# Identification of antimalarial compounds from *Leucas calostachys*, using High Performance Liquid Chromatography and Electrospray Ionization Mass Spectrometry

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***Leucas calostachys*** **is widely used in traditional medicine in Kenya for management of various ailments including malaria. Bio-assay guided fractionation of *Leucas calostachys* extracts was carried out using *in-vitro* antiplasmodial and β-hematin inhibition assays with semi-preparative high performance liquid chromatography (HPLC). The active methanol fraction was subjected to liquid chromatography tandem mass spectrometry to identify constituent compounds. A total of twenty compounds consisting of eight flavonoids and 12 phenylethanoids were identified from this fraction. The flavonoids included, isorhamnetin, luteolin-7-O-glucoside, luteolin-4′-O-glucoside, luteolin diglucoside, apigenin-O-glucoside, genistein-O-glucoside, chrysoeriol-7-O-glucoside, and chrysoeriol-7-O-glucuronide. Seven of the phenylethanoids were identified as acteoside, isoactoeside, hydroxyacteoside, forthsoside B, samioside, alyssonoside and leucoseptoside A. The antimalarial activity of *Leucas calostachys* could be linked to presence of flavonoids and phenylethanoids.**

**Keywords:** *Leucas calostachys*, antimalarial, flavonoids, phenylethanoids, HPLC-MS, β-hematin

## INTRODUCTION

Malaria is still a major public health concern in Kenya, especially in the Lake Victoria basin, western Kenya and coastal regions. Although artemisinin-based combination therapy (ACT) regimens have played an important role in effective case management of malaria, there is resistance to these agents in many regions thus, leading to delayed clearance of parasites or treatment failure [1, 2]. This phenomenon is likely to reverse the gains made in the fight against malaria in the last two decades. It is therefore imperative that search for new anti-malarials continue incessantly. In a previous study, the authors reported the plants used in the management of malaria in western Kenya, which is a malaria endemic region [3]. In the present study, the anti-malarial constituents of *Leucas calostachys*, a commonly used plant in management of malaria among the Luhya community of western Kenya is reported.

*Leucas calostachys* Oliv. (Lamiaceae) (Figure 1) belongs to the genus *Leucas* which comprises of at least 130 plant species distributed mainly in eastern Africa and eastern Asia. The plant is endemic to Burundi, Ethiopia, Kenya, Rwanda, Sudan, Tanzania, Uganda and Zaire [4]. It is a small erect shrub with almost square pubescent stalks. It has obovate, opposite petiolate leaves with many hairs on the upper surface.

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The leaves, roots or entire aerial parts of *Leucas calostachys* are used in traditional medicine for management of gastro-intestinal disorders (amoebiasis, diarrhea, stomachache, constipation, heartburn and peptic ulcers), respiratory disorders (pneumonia, coughs, chest pain, flu and colds), wounds, kidney disorders, malaria, muscle pull, skin disorders, cancer, arthritis, as a food substitute as well as East Coast fever in cattle and poultry diseases [5-7].


**Figure 1: *Leucas calostachys* Plant**

(Photo by NAM, October 2017)

*Leucas calostachys* has demonstrated activity against malaria and bacterial infections [8,9]. Phytochemical screening of *L. calostachys* reveals that it contains alkaloids, saponins, glycosides, phenolics (flavonoids, tannins, lignans, coumarins), terpenoids, phytosteroids, and long chain aliphatic compounds [10]. However, no phytochemicals have been identified from this plant. This study sought to identify antimalarial phytochemicals from this plant through bioassay-guided fractionation by means of high pressure liquid chromatography (HPLC) tandem mass spectrometry.

## EXPERIMENTAL

### Materials, reagents and solvents

General grade solvents for extraction were obtained from Kobian Ltd (Nairobi, Kenya) and distilled in the laboratory before use. Analytical/HPLC grade solvents were all obtained from Wako Pure chemical Industries, (Tokyo, Japan). Roswell Park Memorial Institute

(RPMI) 1640 and Albumax II were from Sigma-Aldrich (St. Louis, MO, USA). Phenazine ethosulphate (PES), glucose, sodium bicarbonate ($NaHCO_3$), D-sorbitol and N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) were obtained from Sigma-Aldrich (Burlington, MA, USA). Chloroquine phosphate was a kind donation by Kampala Pharmaceutical Industries Ltd (Kampala, Uganda) while gentamicin was purchased from Sigma-Aldrich (Dorset, UK). Giemsa stain was obtained from TCS Biosciences (Buckingham, UK). Non-infected O-positive whole blood was obtained from the National Blood Transfusion Centre (Nairobi, Kenya). Chloroquine-sensitive 3D7 *Plasmodium falciparum* parasites were donated by the Kenya Medical Research Institute (KEMRI) (Kilifi, Kenya).

Independently, Nonidet P-40 **(**NP-40), chloroquine diphosphate (Nacalai Tesque Inc., Kyoto, Japan), pyridine, dimethylsulfoxide, hemin chloride (Tokyo Chemical Industry Co. Ltd, Tokyo, Japan), HEPES buffer solution (Dojindo, Japan), sodium acetate, glacial acetic acid, acetone (Wako Pure Chemical Industries Ltd, Tokyo, Japan) were used for the β-hematin assays.

Normal phase silica gel of porosity 60Å and particle size 63-200 µm (Sigma-Aldrich GmbH, Seelze, Germany) was used for open glass column chromatography experiments.

**Instrumentation**

A SH-8000Lab microplate reader (Corona Electric Company, Hitachinaka-shi, Japan) was used to measure β-hematin activity. Millipore Filter unit (0.22 µm) MS® PVDF syringe filter was obtained from Membrane Solutions (Tokyo, Japan).

A Hitachi LaCrom Elite L-2000 series (Hitachi, Tokyo, Japan) HPLC system was used for both analytical and semi-preparative work. It was equipped with a L2130pump and a 7125i Rheodyne Model manual injector and a L-2400 single wavelength UV detector. Chromatographic separation was achieved using a YMC Triart C18 analytical column (150 × 3.0 mm) and YMC Triart C18 (250 × 5.0 mm) semi-preparative column C18 column (YMC, Co. Ltd. Kyoto, Japan. Fractions were collected using Gilson FC 203 B fraction collector (Gilson Inc. Middleton, WI, USA). The Hitachi HPLC data acquisition system was supported by OpenLab control software (Agilent Technologies, Lexington, MA, USA).

Thermo Scientific Q Exactive Focus benchtop LC-MS/MS System (ThermoFisher Scientific Inc. Bremen, Germany) was used for mass spectral data acquisition. The HPLC consisted of a Thermo Scientific™ Dionex™ Ultimate™ RSLC 3000 system with a rapid separation quaternary pump, a diode-array detector (DAD), a thermostated rapid separation column compartment and an auto sampler. The mass spectrometer consisted of a quadrupole ion filter and Orbitrap mass analyzer. Thermo Scientific™ Xcalibur™ instrument control and data processing software were used to acquire data.

**Plant material and preparation**

The aerial parts of the *Leucas calostachys* were harvested from their natural habitat in Ileho sub-location, Kakamega East sub-County during the month of August 2017. They were identified by a botanist, Mr. Patrick Mutiso and voucher specimens deposited at the University of Nairobi Herbarium, under the code *Leucas calostachys* (NMA2015/22). The materials were air dried under ambient conditions, coarsely powdered and stored in air tight plastic containers until use.

For *in vitro* antiplasmodial screening experiments, 100 g each of dried and powdered plant material were separately extracted with dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) respectively by cold maceration over 24 hours. Each extract was filtered, concentrated *in vacuo* at 40 °C and stored in amber colored glass bottles under refrigeration until use.

For the bio-assay guided fractionation with β-hematin inhibition assay, one kg of powdered material was extracted three times by cold maceration using dichloromethane - methanol (50:50) mixture over a period of 24 hours. The extracts were combined, filtered and rotary evaporated at 40 °C to give a dark green extract

(20.3 g, 2.0 % yield) which was kept in a refrigerator until bioassay.

### *In vitro* antiplasmodial activity

The chloroquine sensitive *Plasmodium falciparum* 3d7 parasites were cultured based on the procedure described by Trager and Jensen [11] while microscopic assay was based on the WHO *in vitro* Mark III micro test [12]. In this method, samples were examined under a light microscope with ×100 magnification for the presence of parasites. The activity of the plant extracts was measured with respect to the minimum inhibitory concentration (MIC) values.

### β-Hematin inhibition assay

The procedure described by Sandlin *et al.*, [13] was adopted in the assay for β-hematin inhibition activity. The β-hematin inhibition activity for each of the test samples was tested relative to the positive and negative controls. For each of the tests carried out on the extract or drug ($A_{Analysis}$), there were 2 controls; control analysis ($A_{Analysis;\ Blank}$) whose absorption was read without allowing the 6-hour period for the reaction time of pyridine, and a blank control ($A_{CLTBlank;Blank}$) carried out with DMSO only. The absorption arising from unreacted hematin was calculated according to equation 1.

$$Change\ in\ absorption\ (\Delta A_{Analysis}) = A_{Analysis} - A_{Analysis;Blank} \qquad Equation\ 1$$

The residual absorbance ($\Delta A_{CLT;Blank}$) of the plant/drug (not as a result of β-hematin) was calculated using equation 2, while β-hematin synthesis inhibition by the extract/fraction/drug was treated to equation 3.

$$(\Delta A_{CLT;Blank}) = A_{CLT;Blank} - A_{CLTBlank;Blank} \qquad Equation\ 2$$

$$I_{Analysis} = \Delta A_{Analysis} - (\Delta A_{CLT;Blank}) \qquad Equation\ 3$$

A positive $I_{Analysis}$ was indicative of active extract/drug whereas a negative $I_{Analysis}$ indicated inactive extract/drug. The percent β-hematin inhibition activity of the test samples was computed according to equation 4.

$$\frac{I_{Analysis}\ of\ test\ sample}{I_{Analysis}\ of\ standard} \times 100\ \% \qquad Equation\ 4$$

### Column chromatography

A portion of the plant extract (20 g) was re-suspended in dichloromethane - methanol (50:50) and adsorbed onto 17 g of silica gel. The solvent was removed using vacuum rotary evaporator at 40 °C and the dried material loaded onto a column containing 50 g of silica gel. It was gradient eluted with hexane, ethyl acetate-hexane (50:50), ethyl acetate, methanol-ethyl acetate (50:50), and methanol to give 5 fractions as illustrated in Figure 2.

### Semi-preparative HPLC

The semi preparative HPLC was conducted using mobile phase consisting of acetonitrile (ACN)-methanol (MeOH)-0.01% formic acid, with a gradient elution of increasing volume of ACN (60-100 %) on YMC Triart C18 column. The elution time was 30 min at a flow rate of 3.0 ml/min. A volume of 50 µl of the MeOH fraction, derived from the column chromatography, was injected during each cycle. Ultra Violet detection of eluents was performed at a wavelength of 254 nm. Each fraction was collected over a period of two min giving total of 15 fractions (Figure 2). A total of 10 cycles were run and the respective fractions combined and concentrated using a SpeedVac® concentrator. These fractions were subjected to β-hematin inhibition tests.

### HPLC- mass spectroscopy

The *Leucas calostachys* F10 fraction was separated on YMC Triart C18 (150 × 3.0 mm) column using linear gradient of 60%-100% ACN in MeOH as the mobile phase. An injection volume of 2.0 µl was pumped at a flow rate of 0.5 ml/min over run time of 30 min. The pump pressure was kept at 146 bars while column temperature was set at 10 °C. The mass spectra were recorded in both the positive and negative modes in the range of m/z 120-1700.

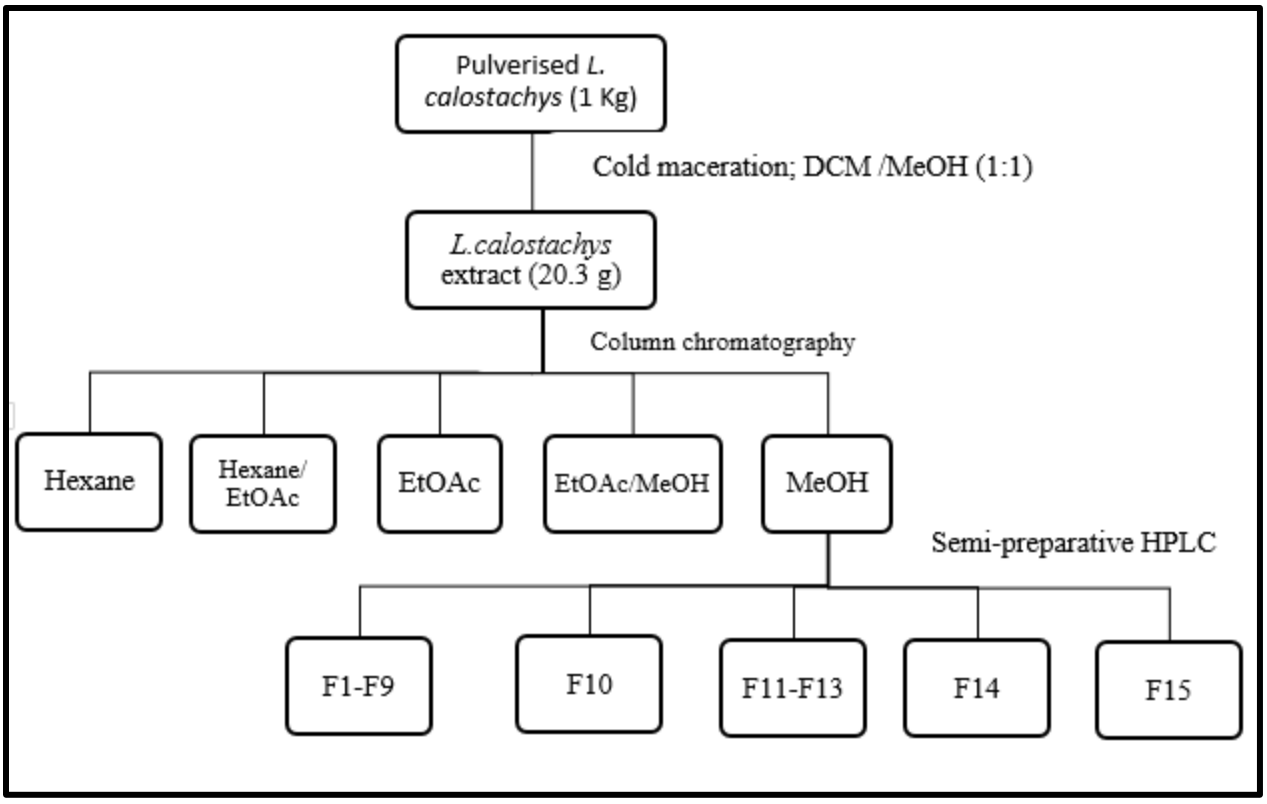


**Figure 2: Extraction and fractionation of *Leucas calostachys***

## RESULTS AND DISCUSSION

### Bio-assays

The MeOH, EtOAc and dichloromethane (DCM) extracts of *L. calostachys* were tested at concentrations between 0-500 µg/ml for their minimum inhibition concentrations (MIC) against chloroquine sensitive *P. falciparum* parasites. The MIC of MeOH and EtOAc extracts was 31.5 µg/ml, whereas that of DCM extract was 62.5 µg/ml.

Micro-fractions derived from the MeOH fraction were tracked for their β-hematin inhibition activity using HPLC and offline activity profiling. In this assay, 5 fractions (Figure 3) were found to be active with positive $I_{Analysis}$ values at a fixed concentration of 0.7 mg/ml.

The *in-vitro* antiplasmodial activity results obtained in this study are in agreement with other reports on this plant. In a previous study, the methanolic and aqueous extracts of *L. calostachys* were active against *P. knowlensi* with an $IC_{50}$ of 3.45 µg/ml and 0.79 µg/ml respectively [9].

*Leucas calostachys* belongs to Lamiaceae (mint) family which is commonly used for management of pain and inflammation [14]. The Lamiaceae family is a rich source of anti-oxidants and anti-inflammatory agents thus making it important in management of malaria [7, 15]. Furthermore, it is postulated that anti-oxidants modulate plasmodial infection [16, 17]. A review of the genus *Leucas* by Chouhan & Singh [7], showed that the genus is rich in phenolic compounds which serve as anti-oxidants. The antiplasmodial activity of the methanol extract of *L. calostachys* in this study may be attributed to presence of phenolic compounds.

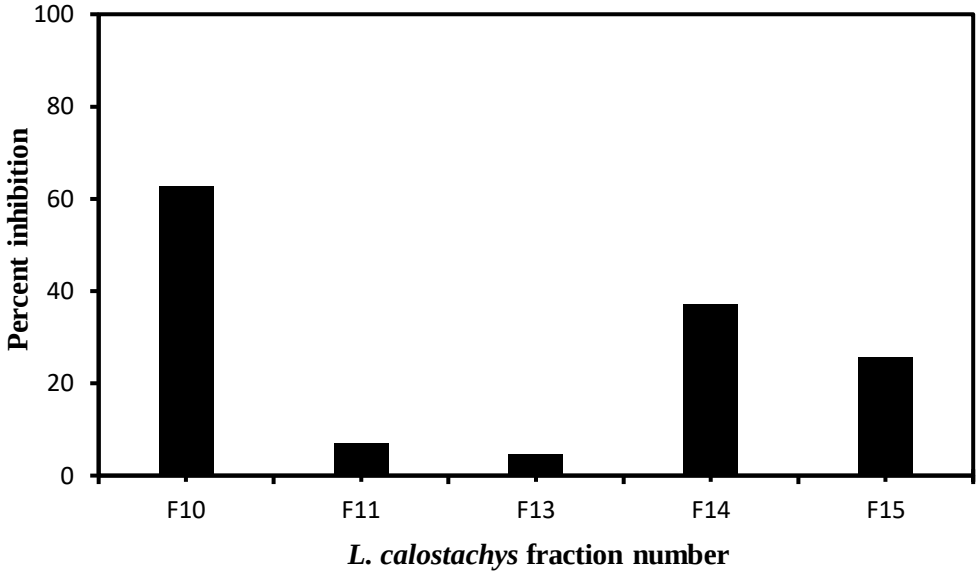


**Figure 3: β-Hematin inhibition assay of *Leucas calostachys* fractions**

**Characterization of compounds**

Fraction F10 which exhibited the highest activity in the hematin assay was analyzed for its phytoconstituents using LC-MS as shown in Figure 4.

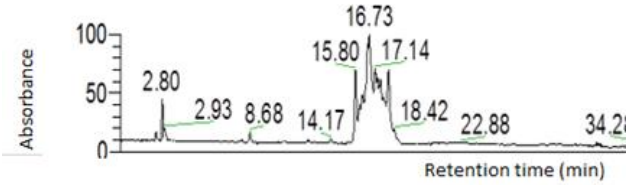


**Figure 4: Chromatographic profile of the phytoconstituents of fraction F10**

Most of the compounds eluted between 14 - 18 minutes retention time. The identification of the compounds was successfully achieved based on accurate mass and fragmentation patterns from previous published literature data. Based on this analysis, the compounds identified were polyphenolics broadly classified into two main groups, namely, flavonoidal glycosides and phenylethanoid glycosides as shown in Table 1. Fragmentation patterns of polyphenolic compounds have been extensively studied and their data were compared with mass spectroscopy data obtained in this study [18-20].

**Table 1: Mass spectral data and identity of compounds from *Leucas calostachys***

| No. | tR | $[M-H]^-$ | Mol. Formula | $MS^2$ | Proposed Identity | References |
|---|---|---|---|---|---|---|
| **1** | 6.10 | 639.1939 | $C_{29}H_{36}O_{16}$ | [639.19], 621.18 (30), 459.15 (10), 179.03 (55), **161.02 (100)** | β-OH-acteoside | [21] |
| **2** | 7.92 | 651.1934 | - | [651.19], 487.14 (10), **179.03 (100),** 161.02 (50) | Unidentified phenylethanoid | |
| **3** | 8.07 | 755.2414 | $C_{34}H_{44}O_{19}$ | [755.24], 593.20 (40), 461.16 (10), 623.20 (5), 179.03 (5), 161.02 (50) | Fortythoside B or Samioside | [22] |
| **4** | 8.60 | 623.1990 | $C_{29}H_{36}O_{15}$ | [623.19], 461.16 (20), 179.03 (5), **161.02 (100)** | Verbascoside (Acteoside) | [21,23] |
| **5** | 9.08 | 637.1779 | - | [637.17], 487.14 (10), 325.09 (5), **179.03 (100)** | Unidentified phenylethanoid | |
| **6** | 9.74 | 735.2210 | - | [735.22], **623.19 (100),** 461.16 (20), 179.03 (5), 161.02 (90) | Unidentified phenylethanoid | |
| **7** | 10.18 | 769.2573 | $C_{35}H_{46}O_{19}$ | [769.25], 593.20 (40), 637.21 (5), 461.16 (10), 193.05 (2), 175.03 (30), 161.02 (10) | Alyssonoside | [24, 25] |
| **8** | 10.38 | 651.1934 | - | [651.19], 623.19 (20), 487.14 (15), 323.07 (25), **179.03 (100),** 161.02 (90) | Unidentified phenylethanoid | |
| **9** | 10.60 | 755.2414 | $C_{34}H_{44}O_{19}$ | [755.24], 593.20 (30), 461.16 (5), 179.03 (5), **161.02 (30)** | Fortythoside B or Samioside | [21,23] |
| **10** | 10.82 | 637.2147 | $C_{30}H_{38}O_{15}$ | [637.21], 461.16 (40), 193.05 (20), **175.03 (100),** 161.02 (40) | Leucoseptoside A | [23] |
| **11** | 11.01 | 623.1987 | $C_{29}H_{36}O_{15}$ | [623.19], 461.16 (30), 179.03 (5), **161.02 (100)** | IsoVerbascoside (Isoacteoside) | [21] |
| **12** | 11.87 | 447.0939 | $C_{21}H_{20}O_{11}$ | [447], 310 (10), 286 (15), 285 (100), 284 (40), 174 (10) | Luteolin-7-O-glucoside (cynaroside) | [26, 27] |
| **13** | 14.13 | 431.0986 | $C_{21}H_{20}O_{10}$ | [431], 311 (3), 269 (40), 268 (100) | Apigenin-O-glucoside | [28] |
| **14** | 14.22 | 803.2414 | - | [803.24], **623.19 (100),** 461.16 (20),175.03 (2), 161.02 (30) | Unidentified phenylethanoid | |

| No. | tR | $[M-H]^-$ | Mol. Formula | $MS^2$ | Proposed Identity | References |
|---|---|---|---|---|---|---|
| **15** | 15.10 | 431.0989 | $C_{21}H_{20}O_{10}$ | [431], 311 (3), 269 (100), 268 (90) | Genistein-O-glucoside | [28, 29] |
| **16** | 15.30 | 447.0915 | $C_{21}H_{20}O_{11}$ | [447], 285 (100), 286 (15) | Luteolin 4′- O-glucoside | [30, 31] |
| **17** | 15.61 | 461.1094 | $C_{22}H_{22}O_{11}$ | [461], 299 (100), 284 (38), 283 (40), 269 (10) | Chrysoeriol 7-O-glucoside | [32,33] |
| **18** | 16.30 | 475.0887 | $C_{22}H_{20}O_{12}$ | [475], 299 (60), 285 (100), 284 (90) | Chrysoeriol 7-O-glucuronide | [34] |
| **19** | 16.80 | 315.0515 | $C_{16}H_{12}O_7$ | [315], 300 (100), 228 (7), 201 (10), 165 (5), 136 (20), 132 (7), 65 20) | Isorhamnetin | [27] |
| **20** | 17.37 | 609.1259 | $C_{27}H_{30}O_{16}$ | [609], 447 (3), 323 (5), 285 (100), 161 (10) | Luteolin diglucoside | [35] |

tR – retention time. Figures in parentheses represent percent ion abundance

This study reports the identification of compounds from *Leucas calostachys* for the first time (Table 1). However, several other flavonoids have been isolated from the genus *Leucas* including baicalein, cirsimaritin, acacetin, chrysoeriol, apigenin, 5-hydroxy-7,4′-dimethoxyflavone, tricin, pillion and gonzalitosin I among others [7]. Flavonoids are widely distributed plant polyphenolic compounds which differ in structures based on the degree of hydroxylation, polymerization and conjugation. They occur in plants as aglycones, methyl derivatives or glycosides.

Flavonoid containing plants are frequently applied in the management of malaria in most ethnomedicine settings. Since, malaria pathogenesis is associated with inflammation and oxidative stress [36], the anti-inflammatory and anti-oxidant activity of flavonoids, coupled with the induction of protective enzymes are important in preventing/slowing the progression of infection. In addition, flavonoids are known to possess direct antiplasmodial effects. They inhibit the growth of malaria parasites as well as potentiating the effects of other antimalarial drugs such as artemisinins [37,38]. They are believed to act by inhibiting the biosynthesis of fatty acid within the malaria parasites [39]. The active extracts from Kenyan antimalarial plants such as *Erythrina abyssinica, Milletia usaramensis* and *Derris trifoliate* are rich in flavonoids [40].

Flavonoids exhibit both *in vitro* antiplasmodial and β-hematin inhibition activities. A study by Vargas [41], demonstrated that apigenin and luteolin exhibited both *in vitro* antiplasmodial and β-hematin inhibition activities. However, some flavonoids such as catechine, epicatechine and morin lacked *in vitro* antiplasmodial activity but showed β-hematin inhibition activities in a study carried out by Bero *et al.* [42]. Among the identified flavonoids in this study, isorhamnetin, luteolin 7-O-glucoside and chrysoeriol 7-O-glucoside have showed moderate *in vitro* antiplasmodial activities against K1 *P. falciparum* in a previous study [43].

Phenylethanoids are widely distributed in medicinal plants where they play an important role both in prevention and treatment of various diseases such as infections, inflammation and tumors. They are also potent anti-oxidants [44]. Several plants used in traditional medicine in the

management of malaria have phenylethanoids. *Stachytarpheta cayennensis* (Verbenaceae) commonly used in Latin America for treatment of malaria is rich in phenylethanoids such as acteoside, isoacteoside, leucosceptoside A, martynoside and jionoside. *Duranta erecta*, a herb used in China and India for management of malaria is also rich in acteoside, in addition to flavonoids and iridoid compounds [45]. Phenylethanoids also contribute to the antimalarial properties of plants such as *Ajuga laxmannii, Stachys lavandulifolia*, and *Grevillea "Poorinda Queen*" [46, 47].

## CONCLUSION

The methanolic extract of *Leucas calostachys*, a commonly used plant in management of malaria, showed both *in-vitro* antiplasmodial activity and β-hematin inhibition activity. The phytochemical evaluation of this fraction revealed presence of flavonoids and phenylethanoids. These compounds should be further investigated for their individual anti-malarial activities.

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## REFERENCES

[1]. K. B. Musyoka; J.N. Kiiru; E. Aluvaala; P. Omondi; W.K.Chege; T. Judah, T.; D. Kiboi; J.K Nganga; F.T. Kimani. Infect Genet Evol. 78, 2020, 104121.

[2]. L. Ndwiga; K.M. Kimenyi; K. Wamae; V. Osoti; M. Akinyi; I. Omedo; D.S. Ishengoma; N. Duah-Quashie; B. Andagalu; A. Ghansah; A. Amambua-Ngwa; S. Tukwasibwe; S. Tessema; C. Karema; A. A. Djimde; A. M. Dondorp; J. Raman; R.W. Snow; P. Bejon; L.I. Ochola-Oyier. Int J Parasitol: Drugs Drug Resist. 16, 2021, 155-157.

[3]. N. Mukungu; K. Abuga; F. Okalebo; R. Ingwela; J. Mwangi. J Ethnopharmacol. 194, 2016, 98-114.

[4]. H. Beentje; J. Adamson; D. Bhanderi. Kenya Trees, Shrubs, and Lianas; National Museums of Kenya, 1994, 312.

[5]. P. Jeruto; C. Mutai; G. Ouma. J Anim and Plnt Sc. 9, 2011, 1192.

[6]. F. Some. J Biodivers Bioprosp Dev. 01, 2014,34-37.

[7]. H. Chouhan; S.J. Singh. Pharmacogn Phytotherapy. 3, 2011, 112-117.

[8]. P. Jeruto; R. Nyangacha; C. Mutai Afr J Pharmacy Pharmacol. 9,2015, 505-510.

[9]. G. Nyambati; Z.L. Osiemo; R. Maranga; S. Mong'are; H. J. Ozwara. Pharm Sci 3,2013, 57-63.
[10]. D. O. Okach; A.R.O. Nyunja; G.T. Opande. Int J Herb Med. 1, 2013, 135-142.
[11]. W. Trager; J. B. Jensen. Science. 193,1976, 673-675.
[12]. World Health Organization (WHO); 2nd rev ed.; Malaria Unit, Ed.; World Health Organization: Geneva, 2001.
[13]. R.D. Sandlin; K.M. Fong; K.J. Wicht; H.M. Carrell; T.J. Egan; D.W.Wright. Int J Parasitol: Drugs Drug Resist. 4,2014,316-322.
[14]. C.M. Uritu; C.T. Mihai; G.D. Stanciu; G. Dodi. Pain Res Manag. 2018, 7801543.
[15]. K. Carović-Stanko; M. Petek; M. Grdiša; J. Pintar; D. Bedeković; M. Herak Ćustić; Z. Šatović. Czech J Food Sci. 34, 2016, 377.
[16]. T. P. Arrey; Atchan, A. P. N.; J.K Kuiate; F.A. Okalebo; A.N. Guantai; G.A. Agbor. Adv Pharmacol Sci. 2013, 678458.
[17]. S. Percário; D.R. Moreira; B.A. Gomes; M.E. Ferreira; A.C. Gonçalves; P.S. Laurindo; T.C. Vilhena; M.F. Dolabela; M.D. Green. Int J Mol Sci. 13, 2012, 16346.
[18]. R.N. Ibrahim; A.M. El-Halawany; D.O. Saleh; E. Naggar; A. E. El-Shabrawy; S.S. El-Hawary. Rev. bras. farmacogn. 25, 2015, 134-137.
[19]. Z.H. Li; H. Guo; W.B. Xu; J. Ge; X. Li; M. Alimu; D.J. He. J Chromatogr Sci. 54, 2016, 805-811.
[20]. K. Ablajan; Z. Abliz; X.Y. Shang; J.M. He; R.P.Zhang; J. G. Shi. J Mass Spectrom. 41, 2006, 352-357.
[21]. J. Gong; H. Miao; X. Sun; W. Hou; J.H. Chen; Z.Y. Xie; Q.F. Liao. Anal Methods. 8, 2016, 6323.
[22]. A. Cardinali; S. Pati; F. Minervini; I. D'Antuono; V. Linsalata; V.J. Lattanzio. Agric Food Chem 60, 2012, 1822.
[23]. I. Grzegorczyk-Karolak; A.K. Kiss. Molecules. 23, 2018, 97-105.
[24]. O. T. Agar; I. Irem TatliCankaya In Recent Advances in Natural Products Analysis; Sanches, S. A., S.F. Nabavi, M. Saeedi, Nabavi, S. M., Eds.; Elsevier: 2020, p 221.
[25]. J. Petreska; G. Stefkov; S. Kulevanova; K. Alipieva; V. Bankova; M. Stefova. Nat Prod commun. 6, 2011, 21-28.
[26]. Z.H. Li; H. Guo; W.B. Xu; J. Ge; X. Li; M. Alimu; D.J.J He. Chromatogr Sci. 54, 2016, 805-813.
[27]. A. Marston; K. Hostettmann In Flavonoids: Chemistry, Biochemistry and Applications; O.M. Andersen and K.R. Markham, E., Ed.; CRC Press: USA, 2005, p 1.
[28]. P. Kachlicki; A. Piasecka; M. Stobiecki; L. Marczak. Molecules. 21, 2016,1494.
[29]. S.T. Soukup; B. Spanier; G. Grünz; D. Bunzel; H. Daniel; S.E. Kulling. PloS One. 7,2012, e46914.
[30]. M. Pikulski; J. S. Brodbelt. J Am Soc Mass Spectrom. 14, 2003, 1437.
[31]. Y. Zhao; X. Li; X. Zeng; S. Huang; S. Hou; X. Lai. Anal Methods. 6, 2014, 1359-1363.
[32]. W. H. Hassan; S. Abdelaziz; H.N.A Yousef. Arab. J Chem. 12, 2019, 377-383.
[33]. M. El-Sayed; A.M. Al-Gendy; D. Hamdan; A.E. Sahzly. J Pharm Sci Res**.** 9, 2017, 375.
[34]. C. Quispe; M. Villalobos; J. Bórquez; M.J. Simirgiotis. J Chem. 2018, 6123850.
[35]. H.J. Chen; B.S. Inbaraj; B.H. Chen. Int J Mol Sci. 13, 2012, 260-267.
[36]. Ty, M. C.; Zuniga, M.; Götz, A.; Kayal, S.; Sahu, P. K.; Mohanty, A.; Mohanty, S.; Wassmer, S. C.; A. Rodriguez. 11,2019, e9903.
[37]. A. Bilia; A. Sannella; F. Vincieri; L. Messori; A. Casini; C. Gabbiani; C. Severini; G. Majori. Nat Prod commun**.** 3, 2008, 1999-2003.
[38]. T. Czechowski; M.A Rinaldi; M.T. Famodimu; M. Van Veelen; T.R. Larson, T.; T. Winzer; D.A. Rathbone; D. Harvey; P. Horrocks; Graham., I. A. Front. Plant Sci. **2019**, 10.
[39]. F. Ntie-Kang; P.A.Onguéné; L. L. Lifongo; J. C.Ndom; W. Sippl; Mbaze., L. M. Malar J **2014**, 13, 81.
[40]. A. Yenesew; S. Derese; J.O. Midiwo; H.A. Oketch-Rabah; J. Lisgarten; R. Palmer; M. Heydenreich; M.G. Peter; H. Akala; J. Wangui; P. Liyala; N.C. Waters. Phytochemistry**.**6 4, 2003, 773.

[41]. S. Vargas; K. Ndjoko Ioset; A.E. Hay; J.R. Ioset; S. Wittlin; K. Hostettmann. J Pharm Biomed Anal. 56, 2011, 880.
[42]. J. Bero; M. Frédérich; J. Quetin-Leclercq. J Pharm Pharmacol. 61,2009. 1401.
[43]. W.H. Pan; X.Y. Xu; N. Shi; S.W. Tsang; J.H. Zhang. Int J Mol Sci. 19, 2018, 412-414.
[44]. Z. Xue; B. Yang. Molecules. 21, 2016, 345-349.
[45]. M. Srivastava; M. Singh; P. Maurya; N. Srivastava; N. Gupta; K. Shanker. J Pharm Biomed Anal. 174, 2019,711.
[46]. I. Atay; H. Kirmizibekmez; M. Kaiser; G. Akaydin; E. Yesilada; D. Tasdemir. Pharm Biol. 54, 2016, 1808.
[47]. S. P.Ovenden; M. Cobbe; R. Kissell; G.W. Birrell; M. Chavchich; M.D. Edstein. J Nat Prod. 74, 2011, 74-81.