



## ***In vitro* Anti-malarial Activity Evaluation of Extracts from Plants often used in the East African Region**

**Janvier Habinshuti<sup>1\*</sup> and T. Akenga<sup>2</sup>**

<sup>1</sup>*Department of Chemistry, College of Science and Technology, University of Rwanda, Avenue de l'armée, P.O.Box 3900 Kigali, Rwanda.*

<sup>2</sup>*Department of Chemistry and Biochemistry, University of Eldoret, P.O.Box 1125-30100 Eldoret, Kenya.*

### **Authors' contributions**

*This work was carried out in collaboration between both authors. Authors JH and TA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. They managed the analyses of the study, literature searches, read and approved the final manuscript.*

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### **ABSTRACT**

Biodiversity plays vital roles in maintaining human and animal health. A wide variety of plants, animals, and fungi are used as medicine, essential vitamins, painkillers. Natural products have been recognized and used as medicines by ancient cultures all around the world. About 119 pure chemicals are extracted from less than 90 species of higher plants and used as medicines throughout the world, for example, artemisinin and quinine for treatment of malaria. Malaria is the most important public health problem in tropical and sub-tropical Africa, and it is becoming more and more difficult to control. Although several attempts have been made on vaccine development, chemotherapy and vector control are currently the mainstays of malaria control. However, with increasing cases of drug-resistant strains of malaria parasites and expensive anti-malarial drugs coupled with the poor distribution of modern health facilities, there is a resurgence in use of herbal remedies to treat malaria and other infectious diseases, before seeking the conventional western remedies. Although the use of herbal preparations for malaria is widespread in the Lake Victoria basin, there has been no previous validation of their efficacy and safety. Furthermore, there are no

\*Corresponding author: E-mail: [janvierhabinshuti@gmail.com](mailto:janvierhabinshuti@gmail.com);

standard practices for quality assurance in sourcing of the herbal anti-malarial drugs. In this paper, a survey of the plants used for treatment and management of malaria in the Lake Victoria was carried out. Organic and water extracts from these plants were subjected to *in vitro* anti-plasmodial assays using W2 (CQ resistant) and D6 (CQ sensitive) strains. The results obtained to authenticate the use of these plants as anti-malarial herbs. A set of compounds have been isolated and characterized from the plant species that exhibited high anti-plasmodial activity.

**Keywords:** Anti-malarial; in-vitro; plant extracts; malaria therapy.

## 1. INTRODUCTION

The relationship of biodiversity to human health has relevance to all eight Millennium Development Goals (MDGs) [1,2] but it has special and fundamental importance for goals 1, 4, 5, 6, and 7. Human health is dependent on biodiversity and the natural functioning of healthy ecosystems. Biodiversity supports human life and promotes health through many ways. These include: providing, at the most basic level, ecosystem services; medicines from plants, animals, and microbes; Providing models for medical research that help us understand normal human physiology and disease; Supporting agriculture and the marine food web; Reducing the risk of contracting some human infectious diseases through the "dilution effect"; by controlling populations of vectors, hosts, and parasites; and by other means. This paper focuses on the provision of medicines from plants as an important role of biodiversity. Plants have been utilized as medicines for thousands of years [3]. In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19<sup>th</sup> Century [3,4]. Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use [3,5,6]. Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products, and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs) [5–7]. In both 2001 and 2002, approximately one-quarter of the best selling drugs worldwide were natural products or derived from natural products [6]. There are also a new set of medicinal plant-derived drugs that have been recently introduced to treat chloroquine resistant malaria. These drugs (artemether and arteether) are

derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae), a plant used in traditional Chinese medicine [8–11].

## 2. THE LAKE VICTORIA REGION AND MALARIA

The lake is shared between Kenya (6%), Tanzania (51%) and Uganda (43%). The lakes' catchment area covers 193,000 km<sup>2</sup> (Tanzania 44 %, Kenya 22 %, Uganda 16 %, Burundi 7 %, Rwanda 11 %). The human population is high, estimated to be about 30 million [12–15] growing rapidly, and heavily concentrated near the lake [16–21]. The lake basin supports one of the densest and poorest rural populations in the world [15,22–25]. Inter-governmental Panel on Climate Change [26–28] studies show that more than 90% of global mortality due to malaria occurs in Africa, and it is the number one killer of children, pregnant women and the elderly on the continent [12,29–33]. It is also the leading cause of infant mortality (20%) and constitutes ten per cent of the continent's overall disease burden [34–37]. The disease deprives Africa of US\$ 12 billion every year in lost Gross Domestic Product (GDP).

In Rwanda malaria cases and deaths in children < 5 years old reported to be more that 60% although declined in recent times due to the Nationwide execution of long-lasting insecticidal nets (LLIN) and artemisinin-based combination therapy (ACT) disseminated countrywide by 2007 [38–40]. While in Kenya, 40,000 infants' deaths are attributed to malaria every year. In 2002 and 2003 in Uganda, there were 5.7 and 7.1 million cases of malaria resulting in 6,735 and 8,500 deaths respectively. In Tanzania, malaria causes between 70,000 and 125,000 deaths annually and accounts for 19 percent of the health expenditure [41,42]. Thus in the East African countries, malaria is ranked as the first cause of morbidity and mortality in both children and adults. The disease is endemic in the

lowlands but unstable in the highlands of the Lake Victoria region. Several factors have been identified as contributing to the emergence and spread of malaria. These include environmental and socioeconomic change, deterioration of health care and food production systems, and the modification of microbial/vector adaptation [29,32,43–46]. For example, stresses on productive land force farmers to clear forests and reclaim swamps, affecting the biodiversity. Papyrus, found in many of the swamps in valley bottoms of the East African highlands, excrete oil and provide shade, which inhibits *Anopheles gambiae* reproduction [47] but the swamps are at a threat from human activity. The adaptations to malaria commonly applied by communities in this region include traditional curative measures, use of bed nets and more recently, the use of ITNs [22,48–50]. Several factors account for the use of local herbs, well known and familiar to most people with easy availability. These herbs are less expensive, and effective as first aid before taking the patient to hospital or health center. There are several plants, which have been used by people living in East African region particularly in malaria endemic zone, to manage and treat malaria [51–58]. Although the use of herbal preparations for malaria is widespread in the region, there is no evidence of their efficacy and safety. There is also no standard practice, quality assurance in sourcing of the herbal anti-malarial drugs. These problems need to be addressed through systematic and scientific research. There is need to carry out a detailed survey of and document anti-malarial plants used in the wetlands not only the northern Lake Victoria basin (Kenya & Uganda), but in East-African region in a broad-spectrum. There is need to use modern scientific methods to carry out systematic investigations to establish the efficacy and toxicity of the plants with the overall aim of preserving and conserving the plant species to avoid loss of such plants.

### 3. MATERIALS AND METHODS

#### 3.1 Study Sites

Reported traditional anti-malarial plants were collected from several wetlands in the Lake Victoria region. Anti-malarial plants were collected from wetlands in Kenya (Suba, Homa Bay, Migori Rachuonyo and Bondo Districts) and Ugandan (Mbarara, southern and southwestern Districts). The plants were identified, and voucher specimens

deposited in the herbaria at the Botany Departments at the University of Nairobi and Makerere University.

#### 3.2 Plant Collection, Extraction, Isolation, Purification and Structure Determination

Plants for the study were sampled according to the documented ethnobotanical practices. The plants were collected and dried under shade, ground into powder, extracted sequentially with solvents of increasing polarity and analyzed through *in-vitro* anti-malarial assays. Plant species with promising *in-vitro* anti-plasmodial activity were subjected to bulk extraction, and fractionation of the active principles was done using column chromatography. The *in vitro* anti-malarial assays were done on each fraction and the active ones purified to single compounds using a combination of column chromatography (CC), preparative thin layer chromatography (PTLC), medium pressure liquid chromatography (MPLC) and high-performance liquid chromatography (HPLC). The structures of pure compounds were determined using spectroscopic techniques, like ultra violet (UV), infrared (IR), mass spectroscopy (MS) and 1D ( $^1\text{H}$ ,  $^{13}\text{C}$ , DEPT, NOE) and 2D (COSY, NOESY, ROESY, HMQC HSQC & HMBC) nuclear magnetic resonance (NMR) techniques.

#### 3.3 *In vitro* Screening for Antiplasmodial Activity

The anti-plasmodial assays were done by the *in vitro* technique that measures the ability of the extracts or compounds to inhibit the incorporation of [ $\text{G-}^3\text{H}$ ]-hypoxanthine into the DNA of malaria parasites through assessment of the inhibitory concentration 50s ( $\text{IC}_{50\text{s}}$ ) according to [59,60]. Chloroquine-sensitive and sulfadoxine-pyrimethamine-sensitive and resistant strains of *P. falciparum* were used. The data were analyzed according to the method of [61]. The stock parasites were maintained according to [62–64].

### 4. RESULTS AND DISCUSSION

#### 4.1 Plants Identified

Thirty-three traditional anti-malarial plants belonging to 19 families were collected (Table 1).

**Table 1. List of collected traditional anti-malarial plants from the Lake Victoria region**

Plant species	Family	Plant species	Family
<i>Hypoestes forskolii</i>	Acanthaceae	<i>Strychnos usambarensis</i>	Loganiaceae
<i>Carrisa edulis</i>	Apocynaceae	<i>Azadirachta indica</i>	Meliaceae
<i>Periploca lineriaefolium</i>	Asclepiadiaceae	<i>Cissampelos mucronata</i>	Menispermaceae
<i>Cryptolepis sanguinolenta</i>	Asclepiadiaceae	<i>Stephania abyssinica</i>	Menispermaceae
<i>Cryptolepis hypogleuca</i>	Asclepiadiaceae	<i>Fiscus sur</i>	Moraceae
<i>Kigelia africana</i>	Bignoniaceae	<i>Myrica salicifolia</i>	Myricaceae
<i>Maytenus heterophylla</i>	Celastraceae	<i>Parinari curatelifolia</i>	Rosaceae
<i>Microglossa pyrifolia</i>	Compositaeae	<i>Fagaropsis angolensis</i>	Rutaceae
<i>Aspilia pluriceta</i>	Compositaeae	<i>Teclea nobilis</i>	Rutaceae
<i>Cyperus dives</i>	Cyperaceae	<i>Zanthoxylum usambarensis</i>	Rutaceae
<i>Cyperus sp A</i>	Cyperaceae	<i>Harrisonia abyssinica</i>	Simaroubaceae
<i>Neoboutonia macrocalyx</i>	Euphorbiaceae	<i>Withania somnifera</i>	Solanaceae
<i>Lippia javanica</i>	Labiataeae	<i>Clerodendrum myricoides</i>	Verbenaceae
<i>Acacia nilotica</i>	Leguminoseae	<i>Clerodendrum rotundifolia</i>	Verbenaceae
<i>Indigoferae emarginella</i>	Indigoferaeae	<i>Strychnos stuhlmanii</i>	Loganiaceae
<i>Senecio discifolius</i>	Asteraceae	<i>Ansellia africana</i>	Orchidaceae
<i>Strychnos heningsii</i>	Loganiaceae		

List of medicinal plants collected used to treat malaria in the region

## 4.2 Extraction and Antiplasmodial Assays

### 4.2.1 Water and methanol extracts

Ten of the plant species were initially subjected to sequential extraction with methanol and water, and *in vitro* anti-plasmodial assays using NF 54 and ENT30 chloroquine-sensitive and resistant *P. falciparum* strains, respectively (Table 2).

Generally, methanol extracts were more active than aqueous ones. Similarly, chloroquine-sensitive *P. falciparum* strains were also more sensitive to plant extracts than resistant ones. Plant species from Rutaceae (*F. angolensis* and *Z. usambarensis*) and Asclepiadaceae (*C. sanguinolenta*) were the most active. A well known traditional anti-malarial plant *H. abyssinica* was inactive *in vitro*. This phenomenon can be explained by the presence of pro-drugs that undergo enzymatic transformations *in vivo* to give the anti-plasmodial compounds. Alternatively, the compounds in these plants may be acting indirectly on effects of infection like fever or through immuno-stimulation.

### 4.2.2 Organic extracts

For each ground plant material (100 g), sequential cold extraction was done for 72 hours using hexane, chloroform (CHCl<sub>3</sub>) or dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), ethyl acetate (EtOAc) and methanol (MeOH). Selected plant extracts were subjected to antiplasmodial assay using D6, W2 and wild strains of *Plasmodium*. Overall

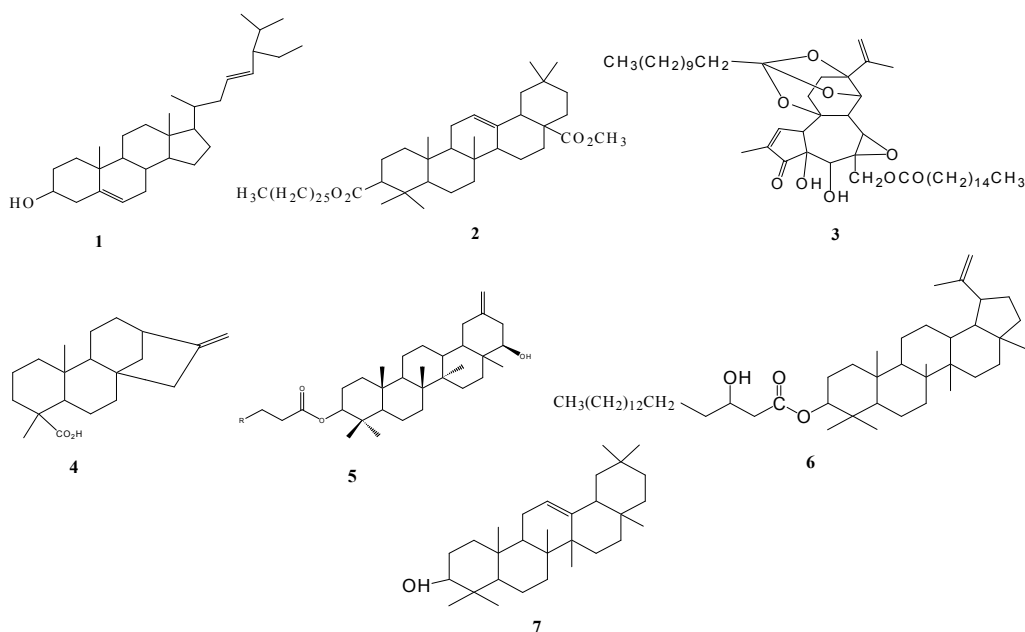
eight plant species were assayed against D6 and W2, while only four species were assayed against the wild strain of *Plasmodium*. The results are shown in Tables 3, 4 and 5.

The activity observed for the crude extracts explain the wide use of these plants to treat malaria in the Lake Victoria region. The values obtained are comparable to those of known plant remedies of various origins [60,65,65–75]. Applying the same system to that used in our study, the IC<sub>50</sub> of crude ethanolic extracts of *Artemisia annua* for the chloroquine resistant strain K-1 has been found to be 3.9 µgml<sup>-1</sup> [60]. The highest antiparasitic activities were detected in hydrophilic methanol extracts of *M. pyrifolia*, *L. javanica*, *C. mucronata*, *S.abyssinica*, *T. nobilis* and *P. linearifolia*. Several classes of plant secondary metabolites are responsible for the anti-malarial activity, but the most important and diverse bio-potency has been observed in alkaloids, quassinoids and sesquiterpene lactones [76,77,77–79]. The plants investigated during this study belong to various families, from which similar metabolites have been isolated. The most active extracts were obtained from six plants, and the phytochemical reports from these plants indicate that they contain similar metabolites. For instance, most plants from the Celastraceae family contain a class of sesquiterpene nicotinoyl alkaloids [80–83]. The alkaloids are characterized by a dihydroagarofuran sesquiterpene bonded as a diester to a substituted nicotinic acid. 1β-Acetoxy-9α-benzoyloxy-2β, 6α-dinicotinoyloxy-β-dihydroagarofuran, and β-amyryn have been

isolated from *M. heterophylla* [84]. The leaf extracts of *P. linearifolia*, of the Asclepiadaceae, contain phytin, anthraquinone, gemnamagenin, gymnemic acid II, betaine, choline and lupeol [85–87]. While *M. pyrifolia*, Compositae (Asteraceae) leaves have been reported to contain several terpenoids such as E-phytol, 1,3-hydroxyoctadeca –9Z, 11E, 15Z trien –oic acid, and 6E-geranylgeraniol –19-oic acid that exhibited the anti-plasmodial activity of IC<sub>50</sub> values between 2.5µg/ml and 13.7µg/ml [76,88–91]. Reported data on *L. javanica*, Verbenaceae, indicate that there are several terpenoids isolated from the flowering tops of this species. The terpenoids exhibit larvicidal, antimalarial, spasmolytic, sedative, hypotensive and anti-inflammatory activities [92–94].

#### 4.2.3 Chemical investigation of selected plants

Chemical investigation of *N. macrocalyx* organic extracts afforded stigmasterol (1) oleanolic acid diester (2), montanin-20-palmitate (3), two tiglane-type and two daphne-type diterpenes. However, none of these compounds exhibited anti-plasmodial activity. *H. forsaken* afforded Kaur-16-en-19-oic acid (4), 3-alkoxy-16-taxasterol (5) and other labdane-type diterpenoids. *P. linearifolia* extract yielded lupeol ester (6), β-sitosterol (7) and β-amyrin (8). The structures of these compounds were confirmed by using spectroscopic techniques (unpublished results) which was then compared with literature data; the results conformed with [95].



**Table 2. Anti-plasmodial activity (IC<sub>50</sub>±SD µg/ml) of plant extracts against NF54 and ENT30 *P. falciparum* strains**

Plant	NF54		ENT30	
	MeOH	H <sub>2</sub> O	MeOH	H <sub>2</sub> O
<i>C. sanguinolenta</i>	< 3.91		<3.91	
<i>Z. usambarensis</i>	3.20±0.45	5.25±0.27	5.54±1.70	14.33±4.42
<i>F. angolensis</i>	4.68±0.009	6.13±1.15	5.04±0.68	10.65±1.23
<i>M. salicifolia</i>	51.07±1.70	66.84±2.88	55.89±2.00	85.97±5.45
<i>S. heningsii</i>	157.91±10.03	67.16±8.70	190.0±16.85	73.39±9.75
<i>A. nilotica</i>	70.33±1.89	153.79±15.79	73.59±2.87	>250
<i>N. macrocalyx</i>	78.40±6.84	84.56±8.98	78.44±2.89	92.85±7.65
<i>H. abyssinica</i>	72.66±1.39	86.56±3.21	79.50±3.30	89.74±8.12
<i>W. somnifera</i>	125.29±1.30	> 250	145.86±2.23	> 250
<i>C. edulis</i>	> 250	148.52±12.63	> 250	>250

Ten plant species assayed against NF54 and ENT30 strains with MeOH: methanol extract; H<sub>2</sub>O and water extract

**Table 3. Anti-plasmodial activity (IC<sub>50</sub>±SD µg/ml) of organic extracts against *P. falciparum* D6 strain**

Plant	C <sub>6</sub> H <sub>14</sub>	CH <sub>2</sub> Cl <sub>2</sub>	EtOAc	MeOH
<i>H. forskolei</i>	14.12±3.75	4.0±1.25	4.11±0.11	4.92±0.44
<i>A. pluriseta</i>	15.98±0.13	10.29±1.44	ND	13.49±2.45
<i>C. mucronata</i>	8.73±1.81	10.09±1.56	< 3.91	19.78±
<i>S. abyssinica</i>	ND	<3.91	5.09±0.04	9.61±0.11
<i>C. myricoides</i>	42.96±5.05	101.0±35.59	37.30±0.75	5.02±0.56
<i>C. rotundifolia</i>	ND	18.73±0.40	5.81±0.47	8.06±0.35
<i>T. nobilis</i>	20.36±0.28	< 3.91	< 3.91	4.46±
<i>F. sur</i>	33.92±0.17	18.26±1.20	15.95±2.16	53.61±10.02

Hexane (C<sub>6</sub>H<sub>14</sub>), Dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), Ethyl acetate (EtOAc) and Methanol (MeOH) plant extracts assayed against D6 and W2 strains

**Table 4. Anti-plasmodial activity (IC<sub>50</sub>±SD µg/ml) of organic extracts against *P. falciparum* W2 strain**

Plant	CHCl <sub>3</sub>	EtOAc	MeOH
<i>M. pyrifolia</i>	30.05±0.97	25.03±0.51	2.50±0.15
<i>K. africana</i>	34.79±0.94	22.63±0.95	27.84±1.29
<i>L. javanica</i>	18.59±0.26	15.80±0.26	1.75±0.17
<i>C. edulis</i> (sb)	32.07±1.14	24.61±0.93	> 50
<i>C. edulis</i> (rb)	41.15±2.40	37.60±1.86	19.50±1.19
<i>S. usambarensis</i>	24.37±0.40	24.00±2.20	27.92±0.70
<i>S. heningsii</i>	16.20±2.38	37.43±0.96	26.25±0.04
<i>M. heterophylla</i>	5.56±0.78	29.04±0.85	19.53±4.40
<i>P. lineriafolium</i>	24.69±0.73	36.13±1.87	3.30±0.18

Chloroform (CHCl<sub>3</sub>), Ethyl acetate (EtOAc) and Methanol (MeOH) plant extract assayed against D6 and W2 strains

**Table 5. Anti-plasmodial activity (IC<sub>50</sub>±SD µg/ml) of extracts from selected medicinal plants against wild *P. falciparum* strains**

Plant	IC <sub>50</sub> ±SD µg/ml			
	H <sub>2</sub> O	C <sub>6</sub> H <sub>14</sub>	EtOAc	MeOH
<i>I. emarginella</i>	≥100.0	≥100.0	≥100.00	22.5 ± 4.9
<i>S. stuhlmanii</i>	69.3 ± 1.3	≥100.0	14.0± 1.4	≥100.0
<i>S. discobolus</i>	≥100.0	≥100.0	24.7 ± 8.1	≥100.0
<i>A. africana</i>	22.7 ± 7.5	≥100.0	9.3± 1.6	23.1 ± 7.5

Four plant species assayed against the wild strain of Plasmodium

## 5. CONCLUSION

The extracts of most plants used for the treatment of malaria in the Lake Victoria Region show antiplasmodial activity. These results support the use of these plants by traditional medicine practitioners (TMPs) for management of malaria. This work has contributed to documentation and scientific experimental verification of the herbalist's claims. We recommend that these plants be studied systematically to identify the active compounds

responsible for the anti-malarial effects observed. For these promising extracts, tested only *in vitro*, we suggest that the *in vivo* anti-malarial activity and several cytotoxicity tests, using mammalian cells, should be evaluated, as well as acute and chronic toxicity. Since many compounds isolated and identified as antimalarials are also cytotoxic and may not be suitable for drugs. There is urgent need to conserve the biodiversity in the Lake region, In addition, an inventory of the various medicinal plants and herbs which are used to treat common diseases should be set up. Local

botanical gardens should be established for the preservation of essential medicinal herbal plants, to ensure a sustainable supply of safe, effective and affordable medicinal herbs. Last, testing laboratories with adequate facilities for the assessment of the efficacy of medicinal herbs, and establishing dosage norms for the most efficacious herbal extracts should be set up.

### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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