Full Length Research Paper

A study of in vivo activity of aqueous and methanolic extracts of plant used as antimalaria

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Extracts from the leaf of *Tithonia diversifolia* used in folk medicine for treatment of various ailments were tested for antimalaria and mosquito repellency properties in experimental animals and human volunteers, under the laboratory conditions. Comparison of the effectiveness of Chloroquine with the aqueous and methanolic extracts from the plant (*T. diversifolia)* showed that Chloroquine was 100% effective in clearing the parasite while the aqueous and methanolic extracts were 50 and 74% effective in clearing the parasites respectively. Both aqueous and methanolic extracts were more effective when administered before the onset of the infection, probably indicating the time-dependency of the antimalaria effects. Earlier application of the extracts at the onset of the malaria symptoms was more effective in reducing the parasitemia within a few days. The administration of the plant extracts during the malaria episode was also effective with longer period of administration. The LC_{50} of the aqueous extract in mice was 1.2ml/100g body weight while the Maximum Tolerated Dose (MTD) was found to be 1.0ml/g. The repellent activity of volatile oil at different concentrations was measured by protection period against the bites of *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*. The volatile oil extract showed higher repellent effect on *A. gambiae* at higher concentrations however its repellent and protective effects at various concentrations on all other species of mosquito tested can not be underestimated.

Key words: *Tithonia diversifolia,* aqueous, methanolic, volatile oil extracts*,* chloroquine, *Plasmodium berghei*, mosquitoes.

INTRODUCTION

Malaria is a public health problem most especially in the tropical countries where majority bear the burden of the disease. It is one of the six killer diseases in the world to - day and it has been estimated that 40% of the world's population is at risk and 500 million people suffer from the disease annually (MIM, 2004). About two million children, mostly less than five years and pregnant women die from malaria related illness each year and nine out of ten cases are found in Sub-saharan Africa (WHO, 2001).

Most vulnerable group in the endemic areas constitutes people in the rural environments who often had little or no access to modern medicine. This situation has been complica-

ted further by the emergence of multidrug-resistance strains of *Plasmodium falciparum* and rapid spread of vector-mosquito resistance to insecticides (Coker et al., 2000, Masaba et al., 2000). Hence, there is an urgent need to find alternative therapies that are not only effective against resistant malaria but are also available and a-fordable to this vulnerable group who are not economically buoyant to afford expensive orthodox medicine or have no access to modern health facilities (Coker et al., 2000).

The presence of active phytochemicals in some herbs used in curing many diseases affecting majority in African communities has been documented (Odetola et al., 1986). More particularly, the phytochemical properties of some of the plants used in folk medicine in Nigeria to treat malaria have been investigated (WHO, 1978)**.** Examples of such plants are *Morinda morindoides* (Onabajo, 1983), *Morinda lucida* (Makinde et al., 1985),

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Azadirachta indica (Ekanem, 1978, Obih et al., 1985) *Enantia chlorantiha, Ocimum gratissiumum* (Agomo et al., 1992), *Adansoni digitata, Alstonia congeensis, Alstonia boonei, Crossopterix febrifga, Cymbopogon citratus* and *Khaya senegalensis inter alia* (Coker et al., 2000). *Tithonia diversifolia* (Hemsl.) A. Gray is considered to be a medicinal plant that is widely used in folk medicine to treat various illnesses, it is commonly known as Mexican Sunflower, tree marigold, shrub sunflower or Japanese sunflower (English), "sepeleba" (Yoruba), pua renga (Cook Island). Ethno botanical surveys have shown that extracts from the plant exhibited antimalaria, antidiarrhoeic, anti-inflammatory, antibacterial, antiproliferation properties and its effectiveness in the treatment of haematomas and wounds had been demonstrated as well (Akobundu et al., 1998; Kuo et al., 1998; Rungeler et al., 1998; Tona et al., 1998; Goffin et al., 2002). The leaf is reported to contain sequiterpene lactones taginin C as an active component against *Plasmodium* (Goffin et al., 2002), diversifolin, diversifolin methyl ether and tirotundin as active components against inflammatory activity (Rungeler et al., 1998). Three new Sesquiterpenoids; 2 alpha-hydroxytirotundin, tithofolinolide and 3 alpha- acetoxydiversifolol along with eight known sequiterpene lactones, 3 beta-acetoxy-8 beta-isobutyryloxyreynosin, tagitinin C,1beta,2 alpha-epoxytagitinin C, 4 alpha, 10alpha-dihydroxy-3-oxo-8beta-isobutyryloxyguaia-11,-en-12,6alphaolide,   3, alpha-acetoxy-4alpha-hydroxy-11,-eudesmen-12-oic acid methyl ester, 17, 20- dihydroxygeranylnerol, taginin A, and tirotundin were evaluated for their potentials as cancer chemopreventive agents(Gu et al., 2002).

In this study, we examined the in vivo activity of the aqueous and methanolic extracts of the plant on *Plasmodium berghei* and repellency of the volatile oil on the mosquito.

MATERIALS AND METHODS

Host animals

Twenty pure strain laboratory adult Swiss albino mice aged 7 - 9 wks with mean weight of 22 g were obtained from Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. They were kept in clean cages in the laboratory and fed on chow diets and water ad-libitum for 2 weeks in order to be allowed to acclimatize to room temperature of $29 \pm 2^{o}C$ before being exposed to the reference drug and plant extracts. The animals were weighed and placed into six groups per assay.

Plant material

Fresh leaves of the plant were collected within the premises of Babcock University campus in March 2005 and July 2006. These were dried in an oven and milled into powder using electric blender/mill grater (model MS-223, Taiwan).

Preparation of extract: 50g powdered plant was soaked in 300 ml-distilled water for 24 h. The resultant mixture was filtered with cheesecloth and the filtrate concentrated under reduced pres-sure at $40^{o}C$ for 20 min using a rotary evaporator (Gallenkamp water

under sterile conditions, the required doses were prepared UK). Having weighed and dissolved the needed amount in distilled accordingly and stored at $4^{o}C$.

Methanolic extraction was carried out in a Soxhlet apparatus set at $60^{o}C$ using 50 g of powdered plant leaves in 300 ml of 96% methanol . This was refluxed at the boiling point of the ethanol. The extract was thoroughly suction-filtered, concentrated in vacuum distillation to thick syrup, and air-dried for 48 h (Sofowora, 1993).

Parasites and inocula

Plasmodium berghei (Anka strain) obtained from NIMR Yaba, Lagos was maintained by blood passaging in white mice and a dose of 10^{5} parasitized red blood cells (RBC) was inoculated intra-peritoneally (IP). The parasite density was assessed using giemsa-stained blood smear preparation from infected animals 6-10 days after inoculation. Infected red blood cells were counted 5 times using the haemocytometer and the mean calculated. The number of parasitized cells per ml was then determined for the corresponding dilutions for the required inocula.

Heamatocrit (HCT)

Heamatocrit (HCT) or Packed Cell Volume (PCV) was determined using the blood collected in heparinized vacutainer tubes, sealed with plasticine at each end and then placed in heamatocrit centri-fuge at 3,800 rev/min for 5 min. The values were read using Hawk-sley microheamatocrit reader.

Administration of chloroquine (CQ) and plant extracts

Chloroquine phosphate powder obtained from NIMR Yaba, Lagos, was thoroughly mixed with distilled water according to the weight range of the mice. The mean body weight of all the animals was used to calculate the amount of reference drug (Chloroquine) to be administered; 0.53 ml/g (full dose) was orally fed to the group A mice (control) for two consecutive days. The next dose (0.27 ml/g) was administered on the third day. The treatment of groups B and C with 1 ml of the aqueous and methanolic extracts respectively started on day 3 (when parasitemia was around 1 to 2%) and conti-nued for 3 consecutive days. Mice in groups D and E were given 1ml of the aqueous and methanolic extracts respectively for 3 con-secutive days before the infection. Group F mice were administered with 5 ml distilled water but neither treated with CQ nor plant extracts before or after the infection.

The extract was administered to four groups of mice (n = 5 each) in doses of 0.5, 0.8, 1.0, and 1.2 m l/g body weight. This was done in order to determine the LC_{50} and Maximum Tolerated Dose (MTD)

Calculation of percentage parasitemia

Percentage parasitemia was calculated on a daily basis by giemsa-stained thin blood films using the blood collected from the tail of each mouse in all the groups with the formula

$$\% \text{ parasitemia} = \frac{\text{No of infected red blood cells}}{\text{Total no of red blood cells}} \times 100$$

The mean % parasitemia recorded for each animal and for each group was used to determine variations in parasitemia level with time of infection.

Repellency assay

The essential oil from *T. diversifolia* leaves was extracted by steam

distillation using the Clevenger apparatus (Dimitrios et al., 2004). The repellency of the essential oils was evaluated by using an arm-in-cage test as described by Schreck et al. (1989) and WHO (1996). The technique involves counting of the number of mosquitoes biting a volunteer's hands introduced into a mosquito cage (35 x 35 x 35 cm) containing 100 hungry female mosquitoes (3 - 5 days old), for the first three minutes of every half-hour exposure. Approximately 0.1 ml of 10, 50 or 100% concentrations of volatile oil was applied to the forearm of each volunteer; each test was repeated three times for each concentration. Four different human volunteers were used per test after obtaining their consent to participate in the experiment. The mosquito species used for the bioassay tests were *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus.* These mosquito species were uninfected laboratory strains which were reared in the insectary of the Nigerian Institute of Medical Research (NIMR), Lagos. At the onset of the experiment, the pentane-treated forearm of volunteer used as control was exposed for 30 seconds and if at least two mosquitoes landed or bit the hand, the repellency test was continued because this means that pentane has no repellent effect on the mosquitoes so it would not affect the repellent activities of the essential oils to be tested. The test continued until at least two bites occurred followed by a confirmatory bite (second bite) in the following expo- sure period. The time between application of the repellents and the second suc-cessive bite was recorded as the protection time.

Statistical analysis

Data were analyzed using Chi-square and ANOVA statistical tests. All the tests were performed at the 95% confidence interval using SPSS version 14.0 Software package. The results of the replica experiments were expressed as mean ± S.E.M.

RESULTS

In group A, the mice treated with chloroquine phosphate showed total clearance by day 7 (Table I) and 100% protection from the malaria parasite (Table 2). The group B mice treated with aqueous extracts on day 3 after infec-tion showed a daily increase in the parasite population from 11% (day 2) to 53% (day 9) (Table 1) and 100% mortality on day 10 (Table 2). The group C mice treated with the methanolic extract on day 3 after infection also showed a daily increase in parasitemia infection from 12% (day 2) to 38% (day 9) (Table 1) and 100% mortality on the day 10 (Table 2). The group D mice treated with aqueous extract 3 days prior infection showed a de-crease in the parasitemia infection from 10% (day 2) to 6% (day 11) (Table 1) and 50% mortality until day 12 (Table 2). The group E mice treated with methanolic extract 3 days prior infection also had 50% mortality till day 13 (Table 2). The parasitemia infection in this group initially increased from 1 to 19% (day 3) but decreased drastically to 3% on day 11. The group F mice (untreated, control) showed an astronomical increase in parasitemia (Table 1) and 100% mortality on the day 7 (Table 2).

There was no significant difference in the parasitemia between groups B and C (p>0.05) but that of groups D and E was statistically different (p<0.05). In addition, a comparative analysis of percentage parasitemia in groups B and C was significantly different from that of groups D and E (p<0.05). The LC_{50} of the aqueous ex-

tract in mice was 1.2 ml/100g body weight while the Maximum Tolerated Dose (MTD) was found to be 1.0ml/g The repellent activity of volatile oil at different concen-trations was measured by protection period against mos-quito bite of *A. gambiae*, *A. aegypti* and *C. quinquefas-ciatus.* The results presented in Table 3 showed that *Anopheles gambiae* was more susceptible to the test solution at 10% concentration with mean protection time of 120 min. However, at 50% concentration, there was no observable difference in the susceptibility of all the mos-quito species tested to the volatile oil but the protection period against the mosquito bite of *Aedes aegypti* was much longer (= 180). Meanwhile, at 100% concen-tration, repellent activity (protection time) was also consi-derably high in *A. gambiae* (= 200).

However, statistical analysis of the results showed that there was no significant difference in protection time against the three mosquito species at 10% concentration (F = 2.4, df = 2, P> 0.05) and no significant difference in number of landing mosquitoes (F = 1.4, df = 2, P> 0.05). Also at 50% concentration, no significant difference was observed in the protection time against the three mos-quito species (F = 4.3, df = 2, P> 0.05). Meanwhile, at 100% concentration, a level of significant difference between the protection time of the three mosquito spe-cies was noticed (F= 9.8, df = 2, P< 0.05) but there was no significant difference in the number of landing mos-quitoes (F = 0.2, df = 3, P> 0.05).

DISCUSSION

The results from this investigation show that both the aqueous and methanolic extracts of *T. diversifolia* leaf contain antimalarial substances with properties that showed both preventive and curative effects on malaria parasites. This study also indicates that the parasite-clearance ability of the extracts is time-dependent. This was evident in our observation whereby the mice treated before the infection had lower parasite count and survived better than those treated after the infection. This could largely be due to the decrease in haematocrit values with infection time especially in the latter groups. In other words, these groups suffered anemia because of red blood cell destruction by parasite multiplication before being treated. This was evident in our observation where-by the haematocrit level in all the groups started normally but reduced drastically as the infection progressed and there was a clear difference between groups A (control) and other groups. The present study supports the earlier reports on the antimalarial activity of *T. diversifolia* (Goffin et al., 2002; Bidla et al., 2004; Elufioye and Agbedahunsi, 2004), however, in addition to these earlier claims, we also showed that the effectiveness of the extract could be time dependent, in other words earlier administration of decoctions is likely to produce better results. This may also help in preventing prolong administration of decoc-tions in order to reduce its toxic-effects on the systems of the body (Oyewole et al., 2007). Meanwhile, previous

Table 1. Summary of parasitemia percentage on a daily examination of the mice in each group.

Group	Mean % parasitemia per day											
	0	1	2	3	4	5	6	7	8	9	10	11
A	0±0.0	12± 0.4	23±2.0	17±2.3	6±0.1	2±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
B	0±0.0	11±0.2	20±0.8	24±5.2	28±0.9	33±3.2	40±9.4	48±5.5	53±10.0	Death	-	
C	0±0.0	12±0.4	19±0.5	26±3.2	28±0.9	30±5,6	33±3.2	34±4.0	36±5.0	Death	-	-
D	0±0.0	10±0.1	15±0.4	20±0.8	18.6±5.4	17.3±3.4	10.1±0.0	9.6±0.1	7.4±3.0	7±0.1	7±0.1	6±0.1
E	0±0.0	13±0.4	13±0.4	19±0.5	18.4±4.3	10.6±3.0	9.2±2.5	9±0.1	7±0.1	5.6±0.0	4±0.1	3±0.0
F	0±0.0	10±0.1	16±1.0	20±0.8	43±10.0	59±12.2	62±12.0	Death	-	-	-	-

Results were mean of three replicates ± SEM

Table 2. Comparative percentage of survivors treated with CQ and plant extracts before and after infection

Group	CQ/ plant extracts	Mode of treatment	Initial number of mice	Survival rate	% Mortality	Survival day after the experiment
A	Chloroquine Phosphate	3 doses after Infection	4	4/4	0	till the end of the exp.
B	Aqueous	3 doses after Infection	4	0/4	100	day 10
C	Methanolic extract	3 doses after Infection	4	0/4	100	day10
D	Aqueous extract	3 doses (before Infection)	4	2/4	50	day 12
E	Methanolic extract	3 doses(before Infection)	4	2/4	50	day 13
F	No treatment before and after infection		4	0/4	100	day 7

Table 3. Repellent activity of the oil in three concentrations (10, 50 and 100%) against three species of mosquitoes under laboratory conditions.

species of mosquitoes	10%								50%								100%							
	Number of landing mosquitoes				Protection time				Number of landing mosquitoes				Protection time				Number of landing mosquitoes				Protection time			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
Culex Quinquefasciatus	6	5	6	6 ± 0.3	90	60	90	80±10.0	4	4	4	4±0.0	120	120	150	130±10.0	6	3	6	5±1.0	150	150	150	150±0.0
Aedes aegypti	6	12	12	10± 2.0	120	90	90	100± 10.0	4	4	4	4±0.0	180	150	180	170±10.0	3	4	4	5± 0.3	180	180	180	180±0.0
Anopheles gambiae	6	6	12	8± 2.0	120	150	90	120±17. 3	4	4	4	4±0.0	150	150	180	160±10.0	4	4	4	4± 0.0	180	180	210	200±10.0

findings indicate that toxic substances in natural products may be relatively independent of those identified for anti- malarial activity. In other words, modification of the appropriate functional groups could be required to permit mitigation of the cyto-toxic potential with concurrent optimization of the therapeutic index (Tane et al., 2005). Hence, further studies can be carried out to prove that the anti-malaria activity and cytotoxicity properties re-

resident in *T. diversifolia* are not directly related and can also be independently optimized.

Repellency activity of oil extracts from plants using human volunteers in preference to laboratory animals is a common practice, this is because using laboratory animals may inadequately simulate the condition of human skin to which repellents will eventually be applied (WHO, 1996; Barnard, 2000; Apiwat et al., 2001). In the present study, the repellent activity was found to be dose dependent. However, similar trend were also reported in previous work by Rajkumar and Jebanesan (2005) in which both oviposition deterrent and skin repellent activity of *Solanum trilobatum* against the malaria vector *Anopheles stephensi* were dose dependent. The repellency activity of different concentrations of volatile oil can be described to be higher in *A. gambiae* than *A. aegypti*, which was also higher than *C. Quinquefas-ciatus.*

The number of landing mosquitoes was expected to reduce with increase in the concentrations of volatile oil but this was not so. Finally the difference in the number of mosquitoes landing on arms treated with different concentrations of the oil, was generally insignificant when compared to the population of mosquitoes used in the experiment. This could be an indication that volatile oil from the leaf of *T. diversifolia* has some repellent properties which is not dose dependent and should be further investigated.

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