

In Vitro Anti-Plasmodial Activity of Crude Extracts of Gardenia Ternifolia, Pittosporum Viridiflorum and Phytolaca Dodecandra Used for Treatment of Malaria in Kericho County, Kenya

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Abstract

Background

Worldwide malaria caused by *P. falciparum* is still the most leading cause of morbidity and mortality. Poor access to treatment in endemic region and parasite resistance to artemisinin drugs with its derivatives has completely affected the efficient treatment of this disease. Herbs offer greatest foundation for development and discovery of new antimalarial agents and a way to ensure that everyone get access to these drugs. The aim of this study was to determine in vitro anti-plasmodial activity of crude extracts of three plants namely *Gardenia ternifolia* roots bark (family Rubiaceae), *Pittosporum viridiflorum* stem bark (family Papilionaceae) and *Phytolaca dodecandra* leaves (family Phytolaceae) used for treatment of malaria by traditional herbal medicine men in Kericho County, Kenya.

Methods

The study aimed to investigate in vitro anti-plasmodial activity of hexane, dichloromethane (DCM), Methanol (MeOH) and 5% H₂O/MeOH extracts of the three plants against mefloquine/ chloroquine resistance (W2) and mefloquine/chloroquine sensitive (3D7) strains of *P. falciparum*. Anti-plasmodial activity of the exudates was measured using a standard in vitro (Micro-Test (MARK III) kit) assay.

Results

Chemosuppression of all the tested plants extracts was high and moderate having their IC₅₀ < 10 µg/ml and expressing a sigmoid curve except the DCM extract of plant *P. viridiflorum*. 3D7 clone was extremely susceptible having the mean IC₅₀ < 5 µg/ mL to all the plant extracts except again DCM extract of plant *P. viridiflorum*. Results trend of extracts were similar to that of Mefloquine and chloroquine suggesting that there was a similar manner of action.

Conclusion

Demonstrated activity in vitro by the three plant species shows the potential of African traditional medicine in treating/preventing malaria, hence it will be imperative to isolate various metabolites of the three tested plants for qualitative and quantitative analysis and further, in-vivo analysis using murine and primate models should be performed with an aim of them being drug candidates for malaria.

Keywords – Anti-plasmodial, in vitro activity, *Gardenia ternifolia*, *Pittosporum viridiflorum*, *Phytolaca dodecandra*.

I. INTRODUCTION

Malaria is a life-threatening tropical disease caused by protozoan parasite under Genus *Plasmodium*. The most virulent parasite in the World Health Organization (WHO) region of Africa out of the four common *Plasmodium* parasites is *P. falciparum* having 99.7% cases of malaria.

According to World malaria report 2019, worldwide 228 million cases of malaria occurred in 2018 with 405 000 deaths having most cases being in the WHO African region [1]. This disease remains to be the major cause of mortality and morbidity in Sub-Saharan Africa [2]

A great challenge has been seen in the control of this disease this is due to the resistance towards the existing antimalarial drugs and insecticides with the lack of access to effective control methods [3]. South East Asia was the second country to report the resistance to Artemisinin combination therapies (ACTs) 30 cases [4] after a single case in Western Cambodia [5]. Adding to that alternative drugs resistance has been reported in many endemic areas of malaria [6]. Therefore there is a need to tirelessly look for new, affordable and safe antimalarial compounds with unique ways of actions. Plants currently in use originated from medicinal plants [7] hence can still play an important role in the discovery of new compounds which have antimalarial activity and can be used to develop new drugs for anti-malaria. In Kericho County scientific investigations show that twenty plants are used in the rural areas to treat and prevent malaria where only three are the most common plants used by these traditional herbalist practitioners [8]. Thus this study evaluated anti-plasmodial activity of the three plants commonly used to treat malaria in Kericho County using strongly polar, moderately polar and strongly non-polar solvent systems.

II. MATERIALS AND METHODS

Collection of plants parts

The plants parts (roots bark, leaves and stem barks) were collected from Kericho County specifically Kapsoit, Kaitui and Fort-Ternan areas then transported to Kisii University Post Graduate laboratory for extraction of its crude extracts.

Plants parts crude extraction

The plant material was air dried under shade for a period of 10–25 days and ground into powder and weighed. 550 g of leaves, 361 g of root barks and 710 g stem barks were extracted with hexane, dichloromethane (DCM), Methanol (MeOH) and 5% H₂O/MeOH at a ratio of 1:1 each using a

rotary evaporator with different boiling points ranging from 60–80°C for 5 hrs. Soaking with the chemicals was done for a period of 24–72 hours. Filtration was done using a Whatman Grade 1 filter paper. Extracts after extraction were concentrated at a temperature of 45°C under a pressure of 22–26 mmHg which was a reduced pressure, with the deposit found being emptied into vials using a spatula and labeled Hexane extracts, DCM extracts, Methanol extracts and 5% H₂O/MeOH extracts for all plants extracts. Additionally for 5% H₂O/MeOH the plant crude extract took another process of mixing the solution with ethyl acetate after the concentration since water had not been extracted from the rotary evaporator. After 24 hours the mixture was separated using a separating funnel where it formed two layers. The upper layer was the organic phase which had all the organic substances while the lower one was the aqua phase which is heavier and carrying the water components. After the removal of the aqua phase the organic phase was passed through the rotary evaporator to remove ethyl acetate which was concentrated at a temperature of 60 °C – 80 °C.

P. falciparum culture

This procedure was done at KEMRI Nairobi. *P. falciparum* culture was done using the procedure described by Trager and Jensen 1976 [9]. *P. falciparum* strains used were W2 and 3D7 clones acquired from KEMRI Nairobi. Parasites were grown in uninfected O+ RBCs as host cells and maintained in RPMI-1640 medium which was supplemented with 2 mg/mL NaHCO₃, 10 µg/mL hypoxanthine, 2 mg/mL glucose, 1% albumax II and 10 µg/mL gentamicin. The culture was maintained at 37 °C in a CO₂ incubator. Parasitaemia was determined qualitatively and quantitatively using a fluorescent microscope (DAPI) and light microscopy (Giemsa stain). Average percentage parasitaemia calculation in each slide was done using the following formula by Kalra[10].

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized Red blood cells}}{\text{Total number of Red blood cells count}} \times 100$$

In vitro anti plasmodial activity

96-well plates were used for the plants crude extracts anti plasmodial activity. Wells having RBC's with parasites only were used as negative control while the ones with cultures of CQ and mefloquine were used as positive controls. Plates containing parasite cultures were incubated for 48 h at 37 °C in a CO₂ incubator. After 48 h of incubation, the plates were frozen overnight at – 20 °C and anti plasmodial activity was assessed using the parasite lactate dehydrogenase (pLDH) assay that was previously described [11]. The pLDH assay generated optical density values at various concentrations of the plant extracts using the Graphpad prism software version 6. A log dose response curve was generated and used to determine IC₅₀. Each product was tested in duplicate and the IC₅₀ values obtained from the duplicates were pooled and

expressed as geometric means and standard deviations. The independent sample p-test was used to compare mean IC₅₀ of anti-malarial activity between plant extracts using ANOVA (a variance of one way analysis) trailed by Tukey's HSD *post-hoc* test which compared the outcome of data within and among collections for differences between final and initial results, where P values < 0.05 were considered statistically significant.

III. RESULTS AND DISCUSSION

The 50% inhibitory concentration (IC₅₀) of the 3D7 clone chloroquine sensitive *P. falciparum* done against preferred plant extracts in Kericho East Sub-County are shown in figure 1 to figure 5, Where else their mean IC₅₀ are shown in figure 6 to figure 8. Effectiveness of these plants extracts was categorized in three forms namely; high (IC₅₀ < 3 µg/ml),

moderate (IC_{50} 3–10 $\mu\text{g/mL}$) and weak (IC_{50} 20–100 $\mu\text{g/mL}$). The 3D7 clone was extremely susceptible (having the mean IC_{50} < 5 $\mu\text{g/mL}$) to all the plant extracts except DCM extract of plant *P. viridiflorum* (Table 1), this plant also never gave out a sigmoid curve nor the IC_{50} (figure 4). As it can be seen in 3D7 plates, the values with the lowest IC_{50} were observed in all extracts of plant *G. ternifolia* (figure 3) and plant *P. dodecandra* extracts of MeOH, 5% H_2O & MeOH (figure 5). The remaining species of plant *P. dodecandra* (DCM and hexane) (figure 8) and all the extracts of plant *P. viridiflorum* (figure 4) showed moderate (IC_{50} 3–10 $\mu\text{g/mL}$) antiparasmodial activity, only the DCM extract was inactive (IC_{50} > 100 $\mu\text{g/mL}$).

For the mean IC_{50} ($X \pm SE$) for plant extracts screened against 3D7 clones only the DCM and MeOH plants extracts

of *G. ternifolia* with the MeOH and 5% H_2O & MeOH of plant *P. dodecandra* extracts had their means < 3 $\mu\text{g/mL}$, (Table 1) hence being categorized as having the highest effectiveness. Generally the IC_{50} for W2 in almost all extracts were not coming out properly since the data were failing to give out the sigmoid curve (figure 6-8) except the extracts of *G. ternifolia* MeOH extract which gave an IC_{50} of (4.6032), 5% H_2O & MeOH (4.246) (figure 6), *P. dodecandra* Hexane (4.5) 5% H_2O & MeOH (2.7744) (figure 8) and lastly *P. viridiflorum* Hexane (4.305), MeOH (3.858) (figure 7) which showed a small sigmoid curve. The results trend of extracts were similar to that of Mefloquine and chloroquine (control drugs), suggesting that there was a similar manner of action (figure 1 and 2).

Table 1: The mean IC_{50} $\mu\text{g/mL}$ ($X \pm SE$) values for plant extracts in different solvents screened against 3D7 *P. falciparum* clones

Plant name / Control drug	Hexane	DCM	MeOH	5% H_2O & MeOH
<i>Gardenia ternifolia</i>	3.166 $\pm$ 0.37 ^a	2.198 $\pm$ 0.152 ^a	2.855 $\pm$ 0.065 ^a	3.579 $\pm$ 0.586 ^a
<i>Pittosporum viridiflorum</i>	3.590 $\pm$ 0.16 ^a		3.120 $\pm$ 0.090 ^a	3.464 $\pm$ 0.122 ^a
<i>Phytolaca dodecandra</i>	3.246 $\pm$ 0.415 ^a	3.435 $\pm$ 0.055 ^a	2.665 $\pm$ 0.125 ^a	2.376 $\pm$ 0.185 ^a
Chloroquine (control test)	1.015 $\pm$ 0.015			

N/B The mean difference is significant at the 0.05 level.

The results were expressed as IC_{50} mean $\pm$ SE of each experiment for four different determinations. The positive control was counted in as a reference drug whereby their IC_{50} were expressed in $\mu\text{g/mL}$.

Key- ^a represents significantly lower inhibition of *P. falciparum* compared to chloroquine (Chloroquine *P. falciparum* inhibition is significantly high compared to plants extract represented with ^a or all plants extract represented with ^a inhibition of *P. falciparum* were significantly lower compared to Chloroquine standard drug $P < 0.05$)

The lower the IC_{50} the higher the activity of the test sample.

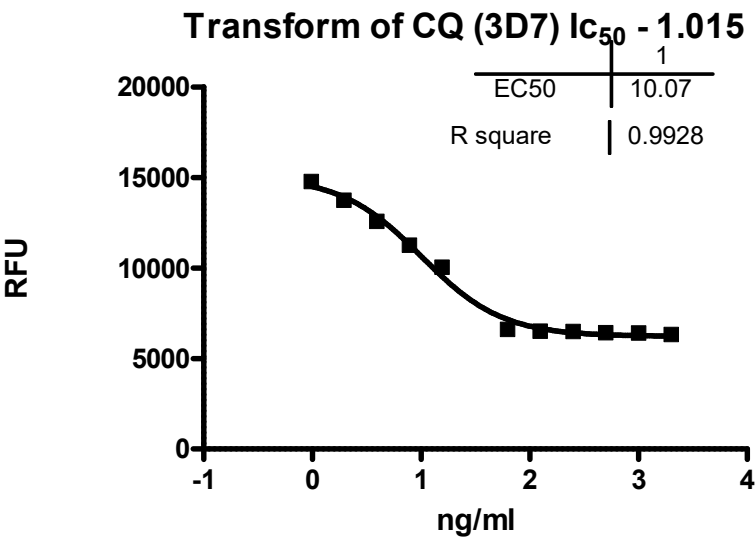


Figure 1: Graph of 3D7 showing the percentage of schizonts vs. logarithm of control drug concentration (chloroquine)

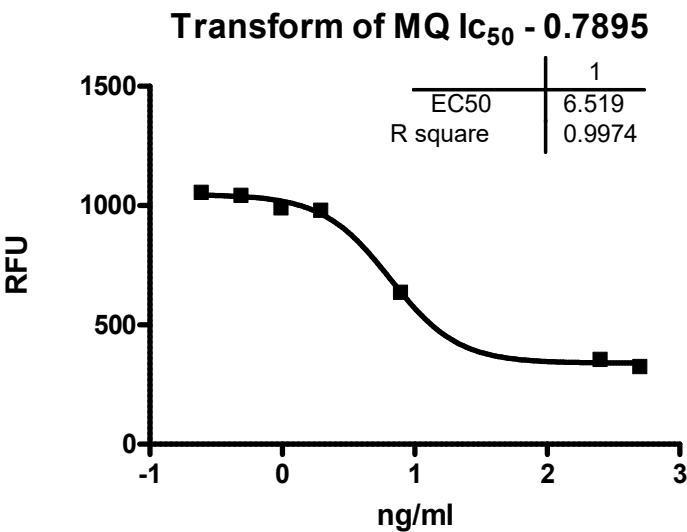


Figure 2: Graph of W2 showing the percentage of schizonts vs. logarithm of control drug concentration (Mefloquine)

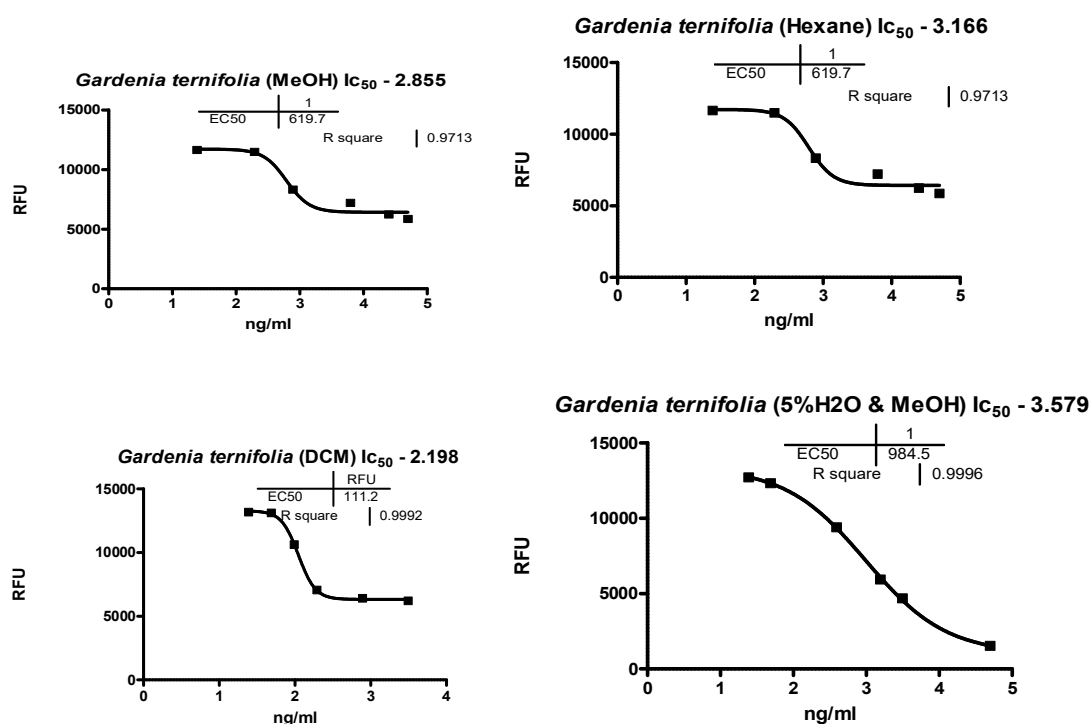


Figure 3: Graphs of 3D7 showing the percentage of schizonts vs. logarithm of drug concentration of *G. ternifolia* different extracts (a plant traditionally used to treat Malaria in Kericho East Sub-County)

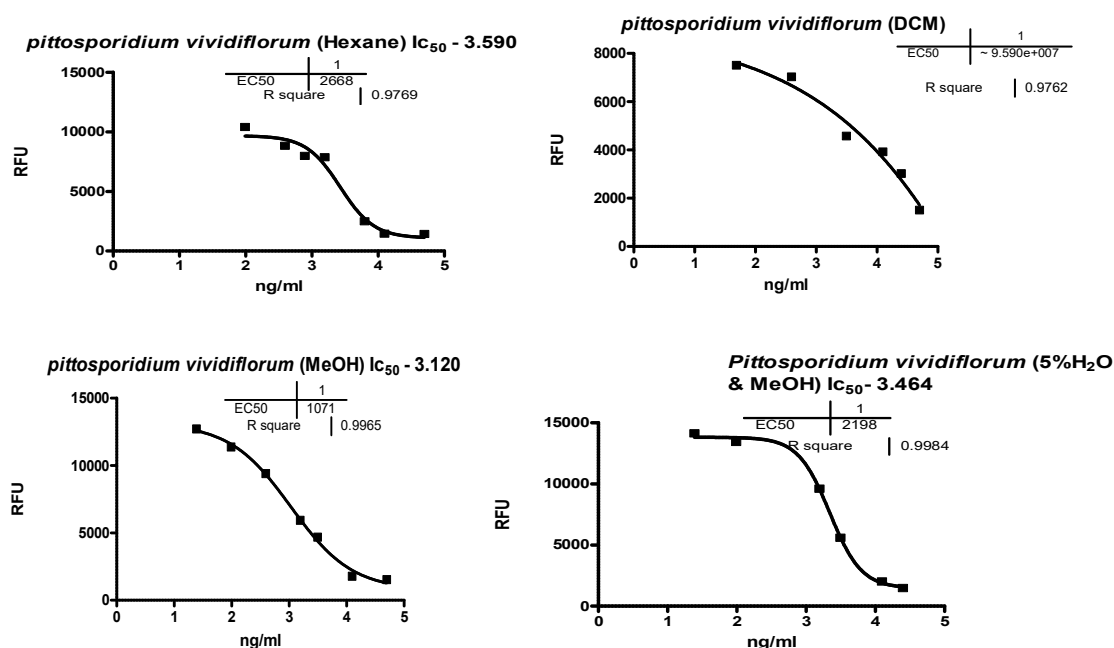
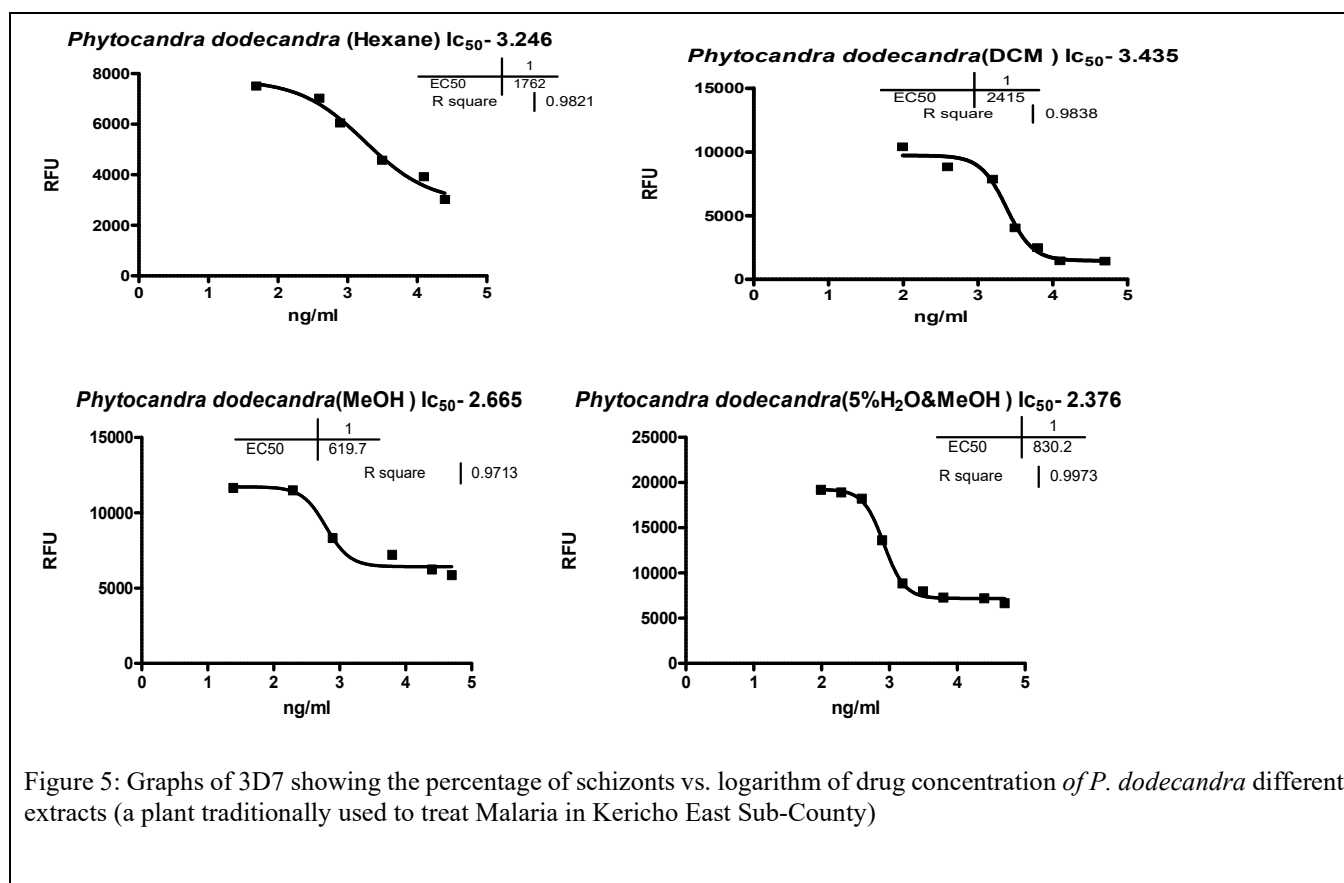


Figure 4: Graphs of 3D7 showing the percentage of schizonts vs. logarithm of drug concentration of *P. vividiflorum* different extracts (a plant traditionally used to treat Malaria in Kericho East Sub-County)



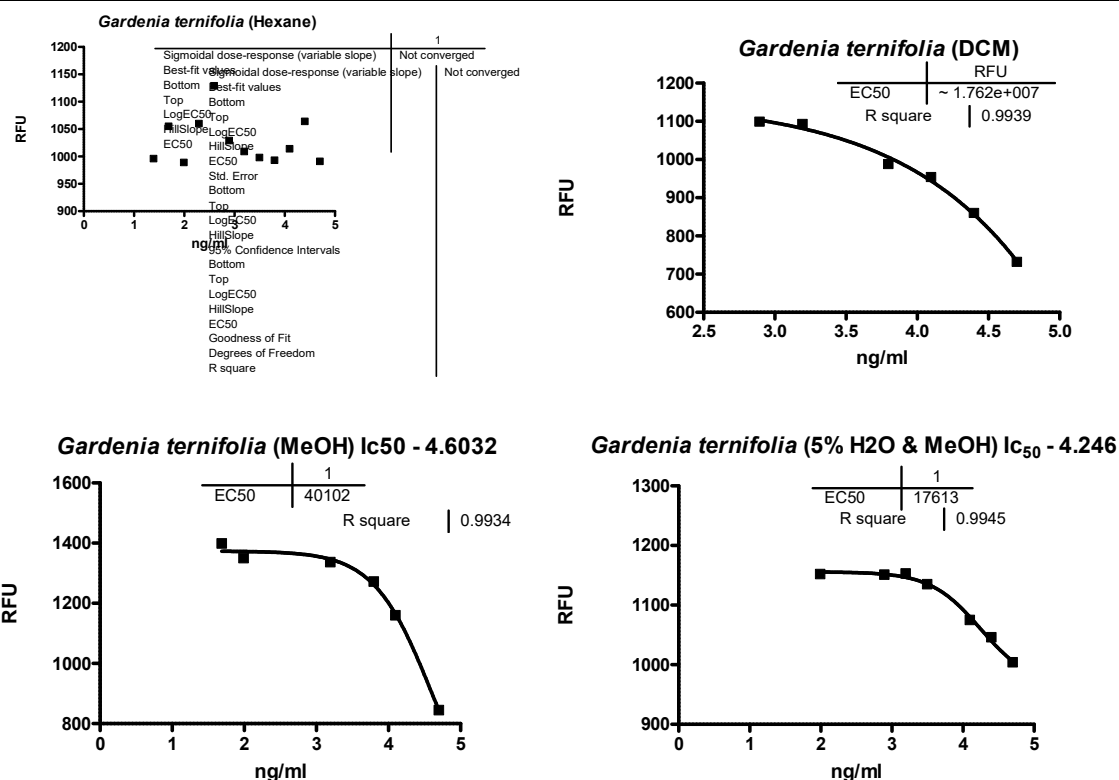


Figure 6: Graphs of W2 showing the percentage of schizonts vs. logarithm of drug concentration of *G. ternifolia* different extracts (a plant traditionally used to treat Malaria in Kericho East Sub-County)

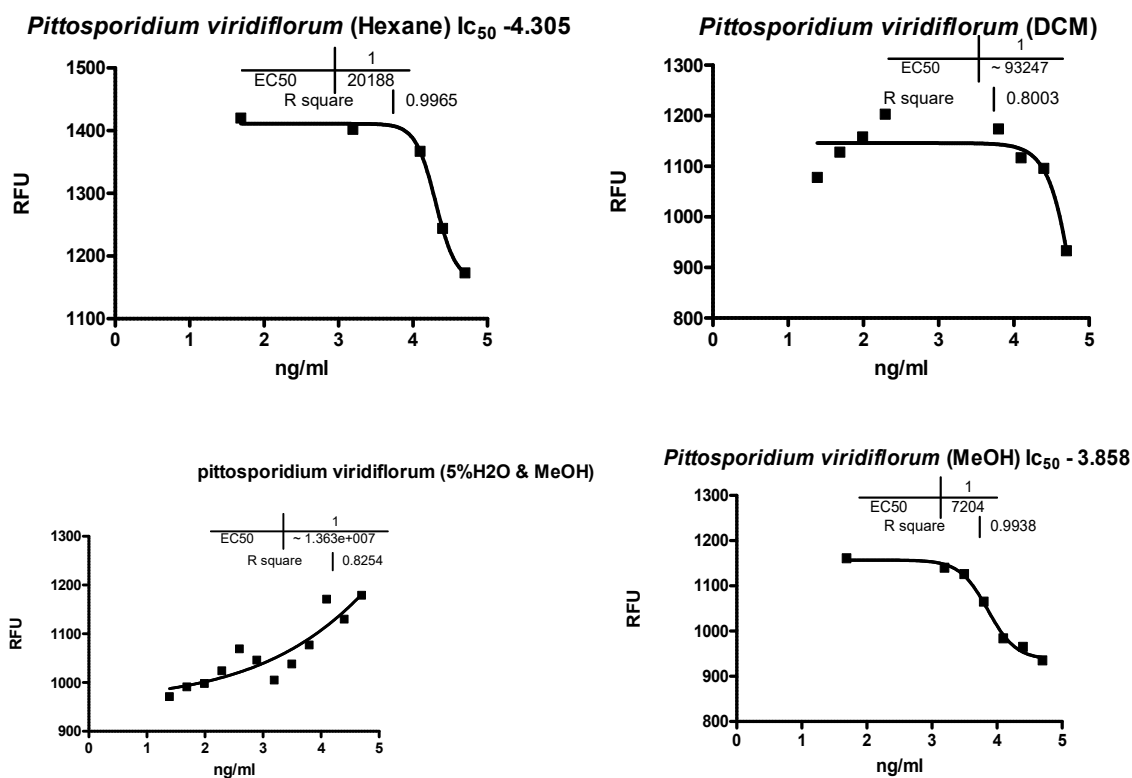


Figure 7: Graphs of W2 showing the percentage of schizonts vs. logarithm of drug concentration of *P. viridiflorum* different extracts (a plant traditionally used to treat Malaria in Kericho East Sub-County)

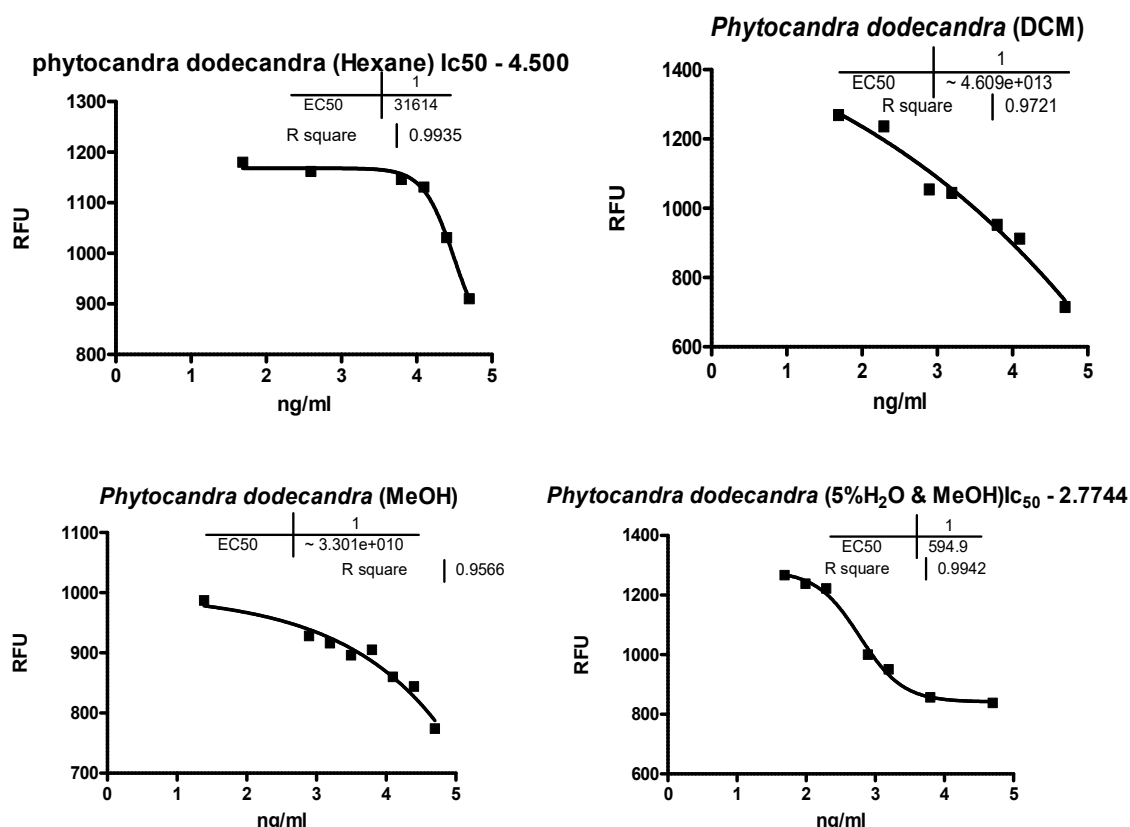


Figure 8: Graphs of W2 showing the percentage of schizonts vs. logarithm of drug concentration of *P. dodecandra* different extracts (a plant traditionally used to treat Malaria in Kericho East Sub-County)

With the help of the above numerous ethnobotanical screening which have taken place on medicinal plants found in Kenya [12, 13, 14, 15, 16], it has led to coming up of programs dealing with isolation of compounds on antiplasmodial activity [17, 18, 19, 20]. Subjection of most of these Kenyan plants species for *in vitro* antiplasmodial activity according to [21] have been using hypoxanthine [³H] combination procedure to radionucleate DNA nucleus of the parasites clones which are alive. Effectiveness of these plants extracts was categorized in three forms namely; high [IC₅₀ < 3 µg/ml], moderate [IC₅₀ 3–10 µg/mL] and weak [IC₅₀ 20–100 µg/ml] because when the concentration was low, inhibition of the growth of parasite indicate selective action opposite to when the concentration is high which is said to be toxic despite that the toxicity level is not specific.

Plants extracts showing effectiveness past the above range and not giving a sigmoid curve were completely considered not effective. Unexpectedly the above combination procedure expressed its plants extracts to have an IC₅₀ below 5 µg/mL, an IC₅₀ which its plant extracts is considered to be highly

effective if isolation of compounds to get the active components is done [22]. *Gardenia ternifolia* Schum & Thonn. is usually known as Powder-bark or Wild gardenia in English [23], "Gambilo" in Amharic [24, 25] "Kambeelloo" in oromifa [26], "Kota" in Gumuz [27], "Gambela" in Sidama [28] "Shigidida" in Gedeo [29], "Duwong" in Anuak [30], "Brmaiya" in Konso [31] "Bodut" in Menit [32] and "Kipulwet" in Kipsigis. From the previous studies in Africa leaves are used to treat malaria [33], arrow poisoning [34], hypertension [35], diabetes [36], syphilis [37] and skin diseases [38]. The fruit is taken to relieve malaria pains with stem being used to halt vomiting [39]. Barks have ethno-medical uses for ascites, leprosy, hepatitis, onchocerciasis, female infertility, wounds, malaria and sexually transmitted disease [40, 41, 42]. Roots are believed to have antirheumatismal [43], anti-jaundice and anti-malarial [44], anti-pain [45], anti-epilepsy, anti-hypertensive [46] and anti-constipation activity [47]. Traditionally, *G. ternifolia* roots are of purgatives, stomachic, anthelmintic, diuretics, and emetics for ascites, and rickets [48].

It is also used to manage kwashiorkor [45], livestock helminthosis [49], black water fever and cough [50]. In Ethiopia root barks macerated in cold water are commonly administered orally for treating malaria by traditional medicine practitioners of the local community [51] which was in conformity with this study.

The ratio of chloroquine (figure 1) and Mefloquine (figure 2) (control drugs) sensitivity was higher compared to all plants extracts sensitivity against both the 3D7 and W2 clone. For W2 crude extracts when compared to 3D7 crude extracts *in vitro* antiplasmodial activity were not giving out the good results for IC_{50} and the graphs showing the concentration of parasitemia against concentration of the drugs used in ng/ml (figure 3-8). This was because W2 is a sensitive clone and its growth was unpredictable. Both the flask culture and dosing in a 96 – well plate took place within the hottest months (January-April) of the year where bacteria's growth was high hence interfering with them. The *in vitro* concentrations in this research were highly considered due to its selective activity of the parasite growth inhibition when in low concentrations unlike the high concentration where toxicity is observed in its non-specific form.

In South Africa the test for *P. viridiflorum* whole plant in MeOH and dichloromethane extract against *P. falciparum* strain D10 (chloroquine sensitive) had its IC_{50} to be 3 $\mu\text{g/mL}$ and 27.7 $\mu\text{g/mL}$ respectively, also for the leaves and flowers the same extracts of dichloromethane and methanol its IC_{50} was 28 $\mu\text{g/mL}$ and 70.5 $\mu\text{g/mL}$ respectively [52]. Another report using the CQ susceptible *P. falciparum* on plant *P. viridiflorum* antiplasmodial activity in the study of [53] using strain K67 showed an IC_{50} of 30 $\mu\text{g/mL}$. *Pittosporum tobira* also showed high antimalarial activities (Hexane 34.4 ± 2.9 $\mu\text{g/mL}$, DCM 44.6 ± 2.4 $\mu\text{g/mL}$, Ethyl acetate 4.8 ± 1.8 $\mu\text{g/mL}$ and in MeOH > 100) [54].

With all these knowledge the attention of *Pittosporum* genus is corroborated being a possible source of drug to treat malaria symptoms. The above results of *Pittosporum* were observed to be lower than in this study signifying the time in which these plants were collected and the area of collection has some influence on the quantity and structure of their effective constituents [55] in addition with the storage and processing [56]. This information was in conformity with the study of [57] who dealt with the ethnobotanical uses of plants in Nandi County, Aldai Division.

IV. CONCLUSION AND RECOMMENDATION

Pittosporum viridiflorum, *Gardenia ternifolia*, *Phytolacca dodecandra* by the Kericho East Sub-County traditional herbalists practitioners showed statistically significant lower activity ($P < 0.05$) compared to chloroquine and mefloquine (standard drug) malaria treatment using 3D7 clone (chloroquine sensitive) and W2 clone (chloroquine resistant) *P. falciparum*. This demonstrated activity *in vitro* by the 3 plant species hence showing the potential of African traditional medicine in treating and preventing malaria.

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