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Evaluation of in *vivo* antimalarial activity of the ethanolic leaf extracts of C*hromolaena odorata* and C*ymbopogon citratus* in mice

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Abstract

Malaria has remained a leading cause of morbidity and mortality in tropical countries of the world due to the resistance posed by malaria parasites to most commonly affordable anti-malarials. The anti plasmodial activities of the ethanolic leaf extracts of *Chromolaena odorata* and *Cymbopogon citratus* on chloroquine sensitive *Plasmodium berghei berghei* in mice was evaluated. *C. odorata* and *C. citratus* exhibited significant ($p < 0.05$) blood schizontocidal activity in a four- day early infection test and in established infection with a considerable mean survival time comparable to that of the standard drug chloroquine (5mg/ kg/ day). The efficacy of both treatments is further indicated in the consistent increase in weight and slight increase in the PCV levels of the treated groups as against those of the untreated groups. The extracts from both plants showed significant ($p<0.05$) anti-plasmodial activity and could serve as possible candidates for the development of new effective drugs for the treatment of malaria.

Key words: *Chromolaena odorata*, *Cymbopogon citratus*, anti-malarial activity, ethanolic leaf extracts

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Introduction

Malaria is unique among diseases because its roots lie so deep within human communities. Malaria remains one of the worst menaces of tropical countries of the world. It is a killer and debilitating disease that affects the physical and economic well-being of people living in endemic areas of Africa (WHO, 2000). The high intensity of the spread of malaria makes it an enormous and leading public health problem especially in Sub-Saharan Africa. African countries are most hard hit by the disease, where it ravages communities (TDR/WHO, 2002).Although the disease has been eradicated in most temperate zones, it continues to be endemic throughout most of the tropics and sub-tropics. Malaria is a threat to more than 40% of the world's population. Out of the more than 300 million acute cases that occur each year, between 1.1 and 2.7 million people die each year (WHO, 2000; RBM, 2002). Malaria, a notifiable, preventable and curable disease is highly endemic in most parts of Nigeria, and is the most common cause of out-patient hospital attendance in all age-groups in all parts of Nigeria (Ogunbona *et al.*, 1990). Malaria constitutes nearly 25% of all childhood mortality in Africa (WHO, 2000). Malaria's cost to human and social well-being is enormous. It is a major cause of poverty and poverty exacerbates the malaria situation (UNICEF, 2001). It has slowed economic growth in African countries by 1.3% per year. Despite measures taken against the ailment, malaria has continued to rank high among the most prevalent and severe diseases of the tropics. Some of the reasons for these include : insecticide resistance in mosquitoes, war and civil disturbances, environmental changes, climatic changes, travel and population increase and the rapid development and sustenance of resistant strains of the parasite to commonly and affordable anti-malarial drugs (Nussebweigh and Levy, 1994).

The main problem for malaria control at present is the resistance of the parasite to the cheapest and most commonly used anti-malarials- chloroquine and sulphadoxine-pyrimethamine (SP) (Schultz, 1994). Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine. Traditionally, plants play an important role in the medical systems in Africa and plant materials remain an important resource to combat serious diseases in the world. Screening of plants for pharmacologically interesting substances are carried out to find novel drugs or templates for the development of new therapeutic agents, as many drugs such as quinine and artemisinin were isolated from plants. The steps to be followed would include, authentication of claims, method of preparation, standardization, and the use of gas chromatography/mass spectrometry among others to find out the actual number of chemical constituents (IJMAT, 2008). Some authors such as Barua *et al*; (1978), Okunji *et al;* (2000), Suksamram *et al;* (2004) and Igboh *et al*; (2009) have reported the presence of flavonoids and terpenes in the leaves and flowers of *C odorata.* Okunji *et al;* (2000) have reported anti-plasmodial activity of *C. odorata* in vitro. The antimicrobial, wound healing and coagulant properties of the plant have also been reported (Suksamram *et al;* 2004). *C citratus* has been reported to contain azaleatin (flavonol) (Obute *et al*; 2007). Nwabuisi, (2002) and Obute *et al*; (2005) reported the antiplasmodial and antidiabetic activities of the leaf extracts of *C. citratus* respectively. This work set out to ascertain the in-vivo anti-malarial efficacy of *Chromolaena odorata* and *Cymbopogon citratus.*

Materials and Methods

Collection of plant materials: From the list of plants most commonly used in the study area (Aba South L.G.A, Abia State, Eastern Nigeria), fresh leaves of *Chromolaena odorata* locally known as (nanimgebi or Awolowo) and *Cymbopogon citratus* locally known as(achara tea or lemon grass) were collected from their natural habitats, and authenticated by Dr. Garuba Omosun of the Department of Biological Sciences, Michael Okpara University of Agriculture, Umudike, Abia State. The leaves of the plant materials were washed, air-dried and then milled with a Phillip Blender to fine powder.

Extraction procedure: The ethanolic extracts were obtained by macerating each of the leaf powder in 95 % ethanol, in the proportion of 100 grams of the powder to 300 mls of ethanol (Ivoke, 2005) for 72 hours. A hundred (100) grams of the powdered materials was fed into the soxhlet apparatus and 300 mls of ethanol was added to it and mixed. The mixture was heated and the ethanolic extract exhaustively extracted at a temperature of 40^0C for about 6 hours. Water bath was used to evaporate the solvent (ethanol). The extract was then left overnight at room temperature (36-38^0C) for the remaining ethanol to evaporate. The procedure was done with the two different powdered materials. Green-coloured pastes were obtained and weighed. The yields were 0.48% and 0.47% respectively.

Phytochemical screening: A preliminary phytochemical screening of the powdered materials of both plants was carried out employing the standard procedures by (Odebiyi and Sofowora, 1978) for detecting the presence of alkanoids, flavonoids, tannins, saponins, glycosides and volatile oils.

Experimental animals: Albino mice (25-32g) of both sexes were obtained from the Department of Veterinary Parasitology, University of Nigeria, Nsukka. The animals were housed in well ventilated plastic stainless cages, and were acclimatized for 7 days prior to their randomization into the various experimental groups. The animals were maintained on standard commercial diets and water was given *ad libitum*. The principle of "Laboratory Animal Care" (National Institute for Health) guidelines and procedures were strictly followed. Prior to the commencement of the experiment, the animals were screened for the presence of blood parasites.

Parasite inoculation: Chloroquine-sensitive *Plasmodium berghei berghei* was obtained from National Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria and maintained by serial passage in mice. The inoculum consisted of 5 X 10^7 *Plasmodium berghei berghei* parasitized erythrocytes per ml. This was prepared by determining both the percentage parasitaemia and the erythrocyte count of the donor mouse and diluting the blood with phosphate buffer saline in proportions indicated by both determinations. Each mouse was inoculated on day zero (0) intra-peritoneally with 0.2 ml of infected blood containing about 1 X 10^7 *Plasmodium berghei berghei* parasitized red blood cells.

Blood sample collection: Blood was obtained from the tail of the mouse to determine the parasitaemia level while the blood used for determining haematological parameters was obtained from the eyes skillfully without puncturing the eyes.

Preparation of thin film: A small drop of blood was collected from the tail of each of the animals in each group (8 groups of 4 animals each), labeled A - H, using clean, non-greasy slides.

Thin films were made accordingly and allowed to air-dry (Cheesbrough, 2000). The thin films were then fixed using few drops of methanol. The films were left for about 15 to 20 minutes to air-dry. Thereafter they were washed off and stained with Giemsa stain for 45 minutes. The stain was washed off and slides left to air-dry, before they were viewed under the light microscope using the oil immersion objective.

Evaluation of parasitaemia: Each of the blood films prepared was mounted and viewed under the microscope using the X100 objective. The number of parasitized red blood cells that were seen per film was counted and recorded.

Evaluation of safe dose and acute-toxicity (LD_{50}): LD_{50} of the extracts were determined using albino mice and the method outlined by Lorke (1983). The mice were treated intra-peritoneally with a single dose of 1.5 g per kg of the individual leaf extracts after being starved for 24 hours. The route was chosen because of its sensitivity and rapid results.

Invivo evaluation of ethanolic leaf extracts of C. odorata and C. citratus for blood schizontocidal activity on early infection (four-day suppressive test): Schizontocidal activity of the extracts of both plants was evaluated using the method described by Knight and Peters (1980). Thirty-two albino mice were used to assess the effect of each plant extract. Each mouse was inoculated in the first day (day zero) intra-peritoneally with 0.2 ml of infected blood containing about 1×10^7 Plasmodium berghei berghei parasitized erythrocytes. The animals were divided into four animals per cage, and a total of eight cages were used for the study labeled A-H. Groups A-C were used to study the effects of C. odorata at three different concentrations of 100, 150 and 200mg/ kg doses per day. Groups D-F were used to study the effects of C. citratus at three different concentrations of 100, 150 and 200mg/kg doses per day, all beginning on the day of inoculation. The group labelled G (positive control group) received 5mg/kg chloroquine per day while group labelled H received distilled water of equal volume for four consecutive days (day 0 to day 3). On the fifth day (day 4) thin blood films were made from the tail of each mouse and the parasitaemia level was determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope.

Average percentage suppression of parasitaemia was calculated using the formula:

$A = (B-C)/B \times 100$

Where A = Average percentage suppression

B = Average percentage parasitaemia in the negative control

and C = Average percentage parasitaemia in the test groups

Evaluation of blood schizontocidal activity of the ethanolic leaf extracts of C. odorata and C. citratus on established infections (Rane test): This was carried out according to the method described by Ryney and Peters (1970). Thirty-two mice were used for the evaluation. Eight groups of four mice each were infected with Plasmodium berghei and left for ten days for the infection to be established. The day the mice were passaged was taken as day-zero (D0). On day-ten (D0+10); different doses of the extract from the two plants were administered. The test groups (A - F) were administered in concentrations of 200 mg/kg, 150 mg/kg and 100 mg/kg. The positive control group (group G) was administered with 5 mg/kg chloroquine for 3 days, while the negative control group (group H) received 0.2ml of distilled water. Thin blood films were made from the tail of each mouse for five consecutive days, starting from the day of treatment. Average percentage parasitaemia was assessed. The number of deaths and the mean survival time (mst) for each group were obtained.

Packed cell volume (PCV): PCV (haematocrit) was determined in micro-haematocrit tubes, sealed at one end with plasticine, and centrifuged in the micro-haematocrit centrifuge at 1,500 (rpm) for 5 minutes. The height of the red cell column in each capillary tube was measured using the micro-haematocrit reader (Dacie and Lewis, 1994). The PCV was noted as the height of the red cell column and expressed in percentage. The PCV was determined by finding the mean PCV in each of the groups.

Body weight: Body weight was measured at the start of the experiment and at different stages using a top pan balance.

Statistical analysis: Data obtained were analysed using student's t-test and one way analysis of variance (ANOVA). Values of $p < 0.05$ were considered significant.

Results

Weight changes: The average weight of the four mice in the positive control (chloroquine) group was 26.2g at the beginning of the study. This increased to 32.9g at the end of the

experimental period, indicating a weight gain of 25.57%. The animals which were infected and treated with *C. odorata* at different doses had average weight changes varying from 27.3g to 27.5g at the peak of the parasitaemia to between 29.5 -31.2g at the end of the treatment period indicating a weight gain of 13.45%. The group treated with *C. citratus* had average weight of between 26.7-27.0g at the peak of the parasitaemia to between 28.7-29.2g at the end of the treatment period indicating a weight gain of 9.36%.The animals in the infected untreated group had weight changes from 26.4g at the beginning of the experiment to 19.2g at the end of the treatment period, indicating a 27.27% weight loss. (Table 1)

Packed cell volume (PCV): The mean PCV of the positive control group at the beginning of the study was 38.9%. This rose to 42.6 % at the end of the treatment period indicating an increase of 9.51%. The animals which were infected and treated with *C. odorata* at different doses had a mean PCV value of between 37.3-37.9% at the beginning of the study and between 38.6-38.9% at the end of the treatment period, indicating a 4.29% increase. The group treated with *C. citratus* had a mean PCV of between 38.4-38.5% at the beginning of the study and between 39.0-39.6% at the end of the treatment period, indicating 2.86% increase. The animals in the infected, untreated group had a mean PCV of 38.2% at the beginning of the experiment, and 28.4% at the end of the treatment period showing a loss of 25.65 %.(Table 2)

Acute toxicity: The extracts from both plants (300-800mg/kg) produced physical signs of toxicity such as writhing, gasping, palpitation, decreased respiratory movement, sluggishness and finally death. The intensities of these effects were proportional to the dose administered. The intra-peritoneal LD_{50} of the extract in mice was calculated to be 425mg/kg body weight.

Anti-malaria activity of C. odorata leaf extract in infected mice (four day test): The ethanolic leaf extract of *C. odorata* produced a dose-dependent, chemo-suppressive effect at various doses employed in the study. The chemosuppressions were 55.02, 72.14 and 78.20% for 100, 150 and 200mg/kg body weight doses per day respectively (Table 3).

Curative test of C. odorata and C. citratus in infected mice: In established infection, it was observed that there was a daily increase in the parasitaemia of the negative control group; a daily reduction in the parasitaemia levels of the extract treated groups (A-F) and that of the positive control (chloroquine treated group G). On day 7, the average parasitaemia for the groups were 31.7, 28.4, 22.0, 8.0 and 86% with *C. odorata* and 49.0, 47.0, 37.0, 8.0 and 86% with *C. citratus* for 100, 150, 200mg/kg/day body weight of the extracts, chloroquine(positive) and negative control respectively. The mean survival time (m.s.t) of the extract treated groups were significantly ($p>0.05$) longer than that of the negative control and was comparable to that of the standard drug, chloroquine(positive control)(Tables 4&6).

Anti-malaria activity of Cymbopogon citratus ethanolic leaf extract in infected mice (four-day test): Ethanolic leaf extract of *C. citratus* produced a dose dependent chemosuppression effect at various doses employed during the study. The chemosuppression was 28.57, 36.99, and 44.76% for 100, 150 and 200mg/kg body weight doses per day respectively. The chemosuppressions produced by the extracts were significant ($P<0.05$) compared to the negative control and incomparable to that of the standard drug (chloroquine, 5mg/kg/day) with a chemosuppression of 88.73 % (Table 5).

Table 1: Weight Changes (g) in the study groups

Group		Days			
		0	3	5	%
		Changes(g)			
100mg/kg	*C. odorata*	27.3	25.4	29.5	8.06
150mg/kg	*C. odorata*	27.5	25.3	29.8	8.42
200mg/kg	*C. odorata*	27.5	25.8	31.2	13.45
100mg/kg	*C. citratus*	26.8	25.8	28.7	7.09
150mg/kg	*C. citratus*	27.0	26.1	28.9	7.04
200mg/kg	*C. citratus*	26.7	25.9	29.2	9.36
Negative Control	(Distilled H_2O)	26.4	23.1	19.2	-27.27
Positive Control	(Chloroquine)	26.2	27.5	32.9	25.57

Table 2: Changes in Packed Cell Volume (PCV)

Group		Days of Experiment			
		0	3	5	(% Increase)
100mg/kg	*C. odorata*	37.9	34.2	38.6	1.86
150mg/kg	*C. odorata*	37.6	35.4	38.8	3.19
200mg/kg	*C. odorata*	37.3	36.5	38.9	4.29
100mg/kg	*C. citratus*	38.4	37.3	39.0	1.30
150mg/kg	*C. citratus*	38.5	36.8	39.5	2.60
200mg/kg	*C. citratus*	38.5	37.5	39.6	2.86
Negative Control	(Distilled H_2O)	38.2	36.3	28.4	-25.65
Positive Control	(Chloroquine)	38.9	37.4	42.6	9.51

Table 3: Antimalarial activity of *Chromolaena odorata* ethanolic leaf extract during four day test in infected mice

Drug/Extract	Dose (mg/kg/day)	Average (%) parasitaemia	Average (%) suppression
C odorata extract	100	17.0 ± 0.71	55.02
	150	11.25 ± 1.40	72.14
	200	9.52 ± 1.59	78.20
Chloroquine(standard)	5mg		88.73
Distilled water (control)	0.2ml	42.30 ± 1.16	

NOTE: Data are expressed as mean ± SEM for four animals per group. P<0.05 when compared to control

Table 4: Mean survival time of infected mice receiving doses of *Chromolaena odorata* ethanolic leaf extract

Drug/extract	Dose (mg/kg/day)	Mean survival time (day)
C.odorata extract	100	10.56 ± 1.39
	150	13.23 ± 0.64
	200	14.58 ± 0.71
Chloroquine(standard)	5	29.0 ± 0.0
Distilled water (Negative control)	0.2ml	8.46± 0.72

Table 5: Antimalarial activity of *Cymbopogon citratus* ethanolic leaf extract during 4-day test in infected mice

Drug/Extract	Dose(mg/kg/day)	Average % Parasitaemia	Average % suppression
C.citratus extract	100	24.75 ± 1.25	28.57
	150	21.84 ± 1.61	36.99
	200	19.14 ± 1.07	44.76
Chloroquine(standard)	5		88.73
Distilled water (Negative control)	0.2ml	42.30 ± 2.16	

Data are expressed as mean ± SEM, n = 4 per group $p < 0.05$ when compared to control.

Table 6: Mean survival time of infected mice receiving doses of *C. citratus* ethanolic leaf extract

Drug/Extract	Dose(mg/kg/day)	Mean survival time
C.citratus extract	100	16.0 ± 1.50
	150	21.0 ± 1.25
	200	26.2 ± 0.00
Chloroquine(standard)	5	29.0±0.00
Distilled water (Negative control)	0.2ml	8.46 ± 0.72

Data are expressed as mean ± SEM, for four animals per group. $P < 0.05$ when compared to control.

Discussions

The *in vivo* anti malaria activity and acute toxicity of the ethanolic leaf extracts of *C. odorata* and *C. citratus* were considered in this study. The intraperitoneal LD_{50} value of 425mg/kg indicated both plants were slightly toxic(Hamburger, 1989).The positive involvement of the treatment can be deduced from the weight status of the experimental animals (Table 1). All the infected groups (A-H) had a loss in weight prior to treatment due to the infection. However as treatment progressed it was observed that the weight of the experimental animals increased, unlike what was observed in the infected untreated group. The increase seen in the infected-treated groups showed that the animals responded to the treatment given. This is in consonance with the findings by Carvalho *et al;* (1991) and Nwabuisi (2002).

The PCV levels of the infected untreated group kept on decreasing as opposed to those of the infected treated groups (Table 2). This was an indication that the level of infection in the treated groups had gradually gone lower than that of the infected untreated groups (negative control). This agrees with the findings by Okpuzor *et al;* (2003).

The leaf extract of *C. odorata* exerted significant ($P< 0.05$) anti-malarial activity as was observed in the chemosupression obtained during a four-day early infection test (Table 3). The activity of the *C. odorota* extract was however observed to be lower than that of the standard drug (Chloroquine, 5mg/kg) at the three different doses studied. The result is similar to the findings by Taleb-contini *et al,* (2004), who reported a 68% chemosuppression, using similar extracts. *C.odorata* extract exerted a significant curative activity although not comparable to that of the standard drug (Chloroquine)(Table 4). This finding agrees with that reported by Okunji *et a*l; (2000).

The leaf extract of *C. citratus* also exerted some antimalarial activity as was observed in the chemosuppression obtained but this was not as effective as the positive control group (Choloroquine standard 5mg/kg) (Table 5). *C. citratus* ethanolic leaf-extract also exerted a significant curative activity on established infection which was not also comparable to that of the standard drug (Choloroquine). This finding agrees with that reported by Tchonmbongnang *et al;* (2005).

C. odorata has been found to contain flavonoids and terpenes while *C. citratus* conatins the chemical compound azaleatin (flavonol). Antimalarial screening of plants have implicated alkanoids, terpenes and flavonoids in this acivity (Phillipson, 1991; Christensen *et a*l, 2001), although the mechanism of action of these extracts has not been elucidated. Some plants are known to exert antimalarial activity either by causing red blood cell oxidation or by inhibiting protein synthesis, depending on their phytochemical constituents (Etkin, 1997).These extracts could have exerted their own action through either of the two mechanisms or by some other unknown mechanisms. These compounds may be acting singly or in synergy with one another to exert the effects observed in this study. Thus, the active principle needs to be identified in subsequent studies. It is evident based on the findings of this work that *Chromolaena odorata* and *Cymbopogon citratus* possess potent antimalarial effects justifying their usage in traditional management of malaria in Nigeria and are good candidates for further studies relating to malaria chemotherapy.

Recommendation

Malaria being a major public health threat all over developing countries makes it imperative for more extensive research to be directed towards the search for new anti-malarial drugs. The candidature of these plants for new anti-malarial drugs should be further investigated.

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