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# *In vitro* Anti-plasmodial Activity of *Rubia cordifolia, Harrizonia abyssinica, Leucas calostachys* Olive *and Sanchus schweinfurthii* Medicinal Plants

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**ABSTRACT**

*Plasmodium falciparum* is becoming increasingly resistant to conventional antimalaria drugs. Rapid increase of parasite resistant strains, resistance of the vector to insecticides and the difficulty in creating efficient vaccines has lead to an urgent need for new anti-malarial drugs. To determine anti-plasmodial activity of *Rubia cordifolia, Harrizonia abyssinica, Sachus schweinfurthii* and *Leucas calostachys* Olive plants. Aqueous and methanolic crude extracts were prepared from *R. cordifolia, H. abyssinica, S. schweinfurthii* and *L. calostachys* plants. The extracts were then prepared into appropriate concentrations for anti-plasmodial activities. In vitro anti-plamsodial activities of herbal drugs were analysed according to the methods of Tona et al., 1999. Methanolic extracts were more efficacious than aqueous extracts. *S. schweinfurthii* and *L. calostachys* had $IC_{50}$ (Inhibition Concentration) of between 1.10µg/ml and 3.45µg/ml and had highest parasite inhibition ranging between 3.5% and 5.2%. *R. cardifolia* and *H. abyssinica* had $IC_{50}$ of between 1.5µg/ml and 3.0µg/ml and it had moderate parasitaemia ranging between 5.20% and 7.22%. *Vernonia lasiopa* and *Erythrina abysinnica* had insufficient yields. *S. schweinfurthii* and *L. calostachys* had the highest parasite inhibition while *R. cardifolia* and *H. abyssinica* had moderate inhibition.

## INTRODUCTION

*Plasmodium falciparum* is the most widespread etiological agent of human malaria. This parasite is becoming increasingly resistant to conventional antimalaria drugs hence necessitating a continuous effort in search of new drugs (Tran *et al.,* 2003). In Sub-Saharan Africa, over 50% of all outpatient visits and 30% - 50% of all hospital admissions are attributed to malaria (WHO, 2005; Muregi, 2007). It is estimated that economic loses due to malaria in Africa is about $12 billion annually (DFID, 2005). Although an effective vaccine is the best long term control option for malaria, current work on vaccine development largely remains at preclinical stage. The declining efficacy of classical drugs due to increase of parasite resistant strains, resistance of vectors to insecticides and the difficulty in creating efficient vaccines has lead to an urgent need for new anti-malarial drugs (Bloland, 2001; Ridley, 2002). While synthetic pharmaceutical agents continue to dominate in research, attention has been increasingly directed to natural products (Atkin, 2003). Anti-malarial properties of Cinchona bark has been known for more than 300 years (Muthaura *et al.,* 2007). Currently, the commonly used anti malarial drugs, quinolines and the peroxide antimalarial (artemisinin derivatives) are modeled upon the plant based compounds, quinine and artemisinin respectively. The success of artemisinin and its derivatives for the treatment of resistant malaria has focused attention to plants as a source of anti-malarial drugs (Tran *et al.,* 1998). Malaria is prevalent among the world's poorest population, who treat themselves with traditional herbal drugs. Herbal drugs are available and affordable to majority of the infected. These drugs are sometimes perceived as more effective than convectional anti-malarial drugs (Merlin, 2004). Studies on anti-malarial activity of Kenyan traditional medicinal plants shows that extracts from plants such as *Hagenia abysinica, Artemisia rehani* and *Ajuga remota* (Kassa *et al.,* 1998) as well as *Withania*

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*somenifer* and *Vernonia amygdalina* (Benoit, 1996) *Vernonia brachycalyx* (Oketch-Rabah *et al.*, 1997; 1998; 2007), have significant anti-malarial activity against *Plasmodium falciparum*. Other *in vitro* studies on African medicinal plants have also indicated promising anti-plasmodial activities (O'Neill *et al.*, 1985; WHO, 1993) although no remarkable *in vivo* studies have been reported so far to strengthen the preclinical study profile.

## MATERIALS AND METHODS

### Collection and preparation of plant materials

Leaves, roots, stem barks and flowers of eight medicinal plants from five families (Table 1) were collected from Transmara, Suba and Kuria districts, Kenya. Voucher specimens of the plant parts were taken to the East African Herbarium in Nairobi for identification and future referencing. The harvested parts were air dried at room temperature for 10 days. The parts were then powdered using food processor/blender (Multi-purpose Kanchan, Tornado) into a fine powder. Plant materials were then packed into air tight plastic containers, stored in the dark before being transported to the phytochemistry laboratory for further processing.

### Extraction of plant crude extracts

The sample extraction procedure was carried out as described by Harbone 1994. Briefly, 150gms of powdered plant material was soaked in 250mls of methanol at room temperature for 48 hours. The materials were filtered with Whatman filter paper No. 1 and were further soaked in 250ml methanol for for 48 hours until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 40°C. The concentrate was weighed and transferred to an air tight sample bottle and stored at -20°C in the cold room until required for bioassay. Aqueous extracts were obtained by weighing 150gms of the sample and soaking in 250ml of distilled water that was placed in a water bath at 60°C for 24 hrs. The extracts were obtained through vacuum filtration using Whitman's filter papers No. 1 after which the aqueous filtrates were lyophilized for 48hours. Drying was achieved through sublimation under vacuum where the extracts were subjected to a temperature of -10$^{o}$C to avoid any qualitative and quantitative change. The extracts were concentrated to crystalline powder form, weighed and stored at 4$^{o}$C in the cold room until required for bioassays (Tona *et al.*, 2004; Bourdy *et al.*, 2004). Stock solutions of 50mg/ml were made with de-ionized water and filtered through 0.45 µm and 0.22 µm microfilters in the laminar flow hood. Insoluble aqueous extracts were first dissolved in 50µl of Dimethylsulfoxide (DMSO) solvent then vortexed for one minute to dissolve the extract. The extract was then dissolved in 50µl RPMI 1640 culture medium.

### *In vitro* anti-Plasmodial Assays *with Plasmodium knowlesi*

Anti-plasmodial assays were carried out at the Institute of Primate Research of the National Museums of Kenya. The *in vitro* anti-plasmodial activities were evaluated according to the method described by Tona *et al.*, 1999. Briefly, assays were performed in duplicate in 96-well microtiter flat-bottomed plates (Coster Glass Works Cambridge, UK). Aliquots of culture medium (100µl) were added to all the wells of the 96 plate. Then, 100µl of the test solutions were added in duplicates to the first well and a Titertik motor hand diluter was used to make two-fold serial dilution.

For assays, serial dilutions were made by first dispensing 100µl of complete RPMI into each well followed by 100µl of 100ug/ml extract concentration as a start concentration in the first well (AI) of a 96-well microtiter plate. Using a micropipette, 100µl was then drawn from the first well and used to make eight-fold serial dilutions down the plate, giving a concentration range of 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.2µg/ml, 1.6µg/ml, 0.8µg/ml, 0.4µg/ml.

A suspension of (10µl, 1.5v/v) of parasitized erythrocytes at 1.6% parasitaemia were dispensed to each well, bringing the total volume per well to 110µl. The plates were transferred into an air tight chamber gassed for 3 minutes then incubated in $CO_2$ condition at 37°C for 24-30 hours. After 48hrs of incubation, contents of the wells were harvested into eppendorf tubes, centrifuged for 3 seconds and the pellet was sucked onto a microscope slide to make a smear. This was Giemsa stained after which, the developed schizonts were counted against the total 2000 erythrocytes. Microscopic examination of Giemsa stained smears was done after every 48hrs to check for the viability of parasites.

The differential counts were done to determine the parasitaemia levels for each extract in each well. The average $IC_{50}$ values were calculated as a fraction of the starting parasitaemia relative to growth in extract free wells *(*Jansen *et. al.* 2006). Negative control consisted of the solvent while positive controls were carried out using WHO approved herbal drug *Artemisia annua* which is used for treatment of malaria.

### Determination of $IC_{50}$ of Extracts on *Plasmodium knowlesi*

The concentration of each herbal extract that inhibited 50% of parasite growth was calculated as a fraction of the starting parasitaemia relative to the growth in the control wells. From the starting parasitaemia of 1.6%, parasitaemia was determined through examination of Giemsa-stained thin smears. The percentage growth in each test well was therefore calculated from individual parasitaemia and growth inhibition determined as the difference between the mean percentage growth in control wells and percentage growth of each test well. The $IC_{50}$ values were selected for extracts that displayed 50% growth inhibitions at very low concentrations.

### Data Analysis

The collected data on anti-plasmodial activity and parasitaemia were transferred into Microsoft Excel spreadsheet which was used to determine the $IC_{50}$. Analysis was done using Chi Square and ANOVA, Windows SPSS, Version 8. *P* value of $< 0.05$ was considered significant.

**Table. 1:** Medicinal plants selected for anti – plasmodial activity.

| Family Name | Plant name | Vernacular name | Part used | Region harvested |
|---|---|---|---|---|
| Compositae | R. cardifolia | Urumurwa (Luo) | Leaves/seeds/stem | Suba |
| Compositae | Vernonia brachycalyx lasiopa (Lam) | Irisabakwa (Kuria)<br>Olusia (Luo)<br>Osiro (Luo) | Leaves/bark/root | Kuria<br>Suba<br>Suba |
| Simaroubaceae | H. abyssinica | Ol-gigiriri(M)<br>Omangoriwe(K) | Bark/roots /stem | TransMara<br>Kuria |
| Libiatae | L. calostachys Olive | Bware (Luo) | Whole plant | Suba |
| Leguminosae | S. schwein furthii | Egesemi (Kuria)<br>Ol-gigiri(M) | Bark/roots | Kuria<br>TransMara |
| Leguminosae | Cassia didymobotrya | Irebeni (Kuria)<br>Bseunete (Maasai) | Leaves/root | Kuria<br>TransMara |
| Canellaceae | Waburgia salutaris | Olosogoni (Maasai) | Bark/roots/ leaves | TransMara |

**Table. 2:** *In vitro* anti-plasmodial effects $IC_{50}$ and percentage parasitaemia inhibition of crude extracts from selected herbs against *Plasmodium knowlesi*.

| Plant name | extracts | $IC_{50}$ µg/ml | Parasitaemia % |
|---|---|---|---|
| *Artemisia annua* | Methanol | 6.8 | 0.01 |
| | Aqueous | 5.18 | 0.00 |
| *Rubia cardifolia* | Methanol | 1.20 | 7.20 |
| | Aqueous | 2.50 | 5.50 |
| *Harrizonia abyssinica* | Methanol | 2.25 | 3.25 |
| | Aqueous | 1.00 | 2.50 |
| *Sanchus schwein furthii* | Methanol | 2.10 | 0.00 |
| | Aqueous | 3.00 | 0.00 |
| *Leucas calostachys* Olive | Methanol | 3.45 | 4.25 |
| | Aqueous | 0.79 | 1.70 |
| Negative control | RPMI 1640 | 23.35 | 29.11 |

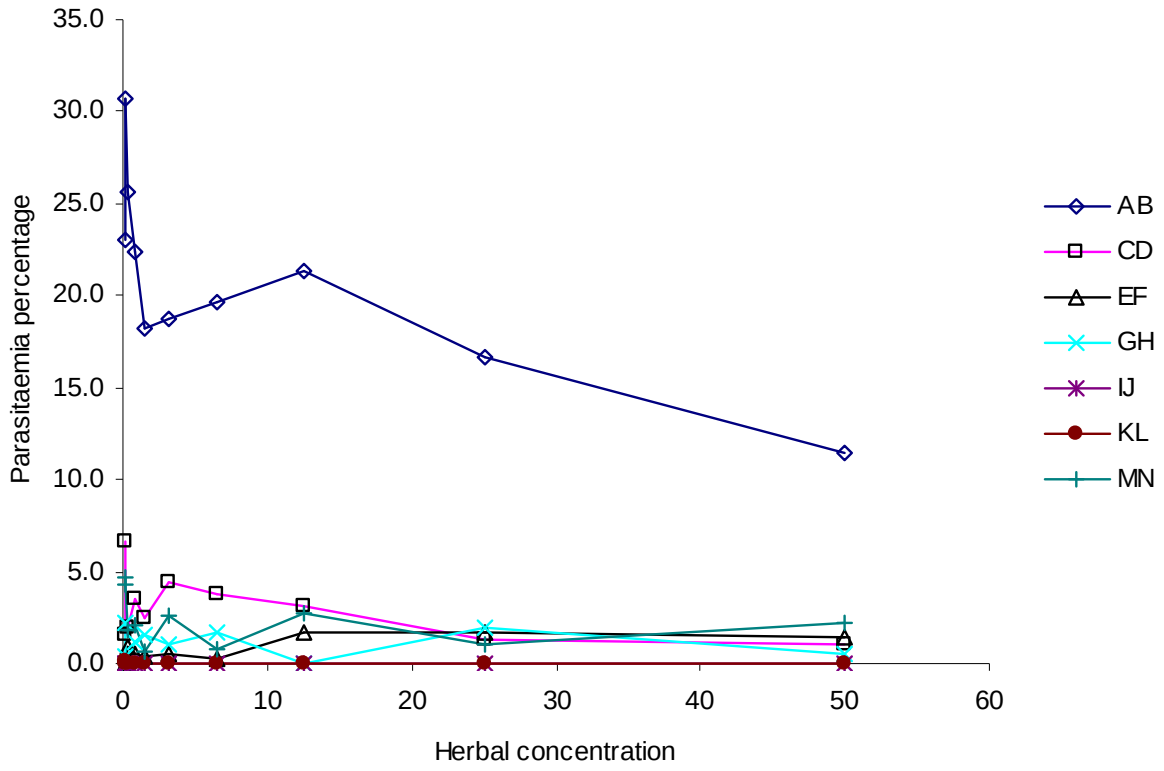


**Fig. 1:** Mean *Plasmodium knowlesi* parasitaemia after treatment with methanol herbal extracts at different concentrations.

*Key*
AB: RPMI 1640 (Negative Control)
CD: *Vernonia brachycalyx* O. Hoffm.
EF: *Rubia cordifolia* L.
GH: *Harrizonia abyssinica* Oliv.
IJ: *Sanchus schweinfurthii* Oliv. & Hiern.
KL: *Leucas calostachys* Oliv.
MN: *Artemisia annua* L. (Positive control)

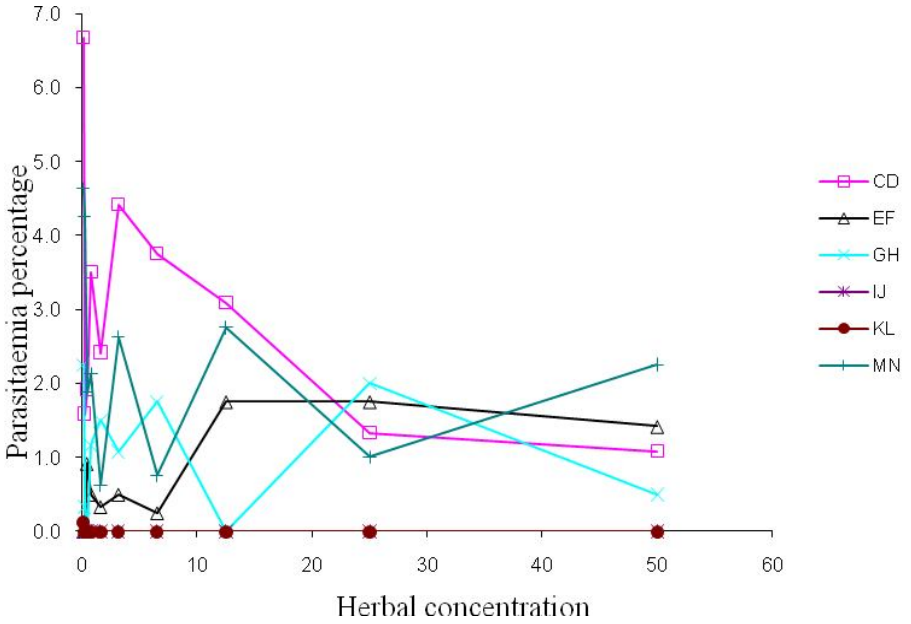


**Fig. 2:** Mean parasitaemia of *Plasmodium knowlesi* treated with aqueous extracts of different herbal plants.

***Key***
AB: RPMI 1640 (Negative Control)
CD: *Vernonia brachycalyx* O. Hoffm.
EF: *Rubia cordifolia* L.
GH: *Harrizonia abyssinica* Olive
IJ: *Sanchus schweinfurthii* Oliv. & Hiern.
KL: *Leucas calostachys* Oliv.
MN: *Artemisia annua* L. (Positive control)

## RESULTS

The crude extracts of *Rubia cordifolia, Harrizonia abyssinica, Leucas calostachys* Olive and *Sanchus schweinfurthii* showed anti-plasmodial activity out of the eight medicinal plants used. Methanolic extract of *R. cordifolia* had $IC_{50}$ of 1.20 mg/ml while *H. abyssinica* had $IC_{50}$ 2.25mg/ml. The $IC_{50}$ for *R. cordifolia* and *H. abyssinica* aqueous extracts was 2.50 mg/ml and 1.00 mg/ml respectively. These crude extracts showed significant effect on *Plasmodium knowlesi* parasites as compared to the positive and negative controls (*Artemisia annua* 6.8 mg/ml and negative control (RPMI 1640) 23.35 mg/ml). However, *R. cardifolia* methanol extract was more effective in the mortality of *P. knowlesi* than *H. abyssinica* extract. Also, the aqueous extracts of *R. cardifolia* were more efficacious on *P. knowlesi* compared to the aqueous extracts of *L. calostachys* and *S. schweinfurthii* although this extract was less effective compared to the methanol extract. There was a significant difference between the various herbal extracts and their anti-plasmodial activities ($P<0.001$). The extracts of *S. schweinfurthii* exhibited properties almost similar to those of *H. abyssinica* but mortality of *P. knowlesi* increased with increase in concentration of the extract. The methanol crude extracts of *S. schweinfurthii* had $IC_{50}$ of 2.30 mg/ml. This was not significant compared to the positive control ($P<0.05$). *L. calostachys* had the least effect on *P. knowlesi* parasites. The methanol crude extract had $IC_{50}$ 3.45 mg/ml (Table 2). Aqueous extracts showed weaker effects as compared to methanol extracts. *S. schweinfurthii* exhibited weaker properties with $IC_{50}$ of 3.00 mg/ml while *L. calostachys* extract had $IC_{50}$ of 0.79 mg/ml. Aqueous crude extracts of *S. schweinfurthii* had moderate anti-plasmodial activity while methanolic extracts had low anti-plasmodial activity. In all the four plant extracts used, parasitaemia decreased with increase in extract concentration. Highest parasite inhibition was seen in *S. schweinfurthii* methanol extract followed by *L. calostachys* methanol extract. Moderate inhibition of the parasites was indicated by *R. cardifolia* and *H. abyssinica* while *V. lasiopa* and *E. abysinnica* had insufficient yields (Fig. 1). Analysis of variance (ANOVA) showed that there was a significance difference between $IC_{50}$ of herbal extracts *S. schweinfurthii*, *L. calostachys* and the anti-plasmodial activity of parasite inhibition ($F_{[13,192]} = 29.747$, $p<0.001$). Significant difference was noted when comparing methanol and aqueous extracts on *Plasmodium knowlesi* parasites. In aqueous extracts, parasitaemia was highest in the parasites treated with *Vernonia brachycalyx* O. Hoffm extracts. Comparable parasitaemia values were obtained when *P. knowlesi* parasites were treated with *Rubia cordifolia* L. extract. *Harrizonia abyssinica* Oliv. extract had a moderate effect on *P. knowlesi* parasites while *Sanchus schweinfurthii* Oliv. & Hiern had the least effect (Fig. 2)

## DISCUSSION

Plants have been used as folk remedies for various ailments and research has shown that these plants have potent

effects. This study shows that extracts from *Rubia cordifolia* had significant anti-plasmodial activity against *P. knowlesi*. This plant has been studied by several groups and some of the results shows that the root extracts of *R. cordifolia* was promisingly cytotoxic and had antitumor activity against myeloid leukemia and Histolytic lymphoma (Parag *et al*., 2010). This finding also explains why *R. cordifolia* high antiplasmodial activities compared to the other medicinal plants used in the study. This plant may have acted on *P. knowlesi* by inhibiting their development or killing them all together. Investigations have shown that medicinal plants used in traditional medicine in various regions of the world as resources that can be relied on to provide effective, accessible and affordable basic healthcare to the local communities (Mainen *et al*.**, (**2010)

$IC_{50}$ analysis showed that methanolic extracts from *L. calostachys* was least effective while aqueous extract from the same herbal plant were most effective against *P. knowlesi.* This result shows that methanol extracted more active compounds than the aqueous extract. This may explain why parasitaemia was highest in the aqueous extracts of *L. calostachys*. This indicates that *L. calostachys* has weak chemotherapeutic properties. However, this medicinal plant is used for treating colds and headache (Okello *et al*., 2010).

Extracts from five herbs showed evident anti-malarial activity with $IC_{50}$ values ranging from 3.5 to 8.1µg/ml; *Kalopanax pictus* Nakai revealed moderate anti-malarial activity of 4.6µg/ml with no cytotoxicity. These findings are similar to what is reported in this study using local herbs as *R. cardifolia* and *S. schweinfurthii*. The herbal medicines used in the assays were effective *in vitro* as they inhibited the growth o*f Plasmodium* parasites. Based on the above findings, both the aqueous and methanol extracts of the four herbal plants tested in this study had $IC_{50}$ of less than10µg/ml thus qualify in gas having good ant-plasmodial activity.

Reports on use of malaria herbal medicines, preparation and administration by herbalists from parts of Tanzania have been documented by Gessler *et al.,* (1994). Similar work from Central Africa on traditional herbs for treatment of malaria has been reported from Democratic Republic of Congo (Mesia *et al*., 2005). In addition the various plants with anti-malarial potency provide a large reservoir for further development of pharmaceuticals against malaria. *In vitro* analysis described here is the first step towards malaria drugs from herbal extracts. It requires that further characterization be done *in vivo* to fully validate anti-plasmodial activity of the crude extracts.

*Sanchus schweinfurthii* had significant anti-plasmodial activity against *P. knowlesi.* Aqueous extracts were more effective than methanolic ones. According to parasitaemia analysis, extracts from. *R. cardifolia* and *S. schweinfurthii* were least and most effective respectively. The herbal medicines used in the assays were effective *in vitro* as they inhibited the growth o*f Plasmodium* parasites. The standards used to determine $IC_{50}$ were similar to Gessler *et al.,* 1994. Studies by Muregi *et al*. (2004), concluded that *Vernonia lasiopus* was locally used by herbalists for treatment of malaria. This shows that communities use more than one plant as source for anti-malaria treatment. Reports on use of malaria herbal medicines, preparation and administration by herbalists from parts of Tanzania have been documented by Gessler *et al.,* (1994) when treatment using multiple as opposed to single herbs was preferred. Similar work from Africa on traditional herbs for treatment of malaria has been reported from Democratic Republic of Congo (Mesia *et al*., 2005), in which three herbs were reported to treat malaria; *Croton mubango, Nauclea pobeguinii and Pyrenacantha staudii.* This suggests that there are plants that can be used synergistically to develop more potent anti-malarials. In addition the various plants with anti-malarial potency provide a large reservoir for further development of pharmaceuticals against malaria.

## CONCLUSION

These results show that some herbal plants used as anti-malarials in Kuria, Suba and Trans Mara districts have potency against malaria. The findings can be used to improve the community's use of herbs by recommending the most efficacious herbal medicines and to contribute towards the development of herbal remedy for malaria in line with WHO concern.

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