatty acid (PUFA). These class of compounds have been implicated in antiplasmodial activity and are reported to increase with the degree of unsaturation, (Kumaratilake et al., 1992). These results are consisted with past reports of antimalarial efficacy of PUFA, where a mixture of Linolenic and linoleic acid showed a 96% chemosupression in mice infected with *P. berghei* (Melariri et al., 2012). 2,4-Di-tert-butylphenol (DTBP), a volatile phenolic compound is reported to possess antioxidant properties (Choi et al., 2013) and *in vitro* antimalarial activity at 100 mM (Kusch et al., 2011; Kulangara et al., 2015). Furthermore, DTBP isolated from a Lactococcus sp demonstrated antifungal, antioxidant and cytotoxicity against cancer cell lines (Kulangara et al., 2015; Jayaseelan et al., 2017). The rest of the compounds have not been reported for antimalarial activity but other pharmacological importance where Hexatriacontane identified in essential oils of *Cassia fistula* showed significant activity against the standard and clinical isolates of Candida species (Irshad et al., 2012). The observed antimalarial efficacy of Lk-3 and by extension the other fractions can be attributed to individual or synergistic activity of compounds with antimalarial activity evidenced herein.

Conclusion

Total organic extracts of *L. kituiensis Vatke* were subjected to fractionation using Chromatographic techniques. Substantive antimalarial efficacy and potential safety of fractions from the plant have been revealed in this study. The classes of compounds attributable to the efficacy have also been made evidenced. Results from this study not only validate the traditional use of extracts of the plant to manage malaria but also highlights the need for research towards isolation and purification of novel compounds from it. Henceforth, it is possible that phytomedicines can be developed for use by local communities to manage malaria. We further recommend comprehensive *in-vivo* safety studies and isolation of compound(s) from fractions of this plant, particularly fraction *Lk-3*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

ANOVA, Analysis of Variance; **D6,** Chloroquine-sensitive; **DCM,** Dichloromethane; **DMSO,** Dimethyl sulfoxide; **FTIR,** Fourier Trans-Infra-Red; **GC-MS,** Gas Chromatography-Mass spectrophotometry; **JKUAT,** Jomo Kenyatta University of Agriculture, Science, and Technology; **KBr,** Potassium bromide; **KEMRI,** Kenya Medical Research Institute; **MeOH,** Methanol; **MTT,** 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; **NM-AIST,** National Institute of Advanced Industrial Science and Technology; **ACUC,** Animal Care and Use Committee; **PUFA,** Polyunsaturated fatty acid; **SERU,** Scientific and Ethics Review Unit; **TLC,** Thin layer chromatography plates; **UV,** Ultra-violet; **W2,** Chloroquine-resistant; **WHO,** World Health Organisation.

ACKNOWLEDGEMENTS

The authors sincerely appreciate the World Bank, through The African Centre for Research, Agricultural Advancement, Teaching Excellence and Sustainability (CREATES) hosted at the Nelson Mandela African Institution of Science and Technology (NM-AIST) for funding this research; Kenya Medical Research Institute for providing research infrastructure; the Jomo Kenyatta University of Agriculture, Science and Technology for their assistance with some aspects of the study; the director KEMRI for allowing the publication of this work and by extension the entire staff Fraternity of the Center for Traditional Medicine and Drug Research for their unwavering support throughout the study.

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