Original Research Article

A study of the antimalarial activity of combined plant extracts traditionally used in Kenya

ABSTRACT

Background

Due to resistance and cost of current antimalarial drugs in use, there is need to continue searching for effective and affordable treatment for this disease. Traditional medicinal plants have been focused on as source of such drugs.

Methodology

For this study, combined crude extracts from six plants used in Kenya were tested *in vitro* against chloroquine resistant *Plasmodium falciparum* W2 strain using the [^{3}H] hypoxanthine uptake assay and *in vivo* using *Plasmodium berghei* on mice. Cytotoxicity tests were done using the MTT based colorimetric assay on Vero 199 and selectivity index used to assess safety of the extracts with the best activity.

Results

In vitro results with combinations involving *Zanthoxylum chalybeum* with four of the extracts indicated activity below IC_{50} 10µg/ml. A combination of *S. pinnat*a and *Toddalia asiatica* extracts exhibited the best activity *in vivo* having a parasite growth suppression of 81.3% at a dose of 500mg/kg. *S. pinnata* and *T.asiatica* combination had CC_{50} values of 102.93±5.96µg/ml and >1000µg/ml with selectivity indices of 9.52 and 15.24 respectively, indicating good therapeutic potential due to their safety margins.

Conclusion

The results obtained from both *in vitro* and *in vivo* tests support the use of the plants selected in traditional treatment of malaria. A combination of methanol fruit extract of *T. asiatica* and methanol whole plant extract of *S. pinnata* showed promising parasite growth suppression against *P. berghei* that was comparable with Artemether and was synergistic on *in vitro* testing against chloroquine- resistant W2 strain of *P. falciparum.*

Key words: Antimalarial activity, combined plant extracts, parasite growth suppression, Selectivity index, CC_{50}.

Background

Medicinal plants have been in use in the management of diseases and maintenance of health by societies for thousands of years. In modern times, the use of plants as medicines has involved isolation of active compounds and has led to isolation of drugs such as cocaine, digitoxin, quinine, morphine, artemesinin, many of which are still in use [1]. WHO avers that 65 to 85% of people in developing countries use plants as remedies for diverse disease conditions [2]. One of the conditions that has benefited greatly from research on medicinal plants is malaria, which informed the current study.

Malaria is a major tropical disease with high morbidity and mortality, affecting about 3 billion of the world population, particularly those residing in Asia, Latin America and sub-Saharan Africa. The sub-Saharan Africa region carries a disproportionate share of 90% of the global malarial load and accounts for 92% of the total malaria deaths [3]. A major setback in the treatment of malaria has been the development of resistance by the parasite to most single molecule allopathic drugs used as well as cost in its management. For example, widespread resistance developed against chloroquine and sulphadoxine-pyrimethamine which were first line drugs in the 1990's [4]. According to the WHO status report of December 2019, Pfkelch 13 mutations, which were identified as the molecular markers of partial artemisinin resistance, had been reported as widespread in the greater Mekong subregion and detected in Guyana, Papua New Guinea and Rwanda [5].

Comment [R1]: Kindly list the 13 mutations

Historically, indigenous communities have managed malaria and fevers using herbal medicine. The most effective single or in combination molecules namely, artemisinin and quinine which continue to be used in modern medicine were isolated from *Artemisia annua* and *Cinchona succirubra* respectively. Furthermore, the use of crude extracts has been demonstrated to cure malaria without the risk of developing resistance. For instance, *A. annua* extract has been in use for thousands of years in Chinese traditional medicine, without development of resistance and studies have shown that the use of the whole plant could help overcome parasite resistance, [6].

Comment [R2]: What is the difference between artemisinin, quinine and Chinese medicine treatment?

Herbal practice across different communities has involved combination of different plant extracts with a view of improving efficacy. Reports from both i*n vitro* and *in vivo* studies

have demonstrated the improvement of antiplasmodial activity by combining different plant extracts [7, 8].

The aim of this study therefore was to evaluate the antiplasmodial activity of combined plant extracts from six commonly used medicinal plants in Kenya. The study also sought to find out the safety profile of the combined plant extracts which was shown to have the best efficacy.

Methodology

Plant materials

An existing database at the Kenya Medical Research Institute, Centre for Traditional Medicine & Drugs Research of plants that had previously been analyzed for antiplasmodial activity was used to select the study plants. Six plants that had demonstrated *in vitro* antiplasmodial activity of IC_{50} less than 10µg/ml were selected. They were: *Carissa edulis, Zanthoxylum chalybeum, Toddalia asiatica, Flueggea virosa, Sckhuhria pinnata and Boscia angustifolia*. These plants were collected from Malindi and Kisumu regions of Kenya and were air-dried, ground and stored in dry manila paper bags in 1kg packs. The ground materials were extracted with distilled water and the extracts freeze dried for the water extracts. Similarly, methanol extracts were obtained and concentrated using a rotary evaporator.

Comment [R3]: Please justify the paragraph

Bioassays

The *in vitro* antiplasmodial assay was carried out according to the method described by Desjardins and coworkers [9]. Stock test solutions were prepared at a concentration of 1mg/ml in DMSO and the working solutions prepared by diluting with RPMI. Serial microdilution technique was used to arrive at the concentrations in each well. A stock solution of Chloroquine phosphate 500µg/ml was similarly prepared in double distilled water.

Plasmodium falciparum W2 strain, which has been demonstrated to be chloroquine resistant was retrieved from cryopreservation storage and thawed. They were cultured using method described by Trager and Jansen [10]. Human erythrocytes from an O+ donor was used as

host cells and culture incubated at 37^{o}C in 3% O_2, 5% CO_2 and 92%N_2 atmosphere. The experiment was conducted in a 96 well flat- bottomed micro-culture plate in duplicates with 500µg/ml chloroquine solution serving as a positive control and parasitized erythrocytes without drug and non parasitized erythrocytes serving as controls for normal growth and no growth respectively. Aliquots of 25µL of the culture medium and test solutions were added to the wells. Two-fold serial dilution of each sample was carried out over a 64-fold concentration range. A suspension of parasitized erythrocytes with 0.4% parasitemia was added to all the test wells followed by incubation at 37^{o}C in 3% O_2, 5% CO_2 and 92%N_2. After 48hours, 0.5µCi radio-labeled Hypoxanthine was added to each well and plate incubated for a further 18hours. The plates were then harvested into glass fibre filters and radioactivity measured using a liquid scintillation counter. Counts per minute (cpm) obtained per well were computed by logarithmic transformation using the formula by Sixsmith *et al.*, 1984, to obtain IC_{50} values for each extract as follows:

$$IC_{50} = \frac{\text{antilog}\,(\log X_1\,[\,(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)])}{(\log Y_2 - \log Y_1)}$$

Where Y_{50} is the cpm value midway between parasitized and non-parasitized 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.

***In vitro* extract-extract interaction experiment**

Two extracts were combined at a ratio of 50:50 and dispensed into 96 well plate and subjected to the *in vitro* antiplasmodial assay procedure above. IC_{50} values obtained for individual extracts and for the combinations were used to determine the level of synergy according to the following formula:

$$SFIC = \frac{Ac}{Ae} + \frac{Bc}{Be}$$

Where: SFIC stands for Sum of fractional inhibition concentration, A_c and B_c are IC_{50} values for combined extracts and A_e and B_e are values for the single extracts respectively.

The results were interpreted as follows: SFIC <1 implies synergism, ≥1 but <2 imply additive interaction and ≥2 implies antagonism [11].

***In vivo* antimalarial evaluation**

The protocol was based on the 4-day suppression test described by Peter's *et al.,* [12], briefly described as follows: *P. berghei* stabilates were thawed and reconstituted in phosphate buffered saline (PBS), injected interperitoneally into naïve recipient passage mice. Once a parasitemia of 20% was achieved, the passage mice were euthanized and blood drawn from the ventricles. The blood was diluted with PBS to a parasitemia of 2% and 0.2ml of this blood injected interperitoneally into sets of experimental Swiss albino mice. Each group comprised of three, 6-week-old mice of the same sex. The negative control group received PBS while the positive control received 10mg/ml Artemether.

The test plant extracts were reconstituted by solubilizing in 10% Tween 80 and 3% DMSO for the methanol extracts and dissolving in distilled water for the water extracts in order to achieve a concentration of 250mg and 500mg per kg body weight. These solutions were then administered to the mice orally at a dose volume of 0.2ml per mouse from day 0 to day 3. On day 4, thin blood smears were made from the tail vein of each mouse, fixed with methanol and stained with 10% Giemsa stain. The slides were examined microscopically at X100 magnification and levels of parasitemia determined for each group of mice. The percentage parasitemia was obtained using the formula given by Kalra *et al.,* [13]:

$$\% \text{ parasitemia} = \frac{\text{No. of parasitized RBC's}}{\text{Total No. of RBC's}} \times 100$$

The value obtained was used to calculate percentage parasite growth suppression according to the formula given by Dikasso *et al*., [14].

$$\text{Percentage chemosuppression} = \frac{\text{Mean parasitaemia in the negative control group} - \text{Parasitaemia in the test group}}{\text{mean parasitaemia in the negative control group}} \times 100$$

Test on acute oral toxicity

Female Swiss albino mice weighing 20±2g were divided into groups of three and administered with fixed doses of 1000, 1500 and 2000mg/kg body weight of the two extracts that had shown the highest *in vivo* efficacy. The mice were observed for signs of toxicity within the first 30 minutes and then periodically within 24 hours. After 24 hours, the numbers of mortality were noted and LD_{50} calculated.

Test on cytotoxicity

Vero cells obtained from the African green monkey were used for this test and the method used was as follows; The cells were seeded at a concentration of 2.5×10^4 cells per well in a 24-well plate and grown in Minimum Essential Medium (MEM) at 37^oC under 5% CO_2 for 48 hours. The culture medium was replaced by fresh medium containing extracts of *T. asiatica* and *S. pinnata*, (two plants extracts that had shown the best *in vivo* antiplasmodial efficacy) at a dose of 1000µg/ml and grown for further 48hours. 10µL MTT working solution was added to each well, plates incubated for 4 hours and the formazan crystals formed solubilized in DMSO for 30minutes. The colour intensity was quantified spectrophotometrically at 550nm. Percentage viable cells were calculated from the absorbance (abs) readings using the formula:

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

A dose response curve was plotted from which CC_{50} was obtained. From this value, Selectivity index (SI) was calculated and used as parameter of clinical significance of the test samples that compares general toxicity and selective inhibitory effect on *P. falciparum* calculated as per the formulae by Wright and Phillipson, [15]:

$$SI = \frac{CC_{50}}{IC_{50}}$$

Results

The studied plants were selected from a database available at KEMRI CTMDR and coded as shown in the Table 1 below. These plants had previously been reported to have high *in vitro* antiplasmodial activity (IC_{50} <10µg/ml) against chloroquine sensitive *P. falciparum*. They had previously been harvested, dried, ground and stored. The plant part and extract used were shown in the Table 1 below.

Table 1: Details and description of plant extracts

Plant	Voucher specimen number	Code	Origin	Plant part	extract
Carissa edulis	JO/JG/IRG/028/2016	CE001	Malindi	Root bark	water
Zanthoxylum chalybeum	JO/JG/IRG/001/2016	ZC002	Malindi	Leaves	water
Flueggea virosa	JO/JG/IRG/027/2016	FV003	Malindi	Leaves	methanol
Schkuhria pinnata	JO/JG/IRG/029/2016	SP004	Malindi	Whole plant	methanol
Boscia angustifolia	JO/JG/IRG/009/2016	BA005	Malindi	Stem bark	methanol
Toddalia asiatica	JO/JG/IRG/015/2016	TA006	Kisumu	Fruits	methanol

Comment [R4]: Why using different extraction method? Why not standardize the extraction method? Extraction method will affect the amount of active ingredients, hence, you will get the bias effect

The activity criteria in the *in vitro* assay for crude extracts were classified as follows: high at IC_{50} ≤10µg/ml, moderate at 10–50µg/ml, low at 50–10µg/ml and inactive at >100µg/ml [7]. Following these criteria, the results obtained were summarized as follows for both single and combined extracts in Table 2 and 3, respectively.

Single plant extracts that were tested *in vitro* showed that the most active extract against the chloroquine resistant W2 strain of *P. falciparum* was SP004 having an IC_{50} of 10.8µg/ml. Moderate activity was exhibited by ZC002, FV003 and BA005 while CE001 and TA006 were observed to be inactive.

Table 2: The antiplasmodial activity of single plant extracts *in vitro*

Extract	CE001	ZC002	FV003	SP004	BA005	TA006
IC_{50} µg/ml	>100	37.7±0.959	19.028±0.392	10.808±0.408	20.257±0.770	65.632±0.876

However, when different extracts were combined at equal ratios of 50:50, different scenarios were observed. The most active combinations were ZC002/FV003, ZC002/SP004, ZC002/BA005, ZC002/TA006 having IC_{50}'s <10µg/ml. Those categorized as having moderate activity were CE001/BA005, FV003/SP004, SP004/BA005, CE001/ZC002, CE001/FV003, CE001/SP004, FV003/BA005, SP004/TA006 and FV003/TA006 having

IC_{50}'s of between 10 and 50µg/ml while inactive combinations were CE001/TA006 and BA005/TA006.

Table 3 gives a summary of the Sum of Fractional Inhibition Concentration (SFIC) which was used to interpret the results obtained on combination as follows: SFIC <1 implies synergism, ≥1 but <2 imply additive interaction and ≥2 implies antagonism [16].

Antagonistic combinations included: CE001/BA005, CE001/TA006, FV003/SP004, SP004/BA005 and BA005/TA006. Combinations that were additive included: CE001/ZC002, CE001/FV003, CE001/SP004, FV003/BA005 and SP004/TA006. Combinations that were synergistic were: ZC002/FV003, ZC002/SP004, ZC002/BA005, ZC002/TA006 and FV003/TA006.

The preliminary *in vitro* results were used to determine combinations that were to be subjected to *in vivo* tests. This selection therefore omitted combinations that were antagonistic.

Table 3: *In vitro* antiplasmodial activity of combined plant extracts

Extracts	**IC_{50} µg/ml**	**SFIC**	**Comment**
CE001/ZC002	41.985	1.53371	additive
CE001/FV003	16.414	1.02676	additive
CE001/SP004	12.665	1.29847	additive
CE001/BA005	46.968	2.78829	antagonistic
CE001/TA006	85.519	2.1582	antagonistic
ZC002/FV003	5.533	0.4376	synergistic
ZC002/SP004	3.23	0.38456	synergistic
ZC002/BA005	9.409	0.71414	synergistic
ZC002/TA006	9.244	0.38613	synergistic
FV003/SP004	39.26	5.69577	antagonistic
FV003/BA005	12.254	1.24893	additive
FV003/TA006	11.392	0.77227	synergistic
SP004/BA005	11.425	6.88314	antagonistic
SP004/TA006	13.471	1.45164	additive
BA005/TA006	59.984	3.87509	antagonistic
Chloroquin phosphate	124.04ng/ml		

Results on *in vivo* antiplasmodial test

Both single and combined plant extracts were tested *in vivo* to enable comparison of their efficacy with those of the *in vitro* tests. The percentage parasite growth suppression was calculated for each extract at different doses from the percentage parasitemia observed in each mouse set compared to the negative control.

Single plant extracts elicited mixed results at different doses as shown in Table 4. Only SP004 and TA006 showed considerable parasite growth suppression at 250mg/kg. At 500mg/kg however, only TA006, showed significant parasite growth suppression. SP004 showed no parasite growth suppression at 500mg/kg. At 250mg/kg CE001, ZC002, FV003 showed no activity at all, with the test mice experiencing higher levels of parasitemia than the negative control. At 500mg/kg dose, there was still no significant change in inactivity of CE001, ZC002, FV003 and BA005 which exhibited negative suppression in the mice. Seeing that SP004 seemed to cause toxicity at higher dose of 500mg/kg, the tests were repeated at 100mg/kg and 250mg/kg where parasite growth suppression of 54.6 and 67.4% were achieved. This could have indicated that the optimal dose of SP004 was below 500mg/kg.

Table 4: Percentage parasite growth suppression of single extracts

Extract	250mg/kg	500mg/kg	100mg/kg
CE001	-64.2	1.5	ND
ZC002	-19.0	-20.3	ND
FV003	-43.6	3.7	ND
SP004	66.3±1.1	-3.8	54.6
BA005	12.7	-11	ND
TA006	51.6	58.9	ND

Key: ND-Not done

Combined extracts

As illustrated in Table 5, at a dose of 250mg/kg, all the combined extracts showed negative parasite growth suppression except CE001/SP004 which suppressed parasite growth slightly by 20%. At 500mg/kg however, there was significant increase in the percentage parasite growth suppression. The combination that showed the most significant parasite growth

suppression was SP004/TA006 at a dose of 500mg/kg giving a percentage parasite growth suppression of 81.3% which was comparable to that of the positive control. This combination was subjected to further testing at a dose of 650mg/kg and a ratio of 1:4 as shown on Table 5. The combination of SP004/TA006 gave a parasite growth suppression of 85.5% which was compared to the positive control of Artemether 10mg/kg with a parasite growth suppression of 88%.

Table 5: Percentage parasite growth suppression results of combined plant extracts

Extract	Percentage parasite growth suppression		
	250mg/kg	**500mg/kg**	**125/500mg/kg**
CE001/SP004	20.0	66.3	ND
ZC002/FV003	-12.2	28.9	ND
ZC002/SP004	-30	43.2	ND
ZC002/BA005	-32.9	24.2	ND
ZC002/TA006	-24.2	49.8	ND
FV003/BA005	-24.2	41.5	ND
FV003/TA006	1.7	46.0	ND
SP004/BA005	-33.6	40.5	ND
SP004/TA006	-61.9	81.3	85.5
Artemether at standard dose of 10mg/kg body wt.	89.3	87.2	88

Key: ND- Not done

Combination of SP004/TA006 at different doses and ratio of 1:4

From the results obtained above, the combination of SP004/TA006 at a combination ratio of 1:4 proved to have the best parasite suppression ability and was tested further at different doses (Table 6). At the highest concentration of 1000mg/kg, there was higher parasitemia compared to the negative control. To the mice which had this dose administered, showed the least survival rate at day 7. Lower doses showed increased survival rate on day 7 and better parasite growth suppression.

Table 6: *In vivo* results of combination of *S. pinnata* and *T. asiatica* extracts at different doses

Dose	**% parasitemia**	**% Parasite growth suppression**	**% Mice survival on day 7**
Artemether (10mg/kg)	10	75.2	100
PBS (negative control)	42.1	0	60
1000mg/kg	69.4	-65	20
750mg/kg	15.3	63.6	40
500mg/kg	12.4	70.6	60
250mg/kg	5.99	85.8	100

Results on cytotoxicity tests

Having selected SP004 and TA006 as the two extracts that gave the best parasite growth suppression, the two were subjected to cytotoxicity tests with the following results; SP004 had a CC_{50} of 102.925±5.96µg/ml while TA006 had CC_{50}>1000µg/ml. Their selectivity indices were found to be 9.52 and 15.24 respectively indicating good therapeutic potential due to their safety margins.

Results on acute toxicity test

The extracts of SP004 and TA006 were subjected to acute toxicity test which showed that both extracts had LD_{50} values >2000mg/kg with no observable changes in mice disposition at that dose.

Discussion

From the *in vitro* tests of single plant extracts, it was found out that water extract of the root back of *C. edulis* and the methanol extract of the fruit of *T. asiatica* did not have activity against chloroquine resistant *P. falciparum*. Extracts from *C. edulis* did not also have any activity in the *in vivo* tests. Extracts from *T. asiatica* however had moderate activity against *P. berghei* validating its use in traditional medicine against malaria. The leaves water extract of *Z. chalybeum* exhibited moderate activity in the *in vitro* test but no activity *in vivo*. The low or moderate activity of *T. asiatica* and *Z. chalybeum* extracts against chloroquine

resistant *P. falciparum* can be hypothesized to be due to the nature of phytochemicals present. Alkaloids are some of the major classes of natural products that exhibit antimalarial activity. *T. asiatica* and *Z. chalybeum* belong to the Rutacea family which is known to contain quinoline alkaloids. Chloroquine is similarly a quinoline alkaloid, hence would explain the relatively low activity against the chloroquine resistant *P. falciparum*. The extract from *S. pinnata* showed both high activity against chloroquine resistant *P. falciparum* and *P. berghei*. It belongs to Astraceae family and is known to contain sesquiterpene lactones just as *Artemisia annua* which has high antiplasmodial activity [17]. This result confirms those from different studies that showed high activity of *S. pinnata* against both chloroquine sensitive and chloroquine resistant *P. falciparum* [8]. Extracts of *B. angustifolia* and *F. virosa* showed moderate activity against chloroquine resistant *P. falciparum* and no or negligible activity *in vivo.*

Combination of extracts *in vitro* resulted in synergy, addition or antagonism, factors which must be considered in making a selection of extracts to combine. *Z. chalybeum* combinations were synergistic with very high activity. However, these combinations only showed moderate *in vivo* efficacy at 500mg/kg warranting further testing. This could be due to the pharmacokinetic principles that affect the disposition of the active ingredients in the extracts.

From *in vivo* studies, moderate activity of combined extracts was obtained at a dose of 500mg/kg. *T. asiatica* extract showed inactivity *in vitro* but moderate activity *in vivo*. A possible explanation could be that some of its active ingredients are prodrugs.

A combination of *T. asiatica* and *S. pinnata* exhibited synergism *in vitro* and high parasite growth suppression *in vivo*, making it the best combination for pre-formulation studies. In terms of toxicity, it was noted that *S. pinnata* was more toxic than *T. asiatica* as indicated by the reduced chemo-suppression at higher doses of 500mg/kg and lower cytotoxicity index. This may explain why optimal *in vivo* efficacy was obtained at a ratio of 1:4 for the former and the later respectively.

Recommendations

Comment [R5]: How you determined the alkaloids content in T.asiatica and Z.chalybeum?

Comment [R6]: What is the mechanism of chloroquine resistant towards malaria?

Comment [R7]: Is it related to the method of extraction?

Comment [R8]: What do you mean by prodrugs?

A combination of *T. asiatica* and *S. pinnata* methanol extracts showed promising parasite growth suppression against *P. berghei* that was comparable with Artemether. It had additive effect against chloroquine resistant *P. falciparum* from the *in vitro* test. Based on the obtained results, it is recommended that further studies be carried out of *T. asiatica* and *S. pinnata* extracts to find optimum efficacious dose combinations that can be formulated into a herbal based preparation for malaria treatment noting that combination of the two is more effective than the use of a single extract. Cytotoxicity of the combination should be carried out and compared to that of the single extracts. Furthermore, chronic toxicity studies on the two extracts should be undertaken to determine their long-term toxicity. Since the plant part selected was the fruit of *T. asiatica*, which from previous studies had elicited the best activity, it would be of interest to subject other parts such as the leaves to a similar study [18]. Different organic fractions of the two plants can also be studied for efficacy.

Consent and Ethical Approval

Ethical Approval was obtained from KEMRI Ethics and Research Committee, Ref. No. SERU/SSC/2928.

NOTE:

The study highlights the efficacy of "natural herbal source" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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