IN-VITRO CYTOTOXICITY OF EXTRACTS OF SELECTED MALARIA MEDICINAL PLANTS USED BY TRADITIONAL HEALERS OF KERICHO EAST SUB-COUNTY, KENYA

ABSTRACT:

Background

Malaria is a fatal disease which affects people of all ages; especially pregnant women, young children<5 years, and the elderly because of their weakened immune systems. The currently used anti-malarial drugs have been linked to a variety of negative side effects including the parasite resistance. Additionally, the costs associated with the conventional malaria management approach are arguably high, particularly for people living in low-income countries, highlighting the need for alternative and complementary approaches. Medicinal plants therefore are a viable alternative since they are arguably less expensive and easily accessible.However, there is limited information on safety and efficacy of the plants.This study was designed to investigate the cytotoxic activities of polar and non-polar crude extracts solvents of selected plants used by traditional healers in Kericho East Sub-County to treat malaria.

Materials and methods

Plants studied included *Pittosporumviridiflorum* (stem barks), *Phytolaccadodecandra* (Leaves), and *Gardenia ternifolia* (roots barks). Plant parts selected were collected from Kericho East Sub-county; Kapsoit, Kaitui, and Fort-Ternan. Their crude extracts were obtained from hexane, dichloromethane (DCM), Methanol (MeOH), and 5% H2O/MeOH. In vitro cytotoxic effects and safety of the studied plants' extracts were identified using mammalian Vero E6 cells.

Results

UNDER PEER REVIEW

24 Most of the plants tested yielded impressive cytotoxicity results, indicating that therapeutic doses
25 could be achieved at safe concentrations. However, *P. viridiflorum* hexane, DCM, MeOH, and
26 5% H_2O/MeOH crude extracts were toxic to the cultured cells expressing the mean CC_{50} ± SE of
27 65.11±0.40, 25.63±0.23, 87.94 ±0.59 and 98.54±0.66 μg/ml, respectively.

28 **Conclusion**

29 *G. ternifolia* and *P. dodecandra* have offered hope in the treatment of malaria since their crude
30 extracts have demonstrated no toxicity. The study found*P. viridiflorum*crude extracts to be toxic
31 but there is the possibility of isolating safe nontoxic compound/s because they were less toxic at
32 lower doses. This study therefore identified potential plants that could be used to develop novel
33 anti-plasmodial agents.

34 **Key words:** Anti-malarial drugs;*Anopheles* mosquito; Medicinal plants; Malaria; Cytotoxicity

35

36 ## 1. Introduction

37 Malaria is a potentially fatal disease caused by a protozoan parasite transmitted through the bites
38 of the female *Anopheles* mosquito.It is estimated that over 241 million people worldwide are
39 affected, with 627 000 deaths occurring each year [1]. Expectant mothers and children aged 0 to
40 5 years are the most vulnerable groups to malarial infections [2]. Malaria is frequently associated
41 with poverty, but it is also a cause of poverty and a significant impediment to economic
42 development. In the year 2021, the World Health Organization (WHO) forecasts over 241
43 million malaria cases, with Sub-Saharan Africa accounting for more than 95% of all cases. [1].
44 In the year 2017, 3,215,116 of *Plasmodium falciparum* cases were reported in Kenya [3].
45 Additionally, the World Health Organization [4] estimates that over 3.2 billion people in 91
46 countries are at high risk of contracting malaria.The high mortality rates of malaria subjects are

UNDER PEER REVIEW

of great concern in light of the rising statistics.Many studies have found that parasites are becoming more resistant to conventional anti-malarials [5, 6]. Furthermore, the toxicities and adverse events associated with conventional antimalarial drugs render them ineffective in malaria treatment, necessitating the urgent need for alternative and complementary approaches [7, 8]. In addition, convectional treatment regimens face the challenge of high production costs, necessitating the search for less expensive, yet effective alternatives [27,28].

For thousands of years, plants have played an important role in the treatment of malaria. Therefore, herbal medicine has been used throughout history, making it the oldest form of healthcare known to humanity. WHO endorses its use and considers it one of the most effective strategies for combating emerging diseases [3]. Plants have been found to contain powerful antimalarial compounds such as quinine and artemisinin. As a result, medicinal plants represent a vast reservoir from which powerful antimalarial drugs can be developed. It is regarded as one of the world's most reliable methods of achieving total health because it is less expensive than conventional medicine. Traditional medicine serves the primary healthcare needs of approximately 80% of the world's population [9]. Affordability, availability, and accessibility are the primary reasons for people's reliance on herbal medicine [10]. Medicinal plant use is also integrated into most African cultures, making it more acceptable than conventional medicine [11]. Because of the numerous bioactive compounds found in herbal drugs, increasing research data has demonstrated their potential [12]. These phytocompounds have been identified as potential leads for some currently used drugs, including anti-cancers, analgesics, and anti-malarials [13].

Despite the tremendous contribution and potential of medicinal plants, particularly in the treatment of malaria, no scientific data exists to validate the claimed antimalarial safety.

UNDER PEER REVIEW

70 Investigated medicinal plantparts and their constituents (*Pittosporumviridiflorum Sims. var.*
71 *viridiflorum*(S.L) local name *Chepngororiot*(stem barks), *Phytolaccadodecandra* L Heritlocal
72 name *Patkawet* (leaves) and *Gardenia ternifolia*Schum.&Thonn local name *Kipulwet* (roots
73 barks) are majorly used by Kipsigis people in Kericho County, Kenya for the management of
74 malaria[14].Their unknown toxicities despite long history of use in anti-malarial and anti-
75 plasmodial activities informed the current study.

76 **Materials and methods**

77 **2.1 Plant parts collection**

78 Plant parts (roots, barks for *Gardenia ternifolia*Schum.&Thonn, leaves for
79 *Phytolaccadodecandra* L Herit, and stem barks for *Pittosporumviridiflorum Sims. var.*
80 *viridiflorum*) were collected in Kericho County, specifically in the Kapsoit, Kaitui, and Fort-
81 Ternan areas with the help of a traditional herbalist. The parts were then transported to the Kisii
82 University laboratory for crude extract removal.

83 **1.2 Crude extractions**

84 The collected plants parts were then air-dried for one week at room temperature in a well-aerated
85 room before being grounded into coarse powder usingKisii University laboratory mill. Crude
86 extracts were then prepared following the procedure stated by [15] with some few
87 modifications.Briefly, 100 g powder of each plant part extract were macerated in 300 ml of each
88 hexane, DCM, MeOH and 5% H_2O/MeOH methanol at room temperature for 72 hours. The
89 extracts were then filtered using double-layer Whatman's number one filter papers and
90 concentrated with a rotary evaporator using different boiling points ranging from 60–80°C for 5
91 hrs. Additionally, for 5% H_2O/MeOH methanol plant crude extracts, it required another process
92 of mixing the solution with ethyl acetate to remove water. After 24 hours, the mixture was

UNDER PEER REVIEW

93 separated into two layers using a separating funnel. The upper layer contained all of the organic
94 substances, while the lower layer was the aqua phase, which was heavier and carried the water
95 components. Following the removal of the aqua phase, the organic phase was passed through a
96 rotary evaporator to remove ethyl acetate, which was concentrated at temperatures ranging from
97 60^oC to 80^oC.

98 **2.3 Cytotoxicity studies**

99 The growth-inhibition assay was performed on actively dividing sub-confluent Vero E6
100 cells(Kurokawa et al., 1995). Vero E6 cells were grown in 25 ml cell culture flasks incubated at
101 37^oC in 5% CO_2 in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY)
102 supplemented with 10% fetal bovine serum (FBS). When the cells reached confluence, they were
103 seeded with 5×104 cells/well in 24-well plates and incubated at 37^oC for 2 days. Positive and
104 negative controls including chloroquine and untreated Vero E6 cells respectively were set up.
105 The culture medium was replaced with fresh MEM containing test extracts at various
106 concentrations, and the cells were incubated for another 2 days. Trypsinization was used to
107 detach cells from each sample in triplicate wells, and the number of viable cells was determined
108 using a tryphan blue exclusion test. To count viable cells, a haemocytometer was used.CC_{50}
109 (concentration required to cause visible alterations in 50% of intact cells) was estimated using
110 inhibition data plotted as dose-response curves using nonlinear regression analysis.Mean CC_{50}
111 (X±SE) < 100 µg/mL was considered toxic. Additionally, at each drug concentration, the results
112 were also recorded as optical density (OD) per well. The data was entered into Microsoft Excel
113 2016 and expressed as a percentage of the untreated controls using the formula described below
114 [16].

115 $$\text{Percentage cell growth inhibition} = \left(\frac{A-B}{A}\right)\times 100$$

UNDER PEER REVIEW

116 Where; A = the OD of the untreated cells

117 B = the OD at each drug concentration

118 The Percentage Yield of the Crude Extract was also calculated using the formula below;

119 $$\text{Percentage Yield} = \frac{\text{weight of extracts obtained}}{\text{weight of powder used for extractions}} \times 100$$

120 **2.4 Data analysis**

121 The statistical significance between the means of the data was analyzed using chi-square where

122 P-value of less than 0.05 was considered statistically significant.

123 **3. Results**

124 **3.1 Crude extracts percentage yield**

125 Following extraction, the respective extracts' percentage yields were calculated. The results

126 revealed that, in general, high percentage yields were recorded in 5% H2O/MeOH of all plants

127 parts followed by MeOH, Hexane and lastly DCM (Table 1).

128

129

130

131 **Table 1: Plants parts and the percentage yield**

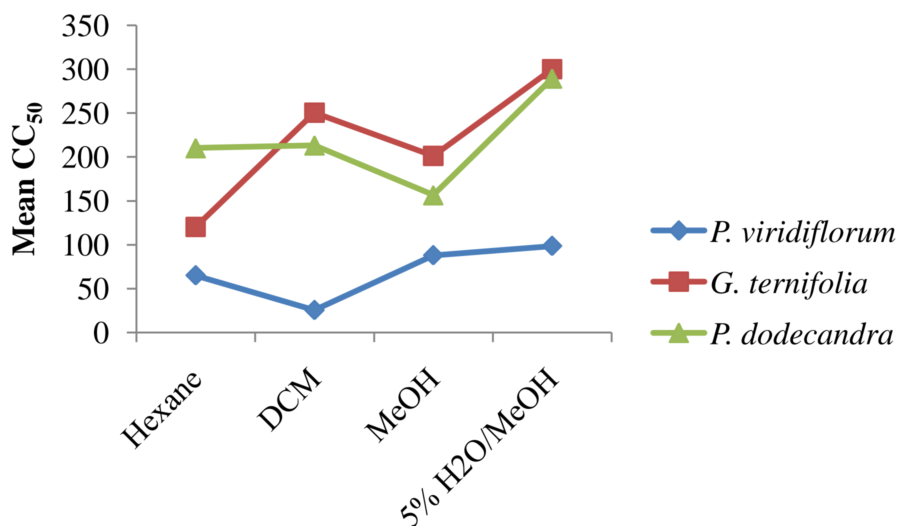
Plant name	Plant parts used	Extraction solvent	% Yield
Gardenia	Roots bark	Hexane	2.43
*ternifolia*Schum.		Dichloromethane (DCM)	0.53
&Thonn.		Methanol (MeOH)	3.65
		5% H_2O/MeOH	6.84
Pittosporumviridi	Bark	Hexane	2.92
florum Sims. var.		Dichloromethane (DCM)	0.94

UNDER PEER REVIEW

viridiflorum(S.L)		Methanol (MeOH)	4.01
		5% H_2O/MeOH	6.03
Phytolacadodeca	Leaves	Hexane	2.36
ndra L Herit		Dichloromethane (DCM)	0.42
		Methanol (MeOH)	2.94
		5% H_2O/MeOH	7.97

132 **3.2 Cytotoxicities of plants crude extracts**

133 *P. viridiflorum* crude extracts showed no toxicity in lower doses (10 µg/ml<) while in high
134 dosage (>10 µg/ml<) it showed the maximum cell harmfulness when exposed to Vero E6 cells
135 expressing the mean CC_{50} ± SE of 65.11±0.40, 25.63±0.23, 87.94 ±0.59 and 98.54±0.66µg/mL
136 Hexane, DCM, MeOH and 5% H_2O/MeOH, respectively (Fig 1).



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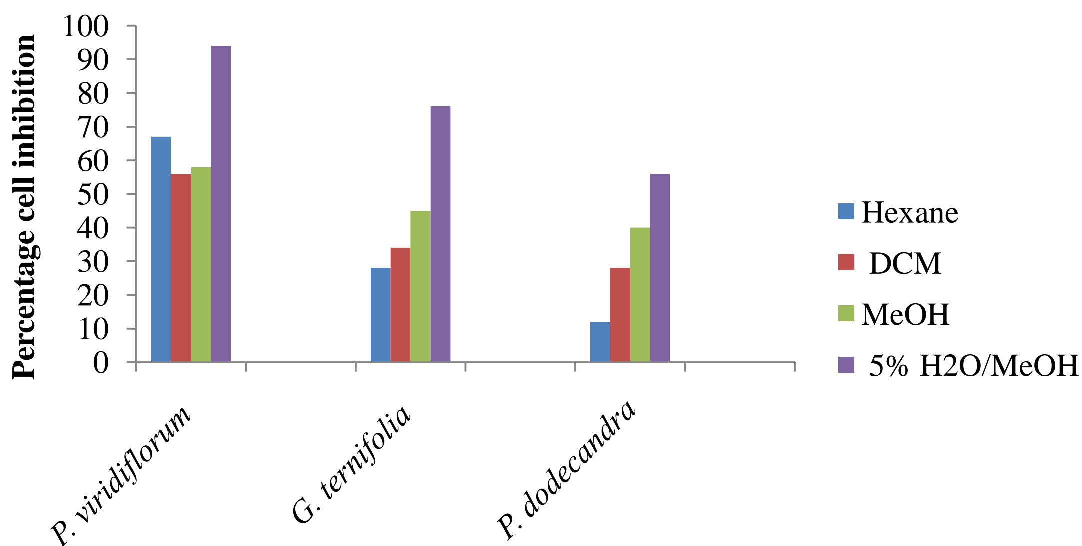
138 **Figure 1: Mean $CC_{50}$of *P.viridiflorum*, *G. ternifolia* and *P. dodecandra* crude extracts**

139 **3.3 Vero E6 cells inhibition**

140

141 *P. viridiflorum* crude extracts had a high percentage of Vero E6 cells inhibited, with 5% 142 H_2O/MeOH showing the highest percentage of inhibition across all concentrations (Fig 2).



143

144 **Figure 2: Percentage cell inhibition of *P.viridiflorum*, *G. ternifolia* and *P. dodecandra* crude 145 extracts**

146 **3.4 Associations between Vero E6 cells inhibitionand plants crude extract concentration**

147 A statistical significant association between the Vero E6 cells inhibition and all plants crude 148 extracts seeded in different concentrationswas observed(χ^2 = 19.91; d.f = 5, P < 0.001), (χ^2 = 149 74.16; d.f = 5, P < 0.001), (χ^2 = 96.22; d.f = 5, P < 0.001), (χ^2 = 84.33; d.f = 5, P < 0.001), (χ^2 = 150 27.56; d.f = 5, P < 0.001), (χ^2 = 67.35; d.f = 5, P < 0.001) in Hexane, DCM, MeOH and 5% 151 H_2O/MeOHrespectively.

152 **Discussion**

153 *P. viridiflorum* crude extracts of Hexane, DCM, MeOH and 5% H_2O/MeOHshowed toxicity in 154 high dosage in the present study despite the plant parts being is used for malaria and fevers in 155 Kerichowith IC_{50} of 3–10µg/ml[14], East Africa [17], and its DCM crude extracts of the whole 156 plant showing an in vitro antimalarial activities in South Africawith IC_{50} of 3 µg/ml[18].

UNDER PEER REVIEW

Additionally, *P. lanatum*, a related species of *P. viridiflorum*plant, had leaves that were toxic to brine shrimp (LC50, 27.4 µg/ml) [19]. It is unclear whether the inhibition is the result of a specific antiplasmodial action or general cytotoxicity, but an in vitro test in the present study with a lower dose less than 10µg/mlshowed no toxicity, hence indicating that the toxic compounds may not be the same as the active constituents. The present study was in agreement with the study of [20] which stated that methanol and water extracts of *P. viridiflorum* had the highest level of cell cytotoxicity on Vero E6 cells in high dosage, with CC_{50} values of 18.08 and 69.21 µg/ml respectively. Furthermore, in the same study [20], mice treated with a 100 mg/kg methanol extract of *P. viridiflorum* died within 24 hours. The present study showed that *P. viridiflorum* 5% H_2O/MeOH was less toxic than DCM, MeOH and Hexane extracts. This means the polar component of *P. viridiflorum*plant is less toxic than the non-polar component. This suggests that traditional herbalist practitioners who prepare plant parts by boiling them in meat soup (water plus fat in the meat) have less toxicity when using the plant as an antimalarial agent than other traditional herbalist practitioners who ferment the plant parts with honey, resulting in the addition of ethanol, an organic (non-polar) solvent. The current study supported the findings of [20], which found that the water extract was less toxic than the methanol extract.The current study contradicted the findings of [21], which found that the stem bark extracts from this plant had no cytotoxicity or brine shrimp lethality. These findings appear to validate their traditional use as antimalarials [22]. In order to continue working on this plant, since the plant have been proven to have antiplasmodial activity, bioactivity guided isolation will allow the separation of active molecules from toxic constituents.

In the current study, *G. ternifolia* and *P. dodecandra* Hexane, DCM, MeOH, and 5% H2O/MeOH crude extract plant roots bark showed no signs of toxicity, similar to the study of

UNDER PEER REVIEW

[23], which found that in an acute oral toxicity test, a hydroalcoholic stem bark extract of *G. ternifolia* did not cause any observable damage in the studied mice at 2,000 mg/kg. Previous research have shown that if the LD_{50} value of a test chemical is three times greater than the minimum effective dose, the extract is considered a good candidate for further investigation [24] & [25].Therefore, *G. ternifolia* and *P. dodecandra* have offered hope in the treatment of malaria since their crude extracts of Hexane, DCM, MeOH, and 5% H2O/MeOH roots bark have demonstrated antiplasmodial activity similar to chloroquine and mefloquine in the study of (15) with no toxicity. The study of [26] disagreed with the present study because it assessed the cytotoxicity activities of a 20.0% aqueous ethanol extract of *G. ternifolia* roots using the brine shrimp lethality test with an LC_{50} value of 54.5g/ml.These differences might be attributed to location and time in which the plants were collected as well as the different physiographic factors which influence plant phytochemicals.

Conclusion

P. viridiflorum extracts were toxic in Vero E6 cells at the test dose, but there is the possibility of isolating safe nontoxic compound/s because they were less toxic at lower doses. Similar to this study, (22) and (20) reported different levels of toxicities in samples of the same plant species collected in different regions, emphasizing the importance of georeference, implying that the location and, most likely, time of plant collection may influence the amount and composition of the toxic components. It is unknown whether such factors influenced the phytochemical composition of the *P. viridiflorum, G. ternifolia* and*P. dodecandra* plants, which has been linked to present or lack of cytotoxicity in East and South Africa.Hence, it would be of interest to isolate active constituents for identification, antiplasmodial evaluation, and further toxicological

UNDER PEER REVIEW

202 screening, where caution should be exercised if these plants are to be promoted as medicinal

203 plants for malaria treatment.

204 **Ethical Approval**

205 Ethical clearance to conduct this research was granted by Kenya Medical Research Institute

206 (KEMRI) ethical review committee and permitted by the National Commission for Science

207 Technology and Innovation (NACOSTI). The research was conducted in accordance with the

208 established SERU guidelines.

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PREVIEW

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UNDER PEER REVIEW

APPENDIX

Appendix I: Photos of *Gardenia ternifolia*Schum.&Thonn., *Pittosporumviridiflorum Sims. var. viridiflorum*(S.L) and *Phytolacadodecandra* L Herit

*Gardenia ternifolia*Schum.&Thonn.

Pittosporumviridiflorum Sims.var. viridiflorum(S.L)

Phytolacadodecandra L Herit