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Anti-Malarial Activity and Toxicological Effect of Combined Corymbia Citriodora, Maytenus Senegalensis and Warbugia Ugandensis as Used in Traditional Medicine in Kenya

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Abstract
Malaria is majorly caused by *Plasmodium falciparum* resulting in thousands of deaths every year. In Africa, it is a key contributor to the disease burden notable in the disability adjusted life years (DALYs). About 243 million individuals are at a risk of contracting the disease and a higher rate of deaths are observed in children under the age of five. Conventional drugs are available at a subsidized rate but the rising problem is the resistance of the plasmodium parasite to these drugs. Hence, there is an urgent need for the development of new and alternative therapeutics for treatment of malaria. In some regions in Kenya, parts of locally available plants are harvested and used for treating malaria. It is estimated that locally, 30% of the population relies on traditional medicine for treating various ailments due to the lack of infrastructure and accessible medical facilities especially in the rural areas. *Warbugia ugandensis*, *Maytenus senegalensis* and *Corymbia citriodora* are amongst the plants used in herbal medicine for the treatment of malaria. However, their combinatorial antimalarial efficacy and safety is yet to be determined hence the aim of this study. The plants were harvested from their natural habitats and transported to the Centre of Traditional Medicine and Drug Research (CTMDR) at the Kenya Medical Research Institute (KEMRI), Nairobi. Antimalarial properties of single and combined extracts were analyzed against *Plasmodium berghei* in vivo. Cytotoxic properties of the plants were carried out against the vero cell-lines in vitro by the MTT assay. Acute oral toxicity was conducted according to the OECD protocol. Effective concentration (ED_{50}), cytotoxicity concentration (CC_{50}) and median lethal dose (LD50) were derived. The result indicated that the combination of *M. senegalensis: W. ugandensis* (1:1) had the most antimalarial activity at ED_{50} of 1.05mg/kg whereas among the single plants *W. ugandensis* had the highest antimalarial activity (ED_{50} of 3.3mg/kg). The combinations of *C. citriodora*: *M. senegalensis*: *W. ugandensis* (1:1:1), *C. citriodora*: *M. senegalensis*: *W. ugandensis* (1:0.5:1) and *C. citriodora*: *M. senegalensis*: *W. ugandensis* (0.5:1:0.5) showed cytotoxicity concentration (CC_{50}) of 101.47±3.17 µg/ml, 213.55±3.47 µg/ml and 575.80±31.40 µg/ml respectively. *A*ll the plants combinations showed no cytotoxic effects. The synergistic antimalarial properties of combined *C. citriodora*: *M. senegalensis*, *C. citriodora*: *W. ugandensis* and *M. senegalensis*: *W. ugandensis* were confirmed as the extracts showed SFIC indexes of 0.67, 0.83 and 0.28 respectively. All the plant extracts demonstrated LD_{50} above 2000 mg/kg with no adverse effects hence recognized as safe. This study confirms the safety and antimalarial activities of these plants and justify their use in herbal medicine practices. The results of this study sets the precedence for the development of an antimalarial herbal formulation that is less toxic and more affordable.
Keywords: Anti-Malarial Activity, Toxicological Effect, Traditional Medicine
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Introduction

Malaria is a mosquito-borne parasitic disease caused by Plasmodium species (Miller *et al.,* 2013; Ghani, 2018). Mammalian hosts are the exclusive targets for *Plasmodium falciparum* and *Plasmodium vivax* with the former causing the most malaria burden globally (Global Health, 2008; Tangpukdee *et al.,* 2009; Clemente and Corigliano, 2012). Various strategies have been enlisted for the treatment, management and eradication of the disease such as vector control, chemosuppression and vaccines. Despite all the established efforts to eradicate malaria, the disease still causes significant morbidity and mortality especially in developing countries. Inaccessibility and unaffordability of conventional healthcare for treatment of malaria has motivated communities to use herbal medicine for the management of malaria. Approximately, 75% of the African population uses traditional medicine for treating human ailments (Clarkson *et al*., 2004; Ogollah, 2015). It has been suggested that the development of direct herbal formulations will provide dosage descriptions leading to cheaper and more affordable sources of drugs to the communities in which they belong. Due to natural origin and prolonged traditional use of herbal medicines they are considered safe (Willcox and Bodeker, 2004). Primarily, mortality and morbidity rates caused by malaria have reduced because of local herbal therapeutics particularly in parts of developing countries where conventional antimalarial drugs are not readily accessible, affordable and available (Kigondu et al., 2011). Despite their continued use, herbal treatments have a number of

deficits such as lack of precise diagnosis from laboratory results, plant extracts identification may be obscure, their chemical constituents are unknown and their efficacy yet validated scientifically (Ancolio, *et al*., 2002). World Health Organization recommends that plant efficacy and safety should be scientifically validated. More importantly based on the scientific background the plants can serve as novel drugs or templates for development of new therapeutic drugs. Hence, the basis of this study.

2.0 Materials and Methods

2.1 Materials

2.1.1 Plant materials

The Plant materials; *Warbugia ugandensis* stem bark from Kinangop, *Maytenus senegalensis* stem bark from Kinangop and *Corymbia citriodora* leaves from Tharaka Nithi, 5kg each were collected in gunny bags from their natural habitat in Kenya. Authentication was done at the East African herbarium, National Museum of Kenya and voucher specimen subsequently deposited at the herbarium. A taxonomist was involved during the collection and authentication process. The plant parts were then delivered to CTMDR, KEMRI.

Botanical Name	Family	Voucher Number	Parts used
Corymbia citriodora	Myrtle	PK03	Leaves
Warbugia Ugandensis	Magnoliidae	TFm11	Stem bark
Maytenus senegalensis	Celestaceae	Fm10	Stem bark

2.1.2 Animals

Swiss mice (18- 25g) were obtained from the animal facility at Kenya Medical Research Institute, Nairobi. The mice were nulliparous and acclimatized to experimental room for a fortnight. The animals were supplied with water ad libitum and food as per experimental procedures. The mice were placed in standard conditions of humidity (40-65%), temperature and 12-hour light/ darkness cycle.

2.1.3 Innoculum

The chloroquine sensitive *Plasmodium berghei (ANKA)* was obtained from Kenya Medical Research Institute Nairobi. A Swiss mouse containing *P. berghei* at a parasitaemia level of 20-30% was used as the donor inoculum. Parasitized erythrocytes were harvested from the donor mouse using a sterile needle and syringe coated with heparin. The blood was then diluted with phosphate saline glucose(PSG) buffer to obtain 1 ml blood containing 1×10^7 parasitized erythrocytes.

2.2 Methods

2.2.1 Plant material

Clean plant parts were air- dried at room temperature (25° C) for a fortnight. Once completely dried, the plant parts were ground using an electric mill into powder form then separately stored in sealed containers at room temperature. A portion (100g) of each of the powdered plant part was put in 1 litre of distilled water and heated in a water bath at 70°C for 90 minutes. The extract was decanted into a clean dry 3 litre conical flask followed by filtering through a cotton gauze. The filtered extract was then lyophilized using an Edwards freeze dryer Modulyo into powder form and then pooled into an air tight bottle, weighed, labeled and stored at 4^0 C awaiting use.

2.2.2 Cell cultures

Vero E6 cell line cells obtained from the American Type Culture Collection were cultured in Minimum Essential Media supplemented with L-Glutamine, 10% heat inactivated fetal bovine serum (FBS), 1% of penicillin and streptomycin then maintained at 37˚C in a humidified atmosphere of 5% CO_2.

2.2.2.1 MTT assay

An amount of 50 mg each of the different extract ratios was dissolved in 5ml double distilled water to give stock solutions of 10mg/ml each. This stock solution was then dissolved in 0.5 ml DMSO Dimethyl Sulfoxide (DMSO). Double distilled water was then added to give a stock solution of 10mg/ml ensuring that the final concentration of DMSO is 1% as it is toxic beyond this percentage. This one-part stock solution was diluted to 99 parts of Earl's Minimum Essential Medium (MEM) (ratio of 1:99), containing 2% Fetal Bovine Serum (FBS, maintenance medium), which was 10µl of the extract in 990 µl of media giving a start concentration of 1000 µg/ ml in 1% DMSO used in the MTT ((3- [4, 5- dimethylthiazol -2- yl) -2,5- diphenyltetrazolium bromide) assay.

In vitro cytotoxicity assay was carried out following a modified rapid calorimetric assay Mosmann (1983). Vero E6 cell line, 1ml, containing 1500-2000 cells was retrieved from liquid nitrogen storage and viability of the cells determined using Trypan blue exclusion assay. The cells were revived and cultured in T-75 flasks with Earl's Minimum Essential Media (EMEM), supplemented with penicillin & streptomycin (1%) and 10% Fetal Bovine Serum maintained at 37˚C in a humidified atmosphere of 5% CO_2 to achieve a monolayer.

Cell monolayer was broken into single cells using trypsin after achieving 70-100% confluence. Cell density count of viable cells was determined. A 96- well micro titer plate was used. A cell suspension (10 µl) containing 2×10^5 viable cells was then seeded onto columns 1,2,4,5,7,8,10,11 whereas 10 µl of media without vero cells

was added onto rows 3,6,9 and 12. The plate was then incubated at 37ºC in 5 % CO_2 for 24 hours to allow the cells to attach. After which the different combination of the drugs was serially added to the cells from rows H to row B.

Treatment protocol for cytotoxicity assay

Treatment Groups	Concentration (µl/ml)
Negative Control (Distilled water)	150
Positive Control	15
Extract Treatments	
i.	1.37
ii.	4.12
iii.	12.35
iv.	37.04
v.	111.11
vi.	333.33
vii.	1000

Different combination ratios of the combined drug extracts of *C.citriodora, M.senegalensis* and *W.ugandensis* was serially added to the wells. The plates were then incubated for 48 hours at 37ºC in 5 % CO_2. Upon incubation, 10 µL of MTT dye was added to all the cells in the plates. The plates were incubated for another 4 hours then entire media plus MTT dye was aspirated off followed by addition of 100 µl of DMSO and shaken for 5 minutes to dissolve the formazin.

The plates were read on a scanning multi well spectrophotometer (Multiskan Ex Labssystems) at 562 nm and 690 nm as reference to determine the extent of cell viability.

2.2.1 Four day chemosuppressive antimalarial activity test

The blood was obtained from the second passage and collected in containers coated with heparin. The blood was diluted with phosphate saline glucose buffer(PSG) to obtain 1 ml blood containing 1×10^7 parasitized erythrocytes. A volume of 0.2ml per mouse containing 1x 10^7 parasitized erythrocytes was intraperitoneally injected into 25 mice using a 27-gauge needle. The animals were then randomly divided into six groups each comprising of five animals. After 2 hours, a randomized group of animals was orally treated with the vehicle; distilled water), reference drug (Chloroquine) and plant extracts as shown in table 3.2 using a 26-gauge stainless steel cannula. The group treated with the vehicle served as the negative control while chloroquine treated group was the positive control. On the 4^{th} day, the mice were pricked at the tail tip and drops of blood collected on a microscope slide. The collected blood was used to prepare thin smears which were stained with giemsa dye then viewed under a microscope to observe and count the number of infected red blood cells. Parasitemia was determined microscopically by counting four fields on the microscope slide having approximately 100 erythrocytes per field. The number of infected erythrocytes against the number of total erythrocytes in the field was recorded.

$$\%\text{Parasitemia} = \frac{\text{Number of Parasitized RBCs}}{\text{Total number of RBCs}} \times 100$$

$$\text{Chemosuppression} = \frac{\text{Parasitemia in Negative Control- Parasitemia in treatment group}}{\text{Parasitemia in Negative Control}} \times 100$$

2.2.4 In vivo acute toxicity

The acute oral toxicity test was closely monitored in intervals of 0, 15, 30, 45min, 1hour,6hr,12hr,24hr, 36hr, 48hr according to OECD 425 protocol (OECD, 2005). Fifteen nulliparous and non-pregnant female (8-12 weeks old, 18 - 25g) Swiss mice were acclimatized to laboratory conditions for five days prior to dosing. Five groups (comprised of three mice) were randomly picked and placed in a marked cage. The mice were fasted for 3 hours prior dosing and their weight determined. A single dose of 0.2 ml (1ml/ 100g of mouse weight) containing combined plant extract at 5, 50, 300, 2000 mg/kg body weight doses was orally administered per group of mice using a 20 gauge oral gavage stainless steel cannula. Food was withheld for 2 hours after treatment with the plant extract.

A single group of mice (3 mice) were first administered with 5mg/kg initial dose and monitored for 48 hours. If none or one mouse dies, then a higher dose would be administered to another group of three mice. The first 48 hours for observation is crucial to ensure survival of the group of mice before proceeding to a higher dose. Toxicity was monitored and the vehicle/ negative control (distilled water) was given to the normal group. Signs of toxicity such as changes in breathing patterns, weight loss, reduced activeness and eventual death were checked within the first 48 hours after treatment. At the end of 14 days, weight of the surviving animals was recorded and humanely sacrificed.

3.0 RESULTS AND DISCUSSION

3.1 Plant extract species and yield

Plant species	Part	Weight before yield (g)	Weight after yield
Corymbia citriodora	Leaf	100	11.2
Maytenus senegalensis	Stem bark	93	17.5
Warbugia ugandensis	Roots	100	23.91

The percentage yield of the plant extract Corymbia citriodora was slightly higher than the ethanolic E. citriodora extract of 9.32% by Muhammed et al (D.Muhammed, 2018).

3.2 In vitro cytotoxicity of C. citriodora, M. senegalensis and W. ugandensis

In vitro safety profile of the plant extract was established by determined the concentration that reduced vero cells by 50% (CC50). Chloroquine had the highest cytotoxic effect with a CC_{50} value of 71.13±1.63 µg/ml. *C. citriodora: M. senegalensis: W. ugandensis* (1:1:1) and *C. citriodora: M. senegalensis: W. ugandensis* (1:0.5:1) had CC_{50} value of 101.47±3.17 µg/ml and 213.55±3.47 µg/ml respectively with no observed cytotoxicity to the vero cells. The least CC_{50} was shown by *C. citriodora: M. senegalensis: W. ugandensis* (0.5:1:0.5) at of 575.80±31.40 µg/ml. The cytotoxicity exhibited by chloroquine was statistically higher from the cytotoxicity shown by all the plant extracts.

Table 3.2.1 Cytotoxic effects of various extract combination on vero cells

Treatments	CC_{50} Values (µg/ml)
Chloroquine	71.13±1.63
C. citriodora: M. senegalensis: W. ugandensis (1:1:1)	101.47±3.17
C. citriodora: M. senegalensis: W. ugandensis (1:0.5:1)	213.55±3.47
C. citriodora: M. senegalensis: W. ugandensis (0.5:1:0.5)	575.80±31.40

All values are expressed as Mean±SEM

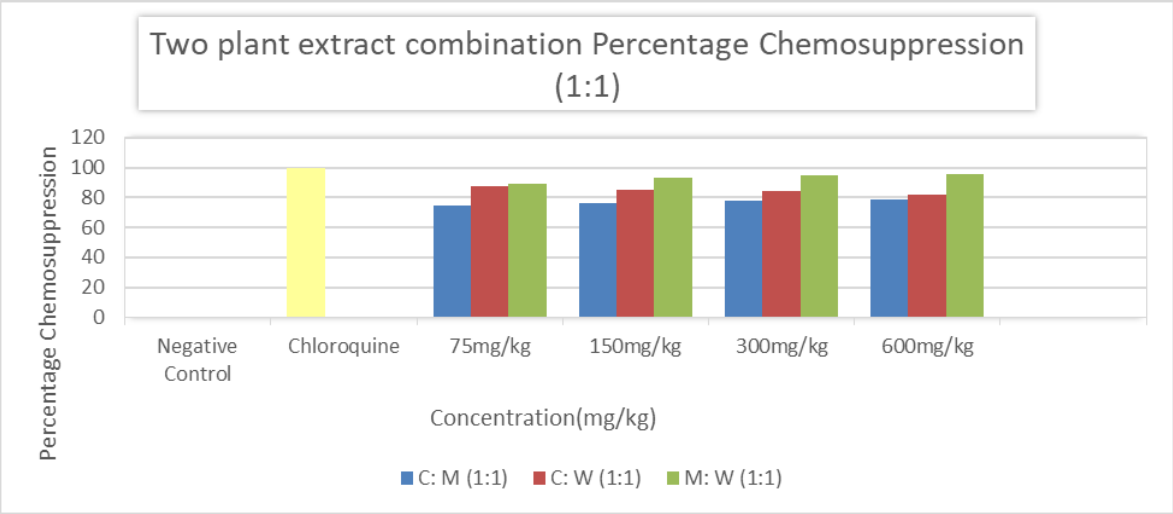
The MTT ([3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide]) assay is used to test for cell viability in which living cells reduce tetrazolium salt in a reaction catalyzed by the mitochondrial dehydrogenase enzyme (Freimoser *et al.,* 1999; Ogollah, 2015). Vero cells are recommended for cytotoxicity assays of *plant extracts and nutraceuticals* in biomaterial research since they mimic normal body cells (Kirkpatrick, 1992; ISO, 1997). The cell line was subjected to extracts treatment upon which the CC_{50} values were determined. Accordingly, *C. citriodora: M. senegalensis: W. ugandensis* (1:1:1), *C. citriodora: M. senegalensis: W. ugandensis* (1:0.5:1) *C. citriodora: M. senegalensis: W. ugandensis* (0.5:1:0.5) showed no cytotoxic effects with CC_{50} values of 101.47±3.17 µg/ml, 213.55±3.47 µg/ml and 575.80±31.40 µg/ml respectively.

Previous findings corroborate the safety properties of the aqueous extract of the plant extracts as observed in the current study. Nabende (2015) corroborated the findings of this study where it was observed that the aqueous leaf extracts of *M. senegalensis* had no cytotoxic effects against the vero cell lines (CC50 values >1000µg/ml). This study also confirms the findings of Nobakht et al. (2017) that *C. citriodora*, *C. torelliana C. citriodora* and *C. torelliana* showed no cytotoxicity activity Vero cells tested using 1000 µg/ml.

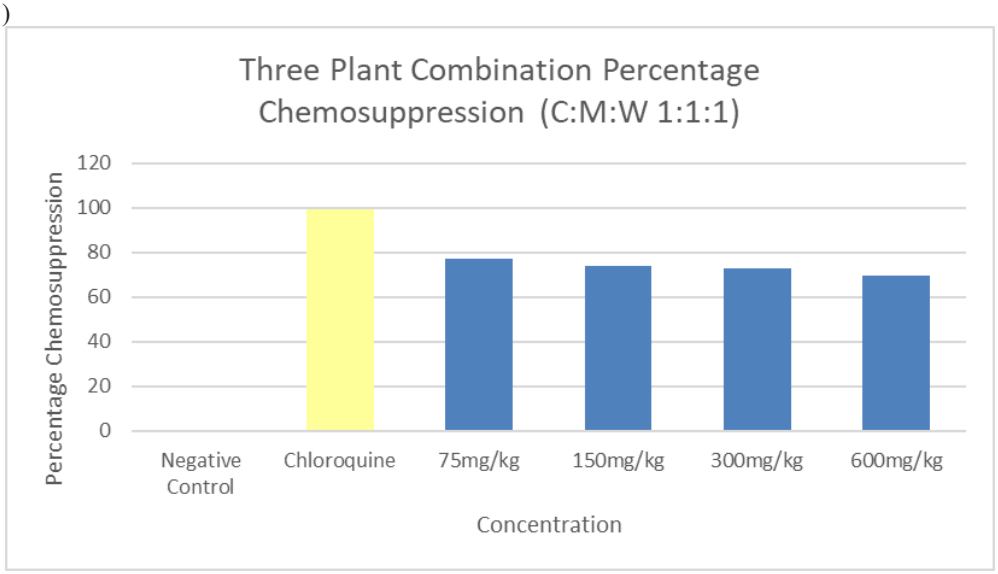
3.3 Four day chemosuppressive antimalarial activity test

Combinations of two plants extracts were tested for antimalarial activity. The combinations tested included *C. citriodora: M. senegalensis* (C: M), *C. citriodora: W. ugandensis* (C: W) *and M. senegalensis: W. ugandensis* (M: W) at the ratio of 1:1. The percentage chemosuppression of *P. berghei* growth by all the extracts was statistically lower than the positive control chloroquine but statistically higher than the negative control.

It was observed that the combinations of *C. citriodora: M. senegalensis* (C: M) and *M. senegalensis: W. ugandensis* (M: W) had dose related antimalarial activity. Correspondingly, as the concentration of the extracts increased, the percentage chemosuppression of *P. berghei* proliferation reduced (table 3.3.1). On the other hand, *C. citriodora: W. ugandensis* (C: W) showed highest chemosuppression (88.80±0.44%) at lowest concentration of 75mg/kg (table 3.3.1). Comparison of the three extracts indicated that *M. senegalensis: W. ugandensis* (M: W) had the highest chemosuppression activity of 95.35% in comparison to Chloroquine. The combination of *C. citriodora: M. senegalensis* (C: M) had the lowest chemosuppression activity of 76.72% in comparison to all the other extracts used in this study.



Further, the three plants (*C. citriodora: W. ugandensis: M. senegalensis*) were combined in the ratio of 1:1:1 and their antimalarial activities determined (table 3.3.2). An inverse dose dependent trend of inhibition was observed. Consequently, the highest percentage chemosuppression (77.06±0.41%) was observed at the lowest concentration (75mg/kg) of the plant extracts. The results also indicated that the combination of the three plants was statistically higher than the negative control group although statistically lower than the standard drug chloroquine. The three plant combination extract showed lower chemosuppression values than the two plant extract combinations. This highlights the need to sensitize the community on the importance of extract combination ratios when using plants for treating ailments to improve efficacy as well as safety (Rasoanaivo et al., 2011)



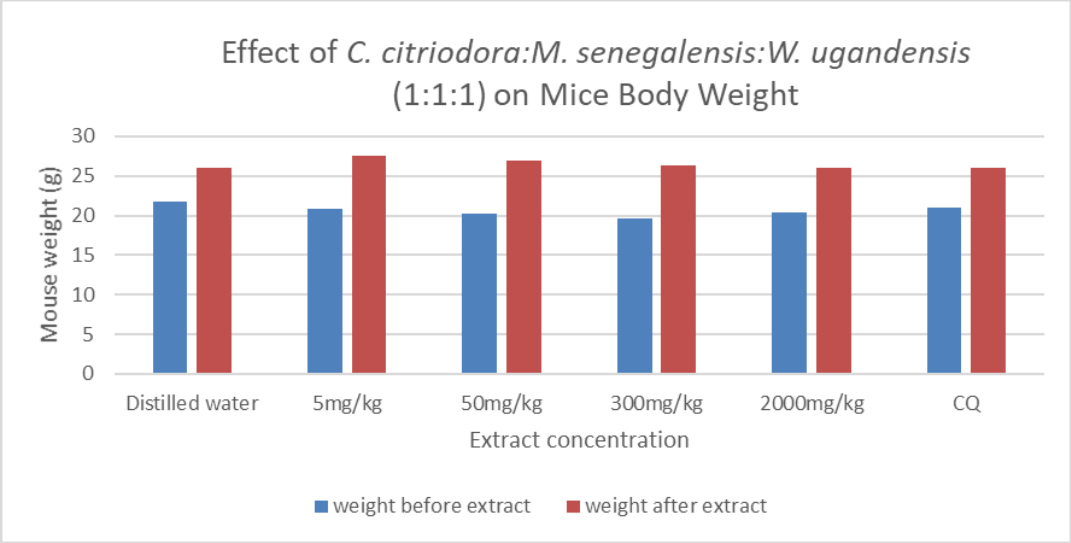
3.5 Acute Toxicity

Acute toxicity of the plant extracts was carried out on Swiss mice and body weights determined. Generally, there was an increment of the animals' body weight after the extract administration hence extract administration did not affect weight gain. Mortality did not occur from onset of drug administration until the 14- day observation period. No animals died at the highest concentration of the treatment hence the lethal dose (LD_{50}) was inferred to be above 2000mg/kg/ oral hence considered safe and nontoxic (Bello et al., 2005). The mice behavioral indicators such as reduced activity, diarrhea, paw licking, convolution and hyperactivity were not observed in this study.

Table 3.5.1 Effects of *C. citriodora*: *M. senegalensis*: *W. ugandensis* (1:1:1) on mice body weight

Concentrations	Weight before extract administration (g)	Weight after extract administration (g)	Percentage Weight change (g)
Distilled water	19.80±0.37[a]	27.00±0.71[a]	27.67±3.17[a]
5mg/kg	20.20±0.66[a]	27.60±0.51[a]	24.60±1.08[a]
50mg/kg	20.20±0.66[a]	27.00±0.45[a]	25.13±2.64[a]
300mg/kg	19.60±0.51[a]	26.40±0.81[a]	24.98±3.35[a]
2000mg/kg	19.40±0.51[a]	26.00±0.32[a]	24.54±2.46[a]
Chloroquine	19.00±0.32[a]	26.00±1.00[a]	26.50±2.05[a]
LD_{50}	**>2000mg/kg**		

All values are expressed as Mean±SEM,



4.0 CONCLUSION

The significant findings from this study supports the antimalarial ethnomedicinal claimed combination of C.citriodora, M.senegalensis and W.ugandensis in treating malaria. Attention to the extract combination ratios is crucial for the effective treatment of malaria. The results show a possibility of developing new anti-malarial novel compound using thorough molecular mechanism and characterization studies.

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ETHICAL APPROVAL

Ethical Approval was obtained from Kenya Medical Research Institute (KEMRI) Scientific Steering Committee (SSC). Ethical Review Committee (ERC) and Animal care and Use Committee (ACUC) before commencement of the study.

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