ANTI-PLASMODIAL ACTIVITY OF SOME MEDICINAL PLANTS USED FOR TREATMENT OF MALARIA AND SYNERGISM OF METHANOLIC EXTRACTS OF *CARISSA EDULIS* AND *ARTEMISIA ANNUA*

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Abstract

The increasing prevalence and distribution of malaria has been attributed to a number of factors, one of them being the emergence and spread of drug resistant parasites. Efforts are now being directed towards the discovery and development of new chemically diverse anti-malarial agents. The present study reports on the *in vitro* anti-plasmodial activity of 15 selected medicinal plants used widely by the traditional healers to treat malaria in the North Rift parts of Kenya. The plant extracts were tested for in vitro activity against chloroquin-senitive (D6) strains of *plasmodium falciparum* parasites. Of the 15 species assayed, 40% showed promising anti-plasmodial activity of $IC_{50} \leq 10$ µg/ml, while another 40% showed moderate activity of IC_{50} between 10 – 50 µg/ml and 20% had weak activity of IC_{50} between 50 – 100 µg/ml. These results justify the correlation between traditional uses of the plants with their bioactivity. Synergism between total extract of *Atemisia annua* and *Carissa edulis* ware also analyzed using combination ratios of 10:90 to 90:10 respectively against *P.falciparum* parasites. This led to the identification of anti-malarial combination therapy of methanolic extracts of the two plants with sum of fraction inhibiting concentration (FIC) of 0.74 at 50% combination ratios. These results support a rational rather than random approach to the selection of anti-plasmodial screening candidates, and identify a number of promising plants for further investigation as plant-based anti-malarial agents.

KEY WORDS: *Carissa edulis, Plasmodium falciparu*m, *Atemisia annua* combination therapy.

INTRODUCTION

One of the biggest challenges of controlling malaria disease is the spread of multi-drug resistance *plasmodium falciparum* parasites (Bloland, 2001). Most of the conventional anti-malarial drugs have developed resistance except for the plant derived drugs that are in use currently. The anti-malarial drugs that are effective are those obtained from medicinal plants used traditionally for treatment of malaria and were developed using plant-derived chemical structure as templates. For example, quinine was developed from *cinchona* sp (Simon *et al.*, 1990) and artemisinin from *Atemisia annua,* where the parent molecule was used as a template and modified to its derivatives artemether, arteether and artesunate that are currently used in combination with conventional drugs for treatment of malaria (Nosten *et al.*, 2000). Therefore there is an urgent need to discover and develop new drug combination therapy.

The use of combination chemotherapy is the current innovative strategy in controlling malaria disease; it involves the use of a short half-life acting anti-malarial drug in combination with long half-life drug. Example being artemether-lumifantrine and artesunate-melfoquine drugs (Nosten, 2000). The short half-life drug kills the parasites and excreted rapidly, resulting in re-emerging of the parasites after a period of time. Therefore use in combination with longer half-life anti-malarial drug result in achieving full eradication of the parasites preventing the recrudescence that occur with use of mono-therapy (Walsh, *et al.,* 2007). Medicinal plants are important sources for exploration in obtaining good combination of drugs.

In Kenya, plant extracts are still widely used in the treatment of malaria (Kokwaro, 2009) but most of these plants have not been subjected to scientific screening for anti-malarial activity. In continuation of the efforts to verify the efficacy of traditional anti-malarial preparations, fifteen medicinal plants used by traditional healers in North Rift Valley of Uasin-Gishu for treatment of malaria were subjected to *in vitro* anti-plasmodial screening. The most active plant was further subjected to *in vitro* analysis in combination with the total extract of *Atemisia annua* in order to obtain drug combination therapy.

MATERIALS AND METHODS

Collection and Solvent extraction

Following a survey on the ethno uses of anti-malarial medicinal plants in Uasin-Gishu District in the Rift-Valley province of Kenya through interviews with traditional health practitioners, fifteen (15) were identified to be the most commonly used medicinal plant for treatment of malaria. Parts used for treatment were collected and botanical identification was done by a taxonomist and voucher specimens were deposited at the Botany Department Herbarium, Moi University, Eldoret - Kenya.

Insert name of taxonomist and the voucher number

The plants parts were chopped into small pieces, air dried at room temperature for two weeks, and grounded into powder form. One kilogram of each powder was soaked in methanol for 3 days to extract the compounds. A dark yellow filtrate (for the roots or bark stem) and green filtrate (for the leaves) were concentrated under reduced pressure using Rota-vapour to a dark brown and green semi-solid substance respectively. The concentrates obtained weighed between 45 – 70 gms of each crude. The methanolic concentrates were then subjected to anti-malarial test.

The most active methanolic extracts was found to be *Carissa edulis* and was further screened in combination with total extract of *Atemisia annua.*

ANTI-MALARIAL TEST

Stock solution

Stock solution (100 µg/ml) for in *vitro* assay of methanolic extracts of the plants parts were prepared in deionized autoclaved water and re-sterilized by passing through 0.22 µm micro-filters in a laminar flow hood. The water-insoluble extracts were dissolved in 100% DMSO (dimethylsulfoxide) from Sigma Chemical Co; St Louis, MO USA, followed by a subsequent dilution to lower the concentration of DMSO to less than 1% to avoid solvent carrying over solvent effect (Dominique *et al.*, 2001). Stock solutions (1 µg/ml) of chloroquine diphosphate were similarly prepared for use as reference drugs. Each drug was filter-sterilized with syringe adaptable 0.22 µml filters into sterile Bijoux bottles and was stored at -20^{o} C until required for bioassay.

Parasite culture

Test samples were screened against *P. falciparum* D6 (CQ-sensitive) strain from Sierra Leone. The strains were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Nairobi. The parasites were cultivated by a previously described *in vitro* technique (Trager *et al.*, 1976). Complete culture medium (CMS) was prepared and consisted 10.4 g/l of RPMI 1640 (Rosewell Park Memorial Institute), 25 mM (5.94 g/l) HEPES buffer and 31.5 mM sodium bicarbonate supplemented with 10% human serum (Schlichtherle *et al.*, 2000). Uninfected human blood group O Rh-positive erythrocytes less than 28 days old served as host cells. The cultures were incubated at 37 ^{0}C in an atmosphere of 3% CO_2, 3% O_2 and 92% N_2 obtained from BOC gas Nairobi.

In vitro anti- plasmodium assay

The *in Vitro* anti-malarial test was based on the inhibition of [G-^{3}H] – hypoxanthine (Amersham International, Buckinghamshire, UK) uptake by *P. falciparum* cultured in human blood (Desjardins, 1979). Twenty five microliters of aliquots of the complete culture medium (CMS) were added to all the wells of 96-well flat bottomed micro-culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), followed by addition in duplicate of 25 µl of the test solution (plant extracts) to the first row wells, a Titertek motorized hand diluter (Flow laboratories, Uxbridge, UK) was used to make serial twofold dilutions of each sample over a 64-fold concentration range. Negative controls treated with solvent (DMSO) alone were added to each set of experiment (Azas *et al.*, 2001) to check for any solvent effects. The susceptibility tests were carried out with initial parasitemia (expressed as the percentage of erythrocytes infected) of 0.4% by applying 200 µl, 1.5% hematocrit, *P. falciparum* culture to each well. Two hundred microliters of culture media without parasites was added into four wells on the last row of each plate to serve as a background (controls). Parasitized and non-parasitized erythrocytes were incubated at 37 ^{0}C in a gas mixture containing 3% CO_2 , 5% O_2 and 92% N_2 for 48 hours after which 25 µl of culture medium containing 0.5 µCi, of [G-^{3}H] – hypoxanthine was added to each well. The culture plates were further incubated for 18 hours. At the end of incubation period, the radio-labeled cultures were harvested onto glass-fiber filters using a 96-well cell harvester. [G-^{3}H] hypoxanthine uptake was determined using a micro-beta trilux liquid scintillation and luminescence counter (Wallac MicroBeta Trilux). The drug concentration capable of inhibiting

50% of the *P. falciparum* (IC_{50}) was determined by logarithmic transformation of drug concentration and radioactive counts per minute (cpm) using the formula:

IC_{50} = antilog (log X_1 +[(logY_{50} – log Y_1) x (log X_2 – log X_1)]/ (logY_2 –log Y_1)).

Where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1, Y_1, X_2, and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints (Sixsmith *et al.,* 1984). The analysis of IC_{50} values was done using the computer software called GraFit-4 database.

Drug Combination bioassay Methanolic extract Carissa edulis *and Total extract of*

Atemisia annua

Template plates were used in preparation of drugs combinations. Test drugs were dispensed into an extra plate in various ratios of blends. The combined test samples were well mixed and transferred to multiple daughter plates or test plates, such that 2 daughter plates were made per two drugs pair. One daughter plate was used for the assay and the other kept at –20 °C for running a repeat test on a different day at the end of which the average of the two experiments was given as the final result.

The method described by Canfield *et al.,* (1995) was adopted. The solutions of intial concentrations 20-50 times the estimated IC_{50} values were combined in various ratios of various drugs. Thus fixed ratios of predetermined concentrations needed to inhibit parasite growth by 50% (IC_{50}) was used to determine the interaction of two drugs. Single and combined drug solutions were dispensed into the 96 well microtitre plates to give nine combinations ratios of 90:10 to 10:90 (extract A:extract B) (Fivelman *et al.,* 1999). Incubation and subsequent procedures were followed as previously described in section of *in vitro* anti- plasmodium assay. Corresponding IC_{50} values were determined for each drug alone and in combination using the method of Sixsmith *et al.,* (1984). The degree of synergy was evaluated according to the method of Berenbaum (1978). Fraction inhibition concentration (sum FIC) was calculated using the formula;

$$A_c/A_e + B_c/B_e = K \quad \text{(sum FIC)}$$

where A_c and B_c are the equally effective concentrations (IC_{50}) when used in combination, and A_e and B_e are the equally effective concentrations when used alone. When FICs < 1 it indicates synergism, sum FICs ≥1and < 2 it indicates additive interaction and sum FIC ≥ 1 it indicates antagonism (Gupta *et al*, 2002).

RESULTS AND DISCUSSION

Anti-plasmodium results of Plants extracts

The anti-plasmodium activity criteria in the *in vitro* assay were defined as high when IC_{50} value was below 10 µg/ml, moderate when between 10 – 50 µg/ml and low when between 50 – 100

µg/ml. Drug samples with IC_{50} > 100 µg/ml was considered to be inactive. The anti-plasmodia activities of the fifteen medicinal plants against CQ sensitive *P. falciparum* (D_6 strain) are summarized in table I

Table I*: In vitro anti-plasmodial activity (IC_{50}) of methanolic extracts of Medicinal plants*

Plant	Plant code	Plant part	IC_{50} (µg/ml)
Momerdica foestida Schumach	MF - R	Roots	23.46
Albizia gummifera (J.F.Gmel) Harms	AG – B	Stem bark	22.12
Erythrina abyssinica DC	EA - B	Stem bark	3.50
Clutia abyssinica Jaud & Spach	CA – L	Leaves	29.19
	CA - R	Roots	7.71
Vernonia amygdalina. Del.	VA - R	Roots	> 100
Cassia didymobotya Fres.	CD – L	Leaves	> 100
	CD - R	Roots	> 100
Clerodendrum myricoides (Hochst).	CM –L	Leaves	41.00
	CM - R	Roots	> 100
*Kanahia laniflora*Forssk.	KL - R	Roots	8.81
Clemantis simensis Fres.	CS - R	Roots	27.29
Toddalia asiatica Lam	TA - R	Roots	7.22
Fagara chellybea Engl.	FC - B	Stem bark	26.41
Zehneria scabra (L.F) Sond.	ZS -R	Roots	16.11
Croton macrostachyus Del.	CMR - L	Leaves	> 100
Teclea nobilis Del.	TN - L	Leaves	5.71
Carissa edulis (Forsk) Vahl	ED - R	Roots	1.95
Chloroquine	CQ		7.49 ng/ml

Six medicinal plants showed promising antiplasmodial activity with IC_{50} of ≤ 10 µg/ml, of which two plants *Carissa edulis* and *Erythrina abyssinica* were found to be highly active with IC_{50} values of ≤ 5 µg/ml (reported by Kebenei *et al*, 2011). Six plants showed moderate activity of IC_{50} between 10 – 50 µg/ml and three plants, *Vernonia amygdalina*, *Cassia didymobotya,* and *Croton macrostachyus* were inactive with $IC_{50} \geq 100$ µg/ml. For *Clerodendrum myricoides* the leaves extract was moderately active but the roots extract is inactive. Therefore the activity varies with the different parts of the plant, meaning different constituents of compounds present in different parts of the plant.

The results of *Vernonia amygdalina* differ from what is reported in literature from a Tanzanian *Vernonia amygdalina* plant with IC_{50} 20 µg/ml (Ohigashi *et al*, 1993) but the Kenyan plant from Uasin Gishu district is in active but widely used traditionally for treatment of malaria. *Toddalia asiatica* is widely used and showed a higher activity of IC_{50} 7.22 µg/ml corresponding to what is reported in literature (Gakunju, 1993). Therefore factors such as environmental parameters, chemo types, harvesting and storage conditions could collectively influence the plant secondary metabolites prior to and following harvesting, which in turn would be reflected in the bioactivity.

Anti-plasmodial Activity of combination of Drugs

Drug combination is one of the effective means to counter parasite resistance in anti-malarial chemotherapy (White and Olliaro, 1996). Combinations also help reduce risk of drug resistance development (Olliaro and Taylor 2003; Anne *et al.,* 2001).

The methanolic extract of *Carissa edulis* was further subjected to synergistic analysis with total extract of *Artemisia annua* and the following results in table 2 were obtained.

Table II: ***Interaction of methanolic extract of Carissa edulis with Total extract of Artemisia annua***

Combination ratios (%)	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.98[a]	0.93[a]	0.88[a]	0.82[a]	0.74[a]	0.73[a]	0.74[a]	0.71[a]	0.62[a]

a – synergistic

The interaction of EDR with *Artemisia annua* extract showed synergism at all combination ratios but decreases with increase in the concentration of *Artemisia annua* extract.

The results of the interaction study of combined test samples showed that some of the *in vitro* effects of plant extracts on parasite development were clearly synergistic these results highlight the interest in combinations of plants for the treatment of malaria. Synergism permits the quantity of each drug to be reduced, enhance efficacy and delay resistance. Drug combination is one of the effective means to counter parasite resistance in anti-malarial chemotherapy (White and Olliaro, 1996). Combinations also help reduce risk of drug resistance development (Olliaro and Taylor 2003; Anne *et al.,* 2001). Therefore combination chemotherapy for malaria should take advantage of synergistic interactions, as these would enhance therapeutic efficacy and lower the risk of resistance emergence since the combined drugs protect each partner against emergence of resistance (Woodrow *et al*, 2005).

CONCLUSION

The methanolic crude extract of the roots of *Carissa edulis* (EDR) with the highest activity of IC_{50} = 1.95 µg/ml among all plant analyzed showed high synergism with total extract of *Artemisia annua* in ratios of combination. Therefore the methanolic crude extract of *Carissa edulis* and *artemisia annua* at ratio of 9:1 respectively has high potential of being developed into a combination therapy for treatment of malaria.

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