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Evaluation of Antiplasmodial Activity and Safety of Flower Extracts of *Chrysanthemum cinerariaefolium*, Singly and in Combination with Chloroquine, Lumefantrine and Piperaquine

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Authors' contributions

This work was carried out in collaboration between all authors. Authors GKW, FWM, JWG and PGM designed the study. GKW, JKN, FMT, LK and BI, wrote the protocol and performed the statistical analysis. Authors GKW and FWM wrote the first draft of the manuscript. Authors FWM and JWG managed the analyses of the study. Author GKW managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Objective: To evaluate the antiplasmodial activity and safety of organic and aqueous flower extracts of *Chrysanthemum cinerariaefolium* from Kenya, singly and in combination with chloroquine, lumefantrine and piperaquine.

Methodology: Antiplasmodial activity of organic and aqueous flower extracts of *C. cinerariaefolium* was assessed in *vitro* by serial micro-dilution assay technique against *Plasmodium falciparum,* and *in vivo* using the 4-day suppressive test as well as the established infection test against *P. berghei* ANKA in mice. To determine the safety of the extracts, cytotoxicity evaluation of extracts against Vero E6 cells and acute toxicity studies in mice were also done.

Results: *In vitro* antiplasmodial assays showed that methanolic extract of *C. cinerariaefolium* flowers was active, petroleum ether extract was moderately active, while the aqueous extract was inactive. Methanolic extract combined with chloroquine (CQ) against CQ-sensitive (3D7) and CQ-resistant (W2) *P. falciparum* showed marked synergy. Both methanol and aqueous extracts (1000mg/kg) showed chemosuppression of >45% (P<0.05) in both 4-day suppression test and established infection test against *P. berghei* ANKA in mice. Lumefantrine (LU) or piperaquine (PQ) combined with either methanol or aqueous extracts showed chemosuppression of >63% (P<0.05) against LU-resistant and PQ-resistant *P. berghei* ANKA strains, indicating synergistic interactions. Methanolic and aqueous flower extracts of *C. cinerariaefolium* had no cytotoxic effect on Vero E6 cells and no overt signs of toxicity in mice.

Conclusion: The findings showed that *C. cinerariaefolium* flower extracts are safe in mammalian systems, have antiplasmodial activity and have potentiation effect of conventional antimalarials. There is need therefore to further explore the plant's bioactive molecules which may serve as template for development of novel, effective and affordable antimalarial agents for management of malaria.

Keywords: Antiplasmodial; Chrysanthemum cinerariaefolium; combination therapy; drug efficacy; drug-drug interactions; drug toxicity.

1. INTRODUCTION

Globally, about 3.2 billion people live in areas prone to malaria transmission and 1.2 billion are at high risk [1]. In 2016, malaria contributed to an estimated 216 million infections and about 445,000 deaths where 91% of the deaths were in the African region and among those about 78% were children under the age of 5 years [1]. About 80% of the Kenyan population is at risk for malaria, 27% (approximately 12 million people) live in areas of epidemic and seasonal malaria transmission where *Plasmodium falciparum* parasite prevalence is usually less than 5%. An estimated 28 million people live in endemic areas, and over 11 million people live in areas where parasite prevalence is estimated to be equal to or greater than 20% [2]. About 26,000 to 34,000 children under the age of five die every year in Kenya [3]. In spite of elaborate interventions in elimination and control of malaria, emergence and spread of resistant *P. falciparum* have made management of malaria difficult and this makes the search for new antimalarial drugs imperative [4].

Currently, artemisinin-based combination therapy (ACT) is the paramount chemotherapy recommended by World Health Organisation (WHO) for managing *falciparum* malaria. The combination contains arteminisin derivatives and long-acting quinoline partners such as lumefantrine (LU) and piperaquine (PQ), which prolong the life-span of artemisinins by reducing the emergence of drug-resistant *Plasmodium* parasites [5]. Reported resistance against ACT is mostly due to decreased sensitivity of those quinolines, which can be restored by drug resistance reversers or chemosensitizers which can be derived from natural products or already existing drugs [6]. It has been reported that some clinical drugs such as probenecid and chlorpheniramine have chloroquine (CQ) chemosensitization effects [7].

Natural products have in the past provided compounds that have served as templates for development of conventional antimalarials. For instance, quinoline drugs which include CQ are derived from quinine, the antimalarial compound isolated from the Peruvian herb *Cinchona ledgeriana* [8]. On the other hand, artemisinin is the bioactive compound of the Chinese herb *Artemisia annua*, from which artemisinin semi-synthetics such as artesunate and artemether are derived [9,10]. Some natural products may

lack direct antiplasmodial activity but they may have the ability to alleviate malaria symptoms like fever, pain and immuno-suppression because they may possess antipyretic, analgesic and immune stimulatory properties [11]. Since 1994, natural products and their derivatives make more than half of approved drugs and over 30% of the drugs in the market [10,12]. For the last 2-3 decades, drug development with the use of natural products has been improving tremendously [10]. Some reports have shown that some plant extracts have potentiation effects in conventional drugs such as CQ against multi-drug resistant *P. falciparum* [13].

Chrysanthemum cinerariaefolium is in the genus *Chrysanthemum* in the family Asteraceae and some of the species in this genus are of medical importance [14]. The hexane extract of *C. cinerariaefolium* flowers has been reported to possess antiprotozoal activity against *P. falciparum* and *Trypanosoma brucei rhodesiense* [15]. The antiviral, antibacterial and antiplasmodial activity of *C. indicum*, *C. morifolium*, *C. coronarium* and *C. cinerariaefolium* have been reported [16,17,18, 19]. Thus, it is necessary for natural products to be investigated for their potential antimalarial activity either when used singly or in combination with conventional antimalarial drugs. Our investigations have shown that flower extracts of *C. cinerariaefolium* have antiplasmodial activity and can work in synergy with selected conventional antimalarial drugs.

2. MATERIALS AND METHODS

2.1 Study Site

The project was done at Centre for Biotechnology Research and Development (CBRD) and Centre for Traditional Medicine and Drug Research (CTMDR), Kenya Medical Research Institute (KEMRI), Nairobi, Kenya.

2.2 Collection of *Chrysanthemum cinerariaefolium* Flowers

Guided by Pyrethrum Board of Kenya, the statutory body that manages pyrethrum farming in Kenya, *C. cinerariaefolium* flowers were collected from West Pokot County, western Kenya. Authentication of the plant was done by a Taxonomist and the Voucher specimen (Tolo/Mwitari/Keter/002) deposited at the University of Nairobi herbarium.

2.3 Preparation of Organic and Aqueous Flower Extracts of *Chrysanthemum cinerariaefolium*

After collection, the flowers were dried in thin layers at room temperature under indirect sunlight in CTMDR, KEMRI and turned frequently in order to avoid fermentation for a period of two weeks. The dry flowers were then grounded into fine powder using laboratory mill (Christy & Norris Ltd., Chelmsford, England) and stored at room temperature in sealed containers until use. For organic extract, 50g of powdered dry flower were separately macerated in 100ml methanol and 100ml petroleum ether for 72 hours. Also, 50g of powdered dry flower was macerated in water, at room temperature under indirect sunlight for 12 hours. After stirring for 3 minutes, the mixture of solvents and flowers was filtered using Whatman filter paper No.1. Filtrates were concentrated using rotary evaporator (Buchi Rota vapor R114) for organic extracts, while aqueous extracts were freeze-dried (Edwards EF4 Modulyo™). The extract was stored at -20°C until use.

2.4 *In vitro* Assays

2.4.1 Cultures of *Plasmodium falciparum*

Chloroquine (CQ)-sensitive (3D7) and CQ-resistant (W2) *P. falciparum* strains were obtained and maintained at malaria culture laboratory, CBRD, KEMRI. Parasites were grown continuously using previously described procedures with subtle variation [20]. The cultures were maintained at between 1% and 10% parasitaemia, at a haematocrit of 6% in a culture containing Roswell Park Memorial Institute medium (RPMI 1640), 25 mM HEPES (N-hydroxyethylpiperazine-N'-2-Ethanolsulfonic acid), 25mM $NaHCO_3$, 10% human serum and human type O^+ erythrocytes (< 28 days old). The serum that was used was pooled from blood groups A, B and O from adult donors who signed the consent form before blood donation. The parasites were incubated in 25 cm^2 culture flasks at 37°C, under microaerophilic conditions constituting 92% N_2, 3% CO_2 and 5% O_2.

2.4.2 Preparation of drugs

One hundred milligrams (100 mg) of aqueous extract was weighed and dissolved in sterile distilled water. The solution was then aseptically filtered through 0.22 µm filters under laminar flow

hood. Subsequent dilutions were done using RPMI 1640 solution to get desired starting concentrations for *in vitro* assays. Also, 100 mg of organic extracts was weighed and dissolved in 100% dimethylsulphoxide (DMSO) and subsequent dilutions were done using RPMI 1640 solution to get desired starting concentrations for *in vitro* assays and also to lower concentration of DMSO in the solution to <0.02% [21]. Artesunate and CQ solutions were similarly prepared and the drugs stored in -20°C until use.

2.4.3 *In vitro* antiplasmodial assays

In vitro serial micro-dilution assay technique that measures the ability of the petroleum ether, methanol and aqueous flower extracts of *Chrysanthemum cinerariaefolium* to inhibit the incorporation of [G-^{3}H] hypoxanthine by *P. falciparum* on 96-microwell plates was used [22]. Culture medium (25 µl) was added to all the wells except in the 2nd row. Also, 50 µl of the extract solutions was added in triplicate in the second row of wells, and a Titertek motorized hand diluter (Flow laboratories, Uxbridge UK) was used to make two-fold serial dilutions of each sample over a 64-fold concentration range. Inoculums of 200 µl *of P. falciparum*-infected erythrocytes (at 0.4% parasitaemia and 1.5% haematocrit) were added to each well with exception of the last 4 wells of the first row which contained 200µl non-infected erythrocytes, which served as the negative control. The first 8 wells in the first row served as drug free controls for normal growth. Reference drugs CQ and artesunate were also included as positive control wells. The plates were incubated at 37°C (3% CO_2, 5% O_2, 92% N_2) for 48 hours, then radio-labelled hypoxanthine was added to each well (0.5 µl in 25 µl of culture medium), and further incubated for 18 hours to allow its uptake by surviving parasites. Each well was harvested onto a glass fibre filter using Betaplate TM cell harvester (Wallac, Zurich Switzerland), followed by washing with distilled water. The dried filters were then inserted into a plastic foil and 10ml of scintillation fluid spread on it and counted in a Betaplate TM liquid scintillation counter (Wallac, Microbeta Trilux). The results were recorded as counts per minute (cpm) per well at each drug concentration. Data was transferred to excel software and expressed as percentage of the untreated controls. 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 minutes (cpm) using the formula;

IC_{50} = 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, X_2, Y_1 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints [23].

2.4.4 *In vitro* drug interaction experiments

The method of Canfield *et al*. [24] was adopted. Methanol extract and CQ were prepared for the drug interaction experiments on the basis of their pre-determined IC_{50}, whereby initial concentrations 5-10 times the pre-determined individual IC_{50} values were combined in various ratios. Single and combined drug solutions were dispensed into the 96-well micro-titre plate containing CQ-resistant and CQ-sensitive *P. falciparum* to give duplicate rows of CQ alone, *C. cinerariaefolium* extract alone, and combinations of extract/CQ (90:10 to 10:90) [25]. Artesunate was used as the reference drug. Incubation and subsequent procedures were followed as previously described in subsection 2.4.3. The degree of synergy was determined using the formula;

K (Sum FIC) =(A_c / A_e) + (B_c / B_d)

Where,

A_c and B_c are the equally effective concentrations (IC_{50}) when used in combination, and A_e and B_d are the equally effective concentrations (IC_{50}) when used alone and K (Sum FIC) is the sum of fractional inhibitory concentrations [26]. Thus,

Sum FIC= (IC_{50} of extract in combination/IC_{50} of extract alone) + (IC_{50} of CQ in combination/ IC_{50} of CQ alone)

Score definition: Sum FICs <1 denote synergism, Sum FICs ≥1 and <2 denote additive interaction, Sum FICs ≥2 denote antagonism [27].

2.5 Toxicity Assays

2.5.1 Cultures of Vero E6 cells

Vero E6 cells originally derived from the African Green monkey kidney cells (ATCC CCL-81) were

obtained from CTMDR, KEMRI. The cells were grown in Eagle's minimum essential medium (MEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), sodium hydrogen carbonate (7.5%), HEPES 1M and 1 percent of antibiotics PCS (10,000 IU/ml penicillin, 25,000 IU/ml colimycin, 10 mg/ml streptomycin; Sigma, USA). They were detached from the flask surface by adding trypsin and sub-cultured in the larger 75cm^2 flasks using 10% media. Media change was done every 48 hours. The cells were maintained in 2.5% FBS media until they attain the confluence and then they were harvested by detaching using trypsin.

2.5.2 Cytotoxicity assays

When the cells attained the confluence, cellular suspensions of Vero E6 cells with 3.5x10^5 cells/ml were seeded in 96-microwell plates and aseptically incubated at 37°C under 5% CO_2 for 24 hours. The culture medium was replaced with fresh media followed by addition of extracts at different concentrations (achieved through 3-fold serial dilution) and incubated for another 48 hours. Row H of the plate contained the highest extract concentration (100 µg/ml) while row B contained the lowest concentration (0.13 µg/ml). Row A served as the untreated control. Columns 1, 2, 4, 5, 7, 8, 10, 11 were filled with media containing cells, while columns 3, 6, 9, 12 acted as control and contained only the media and no cells. Addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (10 µl of 5mg/ml) was done after 48 hours. After 2 hours, 50 µl of 100% DMSO was added to solubilise the formed formazan crystals and to stop the reaction by lysing Vero E6 cells. Optical density (OD) was measured at 540 nm using a spectrophotometer (Spectra Count TM, Packard, USA) with a reference filter of 690 nm. The 50 % cytotoxic concentration (CC_{50}) was defined as the concentration that reduces the OD of treated cells to 50% of that of untreated cells versus the log dose using excel software. Cytotoxicity was also expressed as the percentage of cell destruction (%D);

OD Control – OD Test x 100/ OD Control [28]

OD= Optical density values
Selectivity index (SI) was used as parameter of safety of the extracts calculated as;

SI= CC_{50} (Vero)/ IC_{50} (*Plasmodium falciparum*) [29].

2.5.3 Acute toxicity assays

2.5.3.1 Experimental animals

Male Swiss mice (6-7 weeks) weighing 20-30g were obtained from the animal facility of KEMRI and housed at 24-27°C in a 12 hours light/dark cycle. Mice were fed with pellets and water *ad libitum* in the course of the study. Care and treatment of the mice were in compliance with the guidelines stipulated by Animal Care and Use Committee (ACUC), KEMRI.

2.5.3.2 Acute toxicity test

The acute toxicological study was conducted as previously described [30], and according to OECD Guideline 423 with minor adjustments. After the mice were subjected to 24 hours of fasting but were allowed only water, extracts of plant were administered orally using gastric gavage. Each test group and control group comprised of 6 mice. A total of 24 mice (males) were used. Six mice first received the initial dose of extracts of 2000 mg/kg of body weight and then up-down procedure (UDP) was employed depending on how mice responded to the extract i.e. if more than three mice died the dose would be lowered and if more than three survived, the dose would be increased. In our case, all the mice survived and we increased the dose twice i.e. to 2250 mg/kg and 3000 mg/kg of both aqueous and methanol extracts. The LD_{50} was considered to be greater than 3000 mg/kg because no mortality was noted. The control group received only the water. The mice were individually observed, the symptoms and weight variation were recorded during the first 30 minutes and regularly during the first 24 hours after treatment and daily for 14 days. The symptoms which acted as parameters included changes in skin and fur, eyes and mucous membranes, salivation, diarrhoea, body tremor, lethargy, somnolence or coma and mortality. To avoid severe and enduring distress, mice that showed those signs were humanely killed through suffocation using carbon dioxide. The LD_{50} was calculated using the formula [31];

$LD_{50} = (ab)^{1/2}$

Where,

a= least tolerable dose
b= maximum tolerable dose

2.6 *In vivo* Antiplasmodial Assays

2.6.1 Classical 4-day suppressive test

The *in vivo* antiplasmodial activity of aqueous and methanol flower extracts of *C. cinerariaefolium* was evaluated by the classical 4-day suppressive test [32]. Males Swiss mice (6-7 weeks old, weighing approximately 20-30 g) were used to determine the antiplasmodial activity of *C. cinerariaefolium* flower extracts against CQ-sensitive *P. berghei* ANKA strain. One mouse was inoculated intraperitoneally with *P. berghei* ANKA to act as a donor to experimental mice. *Plasmodium berghei* ANKA strain was obtained from KEMRI, malaria laboratory cryopreserved at -80°C. When parasitaemia level reached 10-15%, the donor mouse was sacrificed and blood obtained through cardiac puncture. The parasitaemia was adjusted downwards using phosphate saline glucose (PSG), and each of the experimental mice was inoculated intraperitoneally with 0.2 ml of approximately 10^5 parasitized erythrocytes. Forty Swiss mice were randomized into 10 groups, each with 4 mice. First 4 groups received 125, 250, 500 and 1000 mg/kg body weight in volumes of 0.2 ml water extract while the second 4 groups received 0.2 ml of methanol extract at the same dosages. The dosages were prepared by weighing the required mass of the water and methanol extracts, dissolving each of them in distilled water and 10% v/v Tween 80 respectively. The other 2 groups served as untreated control (water only) and positive controls (CQ diphosphate- treated with 10mg/kg), respectively. Each dose of extract was administered orally once daily for 4 consecutive days, beginning on the day of infection, starting 4 hours after inoculation until day 3 post-infection (p.i.). Parasitaemia was determined on day 4 p.i. for each mouse, by microscopic examination (x1000) and counting the parasites in 4 fields of approximately 350 erythrocytes per field of thin blood film sampled from the tail of the experimental mice and stained in 10% Giemsa solutions. The mean parasitaemia of mice in each group was used to calculate the percentage chemosuppression (PS) of extract using the formula;

$$PS = [(A-B)/A] X100$$

where PS is percent parasitaemia suppression. A is the mean parasitaemia in the negative control by day 4 p.i, and B the corresponding mean parasitaemia in the test group. Percentage chemosuppression of test groups was determined relative to the untreated controls [33]. Percentage survival rate on day 15 p.i. and mean survival time (days) were also determined.

2.6.2 Established infection test

Established infection study was conducted as previously described with minor adjustments [33, 34]. A set of 40 male Swiss mice was infected with 0.2 ml of approximately 10^5 parasitized erythrocytes. On day 4 p.i, the mice were randomized into 10 groups of 4 mice per group. The first 4 groups received 125, 250, 500, and 1000 mg/kg body weight of *C. cinerariaefolium* flower water extract. The second 4 groups received the same dosages of *C. cinerariaefolium* methanol flower extract. The untreated group received water, while the positive group were treated with 10mg/kg/body weight of CQ diphosphate. Each dose of extract was administered orally once daily for 4 consecutive days, beginning on day 4 p.i. to day 7 p.i. Parasitaemia was determined on day 8 p.i. for each mouse by microscopic examination (×1000) and counting the parasites in 4 fields of approximately 350 erythrocytes per field of thin-blood film sampled from the tail of the experimental mice and stained in 10% Giemsa solutions. Percentage chemosuppression of test groups was determined relative to untreated control. Percentage survival rate on day 15 p.i. and mean survival time (days) were also determined.

2.7 *In vivo* Drug Interactions Experiments

2.7.1 Interaction of piperaquine and lumefantrine with flower extracts of *Chrysanthemum cinerariaefolium* against resistant *Plasmodium. berghei* ANKA

Determining the *in vivo* interactions of *C. cinerariaefolium* flower extracts with lumefantrine (LU) and piperaquine (PQ) was based on the procedures described previously with minor adjustments [33,34,35]. One mouse was inoculated intraperitoneally with LU-resistant *P. berghei* while another was inoculated with PQ-resistant *P. berghei,* which acted as donors. LU-resistant- and PQ-resistant *P. berghei* ANKA strains were a kind donation by Dr Daniel Kiboi from Jomo Kenyatta University of Agriculture and Technology, Kenya. When donor mice had 10-15% parasitaemia level, they were sacrificed and blood obtained through cardiac

puncture. The parasitaemia level was adjusted downward using PSG solution, and each of the experimental mice was inoculated intraperitoneally with 0.2 ml of approximately 10^5 parasitized erythrocytes.

2.7.1.1 Interaction of piperaquine with flower extracts of Chrysanthemum cinerariaefolium against piperaquine-resistant Plasmodium berghei ANKA

Infected mice (44 males) with piperaquine (PQ)-resistant *P. berghei* ANKA were randomized into 8 PQ/extract treated groups, 1 PQ alone treated group and 2 control groups each with 4 mice. The first 8 groups were treated as follows; PQ/Extract (Organic or aqueous); 5/125 mg/kg body weight, 5/250 mg/kg body weight, 5/500mg/kg body weight and 5/1000 mg/kg body weight and one group was treated with 5 mg/kg PQ alone. The drug was orally administered first followed by the administration of the extract through oral route. The other two groups were termed as controls; positive control group received combination of PQ and dihydroartemisinin (DHA) of 4.4 mg/kg and 0.6mg/kg respectively, while the untreated control received distilled water. Treatment was done for 4 days beginning on day 4 p.i. to day 7 p.i. Parasitaemia was determined on day 8 p.i. for each mouse, by microscopic examination (×1000) and counting the parasites in 4 fields of approximately 350 erythrocytes per field of thin blood film sampled from the tail of the experimental mice and stained in 10% Giemsa solution. Percentage chemosuppression of test groups were determined relative to the untreated controls. Percentage survival rate on day 30 p.i and 42 p.i. and mean survival time (days) were also determined.

2.7.1.2 Interaction of lumefantrine with flower extracts of Chrysanthemum cinerariaefolium against lumefantrine-resistant Plasmodium berghei ANKA

Infected mice with lumefantrine (LU)-resistant *P. berghei* ANKA were randomized into 8 LU/extract treated groups, 1 drug alone treated group and 2 control groups each with 4 mice. The first 8 groups were treated as follow; LU/Extract (Organic or aqueous); 10/125 mg/kg body weight, 10/250 mg/kg body weight, 10/500 mg/kg body weight and 10/1000 mg/kg body weight and one group was treated with 10 mg/kg LU alone. The drug was orally administered first followed by the administration of the extract through oral route. The other two groups were controls; positive control group received combination of LU and artemether (AR) of 8.3 mg/kg and 1.7 mg/kg respectively, untreated control received water. Treatment was done for 4 days beginning on day 4 p.i. to day 7 p.i. Parasitaemia was determined on day 8 p.i. for each mouse, by microscopic examination (×1000) and counting the parasites in 4 fields of approximately 350 erythrocytes per field of thin blood film sampled from the tail of the experimental mice and stained in 10% Giemsa solution. Percentage chemosuppression of test groups were determined relative to the untreated controls. Percentage survival rate on day 30 p.i and 42 p.i., and mean survival time (days) were also determined.

2.8 Ethical Considerations

Ethical clearance was sought from Scientific and Ethical Review Unit (SERU, KEMRI) of number KEMRI/SERU/CBRD/142/3152. Mice were handled with a lot of care as stipulated in guidelines set by Animal Care and Use Committee, KEMRI. Animals that died as a result of experimentation were put in bio-hazard bags before being incinerated. To avoid severe and enduring distress, mice that developed the following signs; impaired ambulation which prevented animals from reaching food or water, excessive weight loss and extreme emaciation, lack of physical or mental alertness, difficult laboured breathing and prolonged inability to remain upright were humanely killed through suffocation using carbon dioxide and then incinerated. The ones that remained alive at the end of the experiments were killed humanely by suffocating them with carbon dioxide in a closed chamber and then incinerated. Blood donors (adults between 20-40 years) for parasite cultures were healthy adult volunteers who signed consent forms before donation.

2.9 Data Analysis

In vivo antiplasmodial activities in various assays were analysed by comparing mean percentage parasitaemia, percentage chemosuppression and survival rate of test groups with controls, using student t-test (2-tailed) (IBM SPSS statistics version 20), with $P<0.05$ being considered significant. Effective concentration dose at 50% (ED_{50}) of piperaquine and lumefantrine drugs against both drug-sensitive and -resistant *P. berghei* ANKA was determined using STATISTICA software [36].

3. RESULTS

3.1 *In vitro* Antiplasmodial Assays

Results of *in vitro* antiplasmodial assays of petroleum ether, methanol and aqueous flower extracts of *Chrysanthemum cinerariaefolium* against chloroquine (CQ)-sensitive (3D7) and CQ-resistant *Plasmodium falciparum* (W2) strains are summarized in Table 1. Antiplasmodial activity was classified as follows: inactive extracts with IC_{50} >50 µg/ml; moderate activity at 15-50 µg/ml; active 5-15 µg/ml; highly active at < 5 µg/ml [37]. The methanol extract was highly active against 3D7 and active against W2 with IC_{50} of 4.22±0.41 µg/ml and 6.55±2.33 µg/ml, respectively. Petroleum ether extract was moderately active against both 3D7 and W2 with IC_{50} of 16.24±2.22 µg/ml and 49.51±10.88 µg/ml, respectively. The aqueous extract was inactive against both 3D7 and W2 with IC_{50} of 83.86±22.60 µg/ml and 159.92±13.18 µg/ml respectively.

3.2 *In vitro* Drug Interaction Studies

Methanolic extracts of *C. cinerariaefolium* flowers which had the highest antiplasmodial activity was combined with CQ and tested against 3D7 (CQ-sensitive) and W2 (CQ-resistant) *P. falciparum* isolates. The results are summarized in Table 2. Drug interaction of methanolic extracts with CQ against W2 showed better interaction than against 3D7. All combination ratios of methanolic extracts with CQ against W2 showed synergy, with combination ratio of 60:40 showing strong synergy, with 20:80 combination showing additive interactions. Interaction against 3D7 also showed strong synergy in combination ratios of 70:30, 40:60, and 30:70 but the combinations ratios of 90:10, 20:80 and 10:90 showed additive interactions.

3.3 Cytotoxicity Assays

Results of cell cytotoxicity assays of the *C. cinerariaefolium* flower extracts are summarized in Table 3. Methanol and aqueous extracts exhibited low cell cytotoxicity against Vero E6 cells with CC_{50} of 65.96µg/ml and 2899.19µg/ml, respectively. Water extract showed higher selective index (SI) of 34.57 and 18.13 against chloroquine (CQ)-sensitive (3D7) and CQ-resistant (W2) *P. falciparum* strains, respectively. Methanol extract had slightly lower SI of 15.62 and 10.07 against 3D7 and W2 respectively.

Table 1. *In vitro* antiplasmodial activity (IC_{50}) for selected *C. cinerariaefolium* extracts against chloroquine (CQ)-sensitive (3D7) and CQ-resistant (W2) *P. falciparum* strains

Extract	*IC_{50} value (x ± SD) (µg/ml)	
	CQ-sensitive *P. falciparum* (3D7)	CQ-resistant *P. falciparum* (W2)
Petroleum ether	16.24± 2.22	49.51± 10.88
Methanol	4.22± 0.41	6.55± 2.33
Aqueous	83.86± 22.60	159.92± 13.18
Chloroquine (CQ)	1.40± 0.12ng/ml	45.40± 3.64ng/ml

**Values are mean of two independent experiments carried out in triplicate. Chloroquine (CQ) is used as reference drug. SD, standard deviation*

Table 2. *In vitro* drug interactions of methanolic flower extract of *C. cinerariaefolium* with chloroquine (CQ) against CQ-sensitive (3D7) and CQ-resistant (W2) *P. falciparum* strains

Combination ratio (Methanol extract : CQ)	*Sum FIC	
	CQ-sensitive *P. falciparum* (3D7)	CQ-resistant *P. falciparum* (W2)
90:10	1.35	0.57
80:20	0.89	0.60
70:30	0.01	0.59
60:40	0.71	0.40
50:50	0.76	0.81
40:60	0.39	0.67
30:70	0.13	0.62
20:80	1.05	0.99
10:90	1.59	0.77

**Sum FIC= IC50 of extract in combination/ IC_{50} of extract alone +IC_{50} of CQ in combination/ IC_{50} of CQ alone; Score definition: Sum FICs <1 denote synergism, Sum FICs ≥1 and <2 denote additive interaction, Sum FICs ≥2 denote antagonism [27]*

Table 3. Cytotoxicity activity (CC_{50}) and selective index (SI) of aqueous and methanol flower extracts of *C. cinerariaefolium*

Extract	IC_{50} (µg/ml) against 3D7	IC_{50} (µg/ml) against W2	CC_{50} (µg/ml) against Vero E6 cells	SI against 3D7	SI against W2
Methanol	4.22	6.55	65.96	15.62	10.07
Aqueous	83.86	159.92	2899.19	34.57	18.13

SI was calculated as CC_{50}/IC_{50}

Table 4. Acute toxicity of water and methanol flower extracts of *C. cinerariaefolium* in mice

Extract	No of mice dead per dose				LD_{50} (mg/kg)
	3000mgkg	2250mg/kg	2000mg/kg	Water(control)	
Methanol	0	0	0	0	>3000
Aqueous	0	0	0	0	>3000

There were 6 mice per group. Extracts were administered orally and given once after starvation for 24 hours (with only water). The observation was done within the first 30 min and regularly during the first 24 hours after treatment and daily for 14 days

3.4 *In vivo* Acute Toxicity Studies

In the acute toxicity assay, no death or signs of toxicity was observed in mice at highest dosage tested. The LD_{50} values for methanol and aqueous extracts were above 3000 mg/kg body weight when tested by oral administration.

3.5 *In vivo* Antiplasmodial Assays

3.5.1 Classical 4-day suppressive test

Results of *in vivo* antiplasmodial assay of methanol and aqueous extracts of *C. cinerariaefolium* flowers against *P. berghei* ANKA performed using 4-day suppressive test are summarized in Table 5. chemosuppression of mice that received the highest dosage of extracts was 59.29% and 48.82% respectively, relative to untreated controls. The mean survival time (days) of mice treated with the extracts was 22.00±4.30 and 16.00±7.44 days respectively, relative to survival of the untreated controls (8.00± 1.83 days).

3.5.2 Established infection test

Results of *in vivo* antiplasmodial assay of methanolic and water extracts of *C. cinerariaefolium* flowers against *P. berghei* ANKA performed using established infection test are summarized in Table 5. Chemosuppression of mice that received the highest dosage of extracts was 45.42% and 48.04% respectively, relative to the untreated controls. Their mean survival time (days) was 21.25±5.91 and 22.50±7.72 days respectively, relative to survival of the untreated controls (10.75± 0.5 days).

3.6 *In vivo* Drug Interactions Experiments

3.6.1 Interaction of piperaquine (PQ) or lumefantrine (LU) with methanolic and aqueous flower extracts of *Chrysanthemum cinerariaefolium* against PQ-resistant or LU-resistant *Plasmodium berghei* ANKA using established infection test

Results of *in vivo* antiplasmodial assay of methanolic and aqueous extracts of *C. cinerariaefolium* flowers in combination with piperaquine (PQ) or lumefantrine (LU) against PQ-resistant or LU-resistant *P. berghei* ANKA respectively performed using established infection test are summarized in Table 6. Chemosuppression of highest dosage of methanolic extract/PQ (1000:5 mg/kg), water extract/PQ (1000:5 mg/kg) and PQ (5 mg/kg) alone was 81.02%, 72.29% and 34.33% respectively, relative to the untreated controls. Percentage survival on day 42 p.i of mice that received highest dose (1000 mg/kg) of water and methanolic extracts in combination with PQ (5 mg/kg) was 50%. On the other hand, suppression of highest dosage of methanolic extract/LU (1000:10 mg/kg), water extract/LU (1000:10 mg/kg) and LU (10 mg/kg) alone was 76.42%, 63.11% and 34.21% respectively, relative to the untreated controls. Percentage survival of mice on day 42 p.i of mice that received the highest dose of water (1000 mg/kg) and methanolic extract (500 and 1000 mg/kg) combined with LU (10 mg/kg) was 50%.

Table 5. Percentage chemosuppression and mean survival time (days) of *P. berghei* ANKA-infected mice treated orally with methanolic and aqueous flowers extracts of *C. cinerariaefolium* at selected dosages

Extract	Dosage (mg/kg)	Percentage chemosuppression		Mean survival (days)	
		4-day test	Established infection test	4-day test	Established infection test
Aqueous	125	11.24	9.98	8.00± 1.15	12.25± 1.89
	250	44.00	23.99	9.00± 2.94	15.00± 2.83
	500	45.88	29.77	11.25± 1.50	22.50± 2.08
	1000	48.82	48.04	16.00± 7.44	22.50± 7.72
Methanol	125	15.82	10.68	12.50± 3.87	16.75± 3.40
	250	50.10	25.22	16.00± 1.83	17.75± 4.27
	500	56.12	32.69	18.75± 1.30	20.50± 6.45
	1000	59.29	45.42	22.00± 4.30	21.25± 5.91
Reference drug	CQ	100.00	100.00	>30± 0.00	>30± 0.00
Untreated control	Water	0.00	0.00	8.00± 1.83	10.75± 0.5

Each test group had 4 mice. Mean parasitaemias of mice per group were determined. Observation time post-treatment was 30 days. Water and CQ were used as untreated and positive controls, respectively. Percentage chemosuppression in 4-day test and established infection test were determined on day 4 and 8 p.i. respectively. The dosage for CQ was 10 mg/kg.

Table 6. Percentage chemosuppression and percentage survival (days) of piperaquine (PQ)- or lumefantrine (LU)-resistant *P. berghei* ANKA-infected mice and orally treated with methanolic or aqueous flowers extracts of *C. cinerariaefolium* in combination with PQ or LU at selected dosages

Drug/Extract combination	Extract (mg/kg)	Parasitaemia suppression		Mouse survival (days)	
		PQ-resistant *P. berghei* ANKA	LU-resistant *P. berghei* ANKA	PQ-resistant *P. berghei* ANKA	LU-resistant *P. berghei* ANKA
Drug/Water extract	125	49.27	41.47	0.00	0.00
	250	56.22	44.95	0.00	25.00
	500	63.00	54.21	25.00	25.00
	1000	72.29	63.11	50.00	50.00
Drug/ Methanol extract	125	56.87	54.42	0.00	25.00
	250	64.38	66.11	25.00	0.00
	500	72.00	74.21	25.00	50.00
	1000	81.02	76.42	50.00	50.00
PQ-DHA	4.4/0.6	92.33	-	100	-
LU-AR	8.3/1.7	-	92.26	-	100
PQ	5	34.33	-	0.00	-
LU	10	-	34.21	-	0.00
Untreated control	Water	0.00	0.00	0.00	0.00

Mice infected with PQ-resistant P. berghei ANKA were treated with PQ (5mg/kg) while those infected with LU-resistant P. berghei ANKA were treated with LU (10 mg/kg) mixed with different dosage of extracts as shown in the table. Piperaquine-dihydroartemisinin (PQ/DHA, 4.4/0.6 mg/kg) and lumefantrine-artemether (LU/AR, 8.3/1.7 mg/kg) were used as positive controls. There were 4 mice per group. Post-treatment observation was 42 days.

4. DISCUSSION

Methanolic flower extract of *Chrysanthemum cinerariaefolium* was the most active against both chloroquine (CQ)-sensitive (3D7) and CQ-resistant (W2) *Plasmodium falciparum*, relative to the petroleum ether and water extracts (Table 1). Methanol is known to dissolve both polar and non-polar biomolecules, petroleum ether dissolves only non-polar compounds while water largely dissolves polar compounds. Both polar and non-polar compounds in methanolic extract could have acted synergistically, which may partially explain why methanolic extracts

exhibited better antiplasmodial activity [38]. Unlike aqueous and petroleum ether extracts, the antiplasmodial activity of methanol extract against 3D7 and W2 did not differ significantly (P>0.05) (Table 1). Therefore, methanolic extracts may lack cross-resistance with CQ, a phenomenon that can be attributed to differences in the mode of actions of different bioactive compounds in methanolic extract [39]. But more analysis needs to be carried out to ascertain whether the antiplasmodial activity is solely due to pyrethrins which are highly concentrated in the flowers of *C. cinerariaefolium* or other bioactive compounds that might be present [15]. Methanolic flower extract of *C. cinerariaefolium* can be analyzed further both biologically and chemically to ascertain whether it contains bioactive compounds which can serve as template in the development of affordable antimalarial drugs against resistant *falciparum* malaria.

In drug interaction studies, methanolic extract of *C. cinerariaefolium* flowers potentiated CQ against W2 (CQ-resistant *P. falciparum*) more than against 3D7 (CQ-sensitive *P. falciparum*) (Table 2). This extract seems to have the ability to reverse resistance in W2. It is known that some compounds enhance the influx of CQ in the food vacuole of the malaria parasite [40]. Several chemosensitizers such as promethazine and chlorpheniramine [41,42] have been reported to restore CQ's efficacy in the drug-resistant malaria parasite, both *in vitro* and *in vivo* systems. Exploring mechanisms by which methanolic extract potentiated the conventional drug CQ may lead to the establishment of biochemical tools that can be used to elucidate drug potentiation phenomenon [6,7]. The biological activity of *C. cinerariaefolium* flower extracts may not be attributed to the *in vitro* cytotoxicity since its selectivity index was above 10 [43] (Table 3). Thus, aqueous and organic extracts were not cytotoxic to vero E6 cells. In *in vivo* acute toxicity studies (Table 4), there were no mortality and toxicity signs which were observed when the highest dosage of 3000mg/kg was administered orally to mice and observed for 14 days. This implies that the LD_{50} values for both extracts were above 3000 mg/kg body weight, and thus safe. The extracts thus need to be further explored for bioactive molecules which can be used as template for development of effective and affordable antimalarial agents.

In 4-day chemosuppression test studies, both methanolic extract and aqueous flower extracts of *C. cinerariaefolium* had remarkable chemosuppression of 59.29% and 48.82% respectively against *P. berghei* ANKA, relative to untreated control (P< 0.05) (Table 5). This suggests that the extracts have the ability to reduce the parasite load by almost 50%, therefore, increasing the survival of the treated mice. The mean survival rate in mice that received methanolic extract (P <0.05) is higher than those that received aqueous extract (P >0.05) (Table 5). Methanolic extracts remarkably prolonged the lives of mice more than water extract. The difference in activity between water and methanol flower extracts of *C. cinerariaefolium* is probably due to the fact that methanol is able to dissolve both polar and non-polar biomolecules with high antiplasmodial activity such as alkaloids, flavonoids and sesquiterpenes lactone [38]. Methanolic flower extract of *C. cinerariaefolium* showed higher efficacy both *in vitro* and *in vivo,* unlike water extract which was inactive *in vitro* (Table 1), but had comparatively higher activity *in vivo*. This disparity by water extract probably is due to the fact that bioactive compounds which are inactive *in vitro* can undergo biotransformation *in vivo* into active metabolites [44]. Also, plant extracts may have other pharmacological properties such as analgesic, immune-stimulation or antipyretic, thus increasing the survival rate of mice [11,45, 46]. In established infection chemosuppression test, aqueous extract activity (48.04%) was higher than that of the methanolic extract (45.42%) relative to untreated controls (Table 5), although their difference is not statistically significance (P>0.05). Both chemosuppression and mean survival rate was dose-dependent.

In vivo drug interactions studies indicated that chemosuppression of PQ/methanolic flower extract (81.02%) of *C. cinerariaefolium* is comparable to that of PQ/water flower extract (72.29%) (P> 0.05) against PQ-resistant *P. berghei* ANKA (PQ alone, 34.33%; methanolic extract alone, 45.42%; water extract alone, 48.04%), relative to the untreated controls (Table 5 & 6). Chemosuppression of both combinations were statistically significant, relative to that of PQ alone (34.33%) (P<0.05). Also, 50% of mice that received PQ/methanolic extract (1000 mg/kg) and PQ/water extract (1000 mg/kg) survived the whole of the observation period (42 days), whereas 100% mortality was observed for mice treated with PQ alone. The survival rate was dose-dependent. Chemosuppression of LU/methanolic flower extracts of *C. cinerariaefolium* (76.42%) was comparable

(P<0.05) to that of LU/water flower extracts (63.11%) against LU-resistant *P. berghei* ANKA (LU alone, 34.21%; methanolic extract, 45.42%; water extract, 48.04%), relative to the untreated controls (Tables 5 & 6). Therefore, both extracts in combination with LU possess potentiation properties. Also, 50% of mice that received LU/methanolic extract (500 and 1000 mg/kg) and LU/water extract (1000 mg/kg) survived the whole of the observation period (42 days), while the LU alone-treated mice had 100% mortality. Similarly, just like in PQ-combination studies, the survival rate was dose-dependent. Enhanced chemosuppression and longer survival in combination studies underscore the fact that *C. cinerariaefolium* crude extracts possess bioactive compounds that may not only possess antiplasmodial action but also may act as chemosensitizers.

Chrysanthemum cinerariaefolium flower extracts seem to have the potential of reversing resistance of PQ- or LU-resistant *P. berghei* ANKA and therefore more studies need to be done to explore this phenomenon. Several scientists have reported that combination of crude plants extracts, as well as bioactive compounds with conventional drugs like CQ, have potential to reverse resistance of *P. falciparum* both *in vitro* and *in vivo*, although little is known on mechanism of reversal of resistance [11,13,41,44]

5. CONCLUSION

We have demonstrated that *C. cinerariaefolium* flower extracts seem to have antiplasmodial compounds which are able to potentiate the activity of CQ, LU and PQ against resistant *Plasmodium spp*. The bioactive compounds were also safe in mammalian systems. These bioactive compounds need to be isolated, characterized and evaluated for their antiplasmodial activity, toxicity and potentiation characteristics. Further studies are also needed to understand the mechanism of potentiation that was observed between the conventional drugs CQ, LU and PQ with *C. cinerariaefolium* flower extracts. It is envisaged that further biochemical and chemical investigations of *C. cinerariaefolium* may lead to development of novel, affordable and effective antimalarial drugs for clinical management of malaria.

CONSENT

Blood donors (adults between 20-40 years) for maintaining parasite cultures were healthy adult volunteers who signed consent forms before donation.

ETHICAL APPROVAL

Ethical clearance was sought from Scientific and Ethical Review Unit (SERU, KEMRI) of number KEMRI/SERU/CBRD/142/3152.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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