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Acute Toxicity and Antiplasmodial Activity of Methanol Extracts of *Tetrorchidium didymostemon* in *Plasmodium berghei*-infected mice

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ABSTRACT

The emergence of drug resistance parasite to currently available drugs and the spread of insecticide resistance mosquito vector is worrisome and may result in serious health crisis if it is not addressed. Hence, there is need to develop novel antiplasmodial agent that will be cheap and effective against the *Plasmodium* parasite. This study was aimed at investigating the acute toxicity and antiplasmodial activity of methanol extracts of *Tetrorchidium didymostemon* leaves and stem bark. Acute toxicity of the extracts was investigated using the Lorke's method. Antiplasmodial activity in early infection was tested using the Peter's 4-day suppressive test while Ryley and Peters' curative test was used to investigate the effect of the extracts on established infection. No mortality was recorded in the acute toxicity study. The leaves extract at 250 and 500 mg/kg body weight had significantly higher ($p < 0.05$) chemosuppression (69.84% and 73.11%, respectively) of the *Plasmodium berghei* parasite when compared with the stem bark extract at similar concentrations (55.55% and 49.67 %, respectively). The leaves extract, at 500 mg/kg body weight, showed better curative potential (67.92%) with mean survival time (MST) of 20.4 days than the stem bark counterpart at 500 mg/kg body weight (48.37% and 18.6 days, respectively). This study has shown that methanol extract of *T. didymostemon* leaves contains bioactive compounds with antiplasmodial activity and it substantiates its therapeutic use locally in traditional medicine.

Keywords: Antiplasmodial, *Tetrorchidium didymostemon*, Extracts, Curative, Suppressive, Acute Toxicity

INTRODUCTION

Malaria is a pathogenic disease common in the tropical and sub-tropical regions of the world and it is caused by the parasite, *Plasmodium* (WHO, 2021). It is a global scourge that account for most of the deaths in pregnant women and children (age 0 – 5 years) in sub-Sahara Africa (Rogo *et al.*, 2006). Six countries account for approximately half of all malaria cases worldwide with Nigeria having the highest malaria cases in the order: Nigeria (27%), Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%) and Niger (3%) (World Malaria Report, 2020). Of the 409, 000 deaths from malaria globally, Nigeria alone accounted for approximately 94, 070 (23%) deaths in 2019 (World Malaria Report, 2020). Aside sub-Saharan Africa, malaria is also clustered in Afghanistan, India, Brazil, Thailand, Indonesia, Cambodia, Sri Lanka, China and Vietnam (World Malaria Report, 2020).

Mortality resulting from malaria infection has reduced and success has been recorded in the fight against this disease (World Malaria Report, 2020); however, increasing reports on drug resistant parasites and adulteration of available antimalarial drugs are major setback to this progress. Malaria parasite is transmitted via the bite of a female Anopheles mosquito that is carrying the parasite during blood meal (Tuteja, 2007) and it spends part of its life cycle between two host; man and mosquito. Human malaria is caused by five species of *Plasmodium*: *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malariae* and *Plasmodium knowlesi.* Most of the malaria related deaths are caused by *Plasmodium falciparum* which is the most contagious (Badshah *et al.*, 2018). Despite the progress and successes recorded in the fight against malaria, the disease is still spreading as the parasite particularly *Plasmodium falciparum* is skilled at thwarting traps set by man (Amato *et al.*, 2018). The emergence of drug resistant parasite to currently available drugs is worrisome and may result in serious health crisis. More disturbing, is the spread of insecticide resistance mosquito vector which may eventually lead to drawback in malaria control programs. Artemisinin resistance has been declared in mainland Southeast Asia where it emerged independently and started to spread rapidly (Hien *et al.*, 2012; Phyo *et al.*, 2012). There are also some reported cases of therapeutic treatment failure of artemisinin combined therapy in Nigeria (Ebohon *et al.*, 2019).

The parasite is developing resistance to available drugs and there is still no effective vaccine, the search for novel and effective antiplasmodial agent is pertinent in other to avoid the impeding health crisis that may arise should artemisinin resistance spread to Africa. Medicinal plants have been useful in the treatment and management of malaria infection (Odugbemi *et al.*, 2006). Successful examples are *Cinchona officinalis* and *Artemisia annua* which gave rise to the antimalarial drug quinine and artemisinin respectively and are responsible for the gains and progress enjoyed in malaria treatment and control. Majority of the

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populations in many tropical countries depend on traditional herbal remedies due to limited affordability of orthodox drugs (Zihiri *et al*., 2005). Some individuals in developing countries particularly in Africa have more faith in traditional herbs compared to orthodox drugs as they believe the herbs are more effective and make them closer to their culture. Hence, even when orthodox drugs are available, they rely heavily on traditional herbal medicine or take it concomitantly with orthodox drugs. *Tetrorchidium didymostemon* is one of such herbs utilized in the rural areas for the treatment and management of malaria.

Tetrorchidium didymostemon (Baill.) Pax and K. Hoffm is an evergreen shrub that belongs to the family *Euphorbiaceae* (Burkill, 1994). It has drooping branches and can grow up to 25 meters tall. It is called *Iheni* (Edo-language, Nigeria) and *ofun oke* (Yoruba-language, Nigeria). The tree resides in the wild where it is harvested and used as medicine and source of materials for firewood and charcoal making (Burkill, 1994). The bark serves as antidote, diuretic, emetic, febrifuge, parasiticide and purgative. A stem bark infusion is rubbed on to rheumatic and painful limbs, painful kidneys and to treat oedema (Toirambe, 2008). It is soaked in water or rum when taken as a purgative (Burkill, 1994). Bark scrapings are applied as enema to treat malaria and backache (Toirambe, 2008). Leaf sap is applied to wounds as a haemostatic, to treat fever; and as a purgative, the leaf sap in water or rum, or a stem bark decoction, is commonly used (Toirambe, 2008). It is also used to treat constipation or enlarged spleen in babies, the leaf sap is applied to nipples of nursing mothers or to scarifications (Toirambe, 2008). The sub-acute toxic effects of *T. didymostemon* and its effect on *P.* berghei-induced oxidative stress in mice have been investigated (Ebohon *et al.*, 2020a; Ebohon *et al.*, 2021a). Similarly, we have evaluated the *in vitro* antiplasmodial activity of extracts and fractions from *Tetrorchidium didymostemon* (Ebohon *et al.*, 2021b). Despite the diverse usage of *T. didymostemon*, there is still a shortfall of scientific validation of many of its ethnomedicinal uses. Hence, this study was aimed at evaluating the acute toxicity and *in vivo* antiplasmodial activity of methanol leaves and stem bark extracts of *T. didymostemon*.

MATERIALS AND METHODS

Plant Collection, Authentication and Extraction

Leaves and stem bark of *T. didymostemon* were collected from the wild in Urhokuosa village, Uhumwonde Local Government Area, Benin City, Edo State. Thereafter, the plant was authenticated by Dr. H.A. Akinnibosun at the Department of Plant Biology and Biotechnology, University of Benin, Nigeria. Voucher specimen (UBH_T439) of the plant was deposited at the herbarium of the same department. The leaves and stem bark of *T. didymostemon* were extracted with methanol as described by Ebohon *et al.* (2020a).

Animals for the Study

Healthy male albino mice of the Swiss strain weighing between 19 - 23g were obtained from the Nigerian Institute of Medical Research (NIMR), Lagos State, Nigeria. The mice were housed under standard laboratory conditions, maintained at 27 ± 2ºC with free access to commercial mash and water *ad-libitum* in the vivarium of the Department of Biochemistry, University of Benin, Edo State, Nigeria. The mice were acclimatized for two weeks before the commencement of the experiment. Ethical approval for this study was granted by the Institutional Ethics Review Committee, University of Benin (No: LS19114).

Acute Oral Toxicity Study

Thirty six (36) male albino mice were used for the acute toxicity study. The acute toxicity of the *T. didymostemon* extracts was determined by establishing its median lethal dose (LD_{50}) using the Lorke's method (Lorke, 1983).

Malaria Parasites and Inoculation

Chloroquine sensitive strain of rodent parasite *Plasmodium berghei* NK65 maintained in mice was obtained from the Nigerian Institute of Medical Research (NIMR) Lagos, Nigeria. Inoculation of experimental mice with *P. berghei* was done as described by Ebohon *et al.* (2021a).

Antiplasmodial Study

Suppressive test

The Peter's 4- day suppressive test against chloroquine-sensitive *Plasmodium berghei* NK 65 infection in mice was employed (Peters *et al.*, 1993). Forty two (42) male albino mice were randomly divided into 6 groups of 7 mice each using the Rand function in Microsoft Excel. On the first day of the experiment (termed 'day 0'), all the experimental mice were injected intraperitoneally with standard inoculums of *P. berghei*. Two hours after infection of mice, Group 1 (negative control) was administered 0.2 mL of the vehicle (0.7% carboxy methyl cellulose), group 2 (positive control, CqT) was treated with 10 mg/kg body weight of chloroquine phosphate, group 3 and 4 were treated with 250 and 500 mg/kg body weight leaves extract, respectively while group 5 and 6 were administered 250 and 500 mg/kg body weight stem bark extract, respectively. All administrations were repeated once daily for four consecutive days (day 1 to day 4). On 'day 5' of the experiment, thin film was made on microscopic slide using blood from the tail of the mice. The slides were then examined microscopically using ×100 magnification in oil immersion after staining with Geimsa. The percentage parasitaemia was calculated using the formula as described by Peter and Robinson (1992).

$$\%\ \text{Parasitaemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

The percentage parasitaemia was expressed as mean ± SEM. The average suppression of parasitaemia was calculated using the formula as described by Peters *et al.* (1993).

$$A = \frac{B - C}{B} \times 100$$

Where: A = Average percentage suppression of parasitaemia

B = Average percentage parasitaemia in the negative control group

C = Average percentage parasitaemia in the test group

Curative test

The curative test was used to evaluate the parasiticidal activity of the plant extracts on established infections. The method described by Ryley and Peters (1970) was adopted. On the first day of the experiment (termed 'day 0'), all mice were injected intraperitoneally with standard inoculums of *P. berghei* containing 1×10^7 infected erythrocytes. Seventy-two (72) hours later, Group 1 which was the negative control was not treated while group 2 the positive control was treated with 10 mg/kg body weight chloroquine phosphate (the reference antimalarial drug). Groups 3 and 4 were treated with 250 and 500 mg/kg body weight leaves extract, respectively while groups 5 and 6 were administered 250 and 500 mg/kg body weight stem bark extract, respectively. The extracts and drugs were administered once daily for four days consecutively using an oral cannula. The percentage parasitaemia level was determined as described above. The mean survival time (MST) for each group was determined arithmetrically by finding the average survival time (days) of the mice (post-inoculum) in each group over a period of 28 days (D_0 to D_{27}) as described by White, (2004).

$$\text{Mean survival time} = \frac{\text{sum of days of survival of animals/group}}{\text{total number of animals in the group}}$$

After sample collection, the mice were euthanized via cervical dislocation and death was confirmed. The carcasses of the mice were disposed of appropriately using a burial pit.

Statistical Analysis

The statistical analysis was performed using the statistical package for social science (SPSS) for windows, version 16.0 (SPSS Inc., Chicago, IL, USA). The results obtained were expressed as mean ± SEM. One way analysis of variance (ANOVA) test was used to determine significance differences between the groups and post hoc multiple comparison test was done using Tukey's HSD (honest significant difference). Statistical significance was declared when P value was less than 0.05.

RESULTS AND DISCUSSION

Acute Toxicity of Methanol Extracts of *T. didymostemon* Leaves and Stem Bark

Oral administration of the plant extracts showed no sign of adverse effects (such as hyperactivity, shivering, spasms of both rear legs, salivating, circling, excessive grooming, ruffled fur, sitting in corner of cages etc.) and lethality to mice at all the doses evaluated (Table 1). The absence of death suggests that the plant extracts are relatively safe, non-toxic and the LD_{50} are above 5000 mg/kg body weight. Hodge and Sterner (2005) toxicity scale, suggests that compounds with LD_{50} (rat, oral) of 5000 mg/kg body weight or more are practically non-toxic. *T. didymostemon* may be said to contain bioactive secondary metabolites that are relatively safe or it contains low amount of toxic metabolite that are not enough to result in lethality at the highest dose administered. Aside the beneficial effects of some bioactive compounds present in medicinal plants, these metabolites may sometimes be toxic and result in an unwanted effect in the body. Plant derived substances have been gaining interest due to their diverse applications. These plants are the richest bio-resource of modern medicine, folk medicine, food supplement, pharmaceutical intermediate, nutraceuticals and chemical entities for synthetic drugs (Ncube *et al.,* 2008). The chemical substances produced by these plants are responsible for the definite physiological action they impact on the human body (Edeoga *et al.,* 2005). Acute toxicity study is one of the ways to measure the level of toxicity of a substance. It is used to describe the adverse effect that may result from the administration of test substance over a short period of time (usually 24 hours). During this study, behavioral changes as well as death are recorded. Generally, a substance is considered a good candidate for further study if the lethal dose (LD_{50}) of the test substance is three times higher than the minimum effective dose (MED) (Abdillah *et al.,* 2013).

Table 1: Acute toxicity of methanol extracts of *T. didymostemon* leaves and stem bark administered orally to mice.

Plant	Parts used	Dose (mg/kg. b. wt.)	No of Animals used	Mortality
Phase I	Leaves	10	3	0
		100	3	0
		1000	3	0
	Stem bark	10	3	0
		100	3	0
		1000	3	0
Phase II	Leaves	1600	3	0
		2900	3	0
		5000	3	0
	Stem bark	1600	3	0
		2900	3	0
		5000	3	0

Where, mg/kg. b. wt. = mg/kg body weight

Chemo-suppressive Potential of Methanol Extracts of *T. didymostemon* Leaves and Stem Bark (Suppressive Test)

The first choice for medical care for rural dwellers in Africa, Latin America, and Asian continents are the traditional practitioners and these individuals only visit qualified medical personnel or hospitals in the absence of relief (Adebajo *et al.,* 2014). *In vitro* antiplasmodial studies have been very useful in screening for antiplasmodial compounds. However, it has some limitations due to the absence of metabolism of compounds and its specificity for the erythrocytic stage of malaria parasite. Hence, in order to validate the ethnomedicinal claims of *T. didymostemon* extracts, the *in vivo* antiplasmodial activity against *P. berghei* using the suppressive and curative test models were employed. The evaluation of percentage parasitaemia is the most reliable parameter in the suppressive and curative test (Madara *et al.,* 2010). The results of this study showed that the percentage parasitaemia levels of the extracts treated *P. berghei*-infected mice were significantly ($p < 0.05$) lower than the negative control group (untreated group), but higher than the positive control, chloroquine (Table 2). This indicates that the extract possesses antiplasmodial activity. The antiplasmodial activity of the leaves extracts was in a dose-dependent manner while that of the stem bark extract was not dose-dependent (Table 2). This implies that higher doses of the stem bark extract may not possess more beneficial antiplasmodial effect or it exacerbated the infection. The least antiplasmodial activity was seen in the mice treated with the stem bark extracts (Table 2). Bioactive compounds such as alkaloids, flavonoids, phenolics and terpenoids (known to possess antiplasmodial activity) which were abundant in the leaves extract in comparison to the stem bark extract (Ebohon *et al.* 2020b), may be responsible for its ability to suppress parasite growth. *T. didymostemon* methanol leaves extract had a higher suppressive effect on parasite growth when compared with methanol extracts of *Tapinanthus sessilifolius* leaves and *Striga hermonthica* (Okpako and Ajaiyeoba, 2004). However, the suppressive potential of *T. didymostemon* methanol extracts were lower than aqueous leaves extract of *Ageratum conyzoides* (Victoria *et al.,* 2010), methanol leave extract of *Bombax buonopozense* (Akuodor *et al.,* 2011) and hexane / methanol extract of *Trichilia emetic* (Sulaiman *et al.,* 2015).

Table 2: Chemo-suppressive potential of methanol extracts of *T. didymostemon* leaves and stem bark on *P. berghei*-infected mice (suppressive test)

Extrats/Drugs	**Extracts/Drug concentration** (mg/kg body weight)	**Percentage parasitaemia**	**Percentage suppression**
Negative Control	-	10.71 ± 1.16^{c}	0.00
CqT	10	0.40 ± 0.05^{a}	96.27
Leaves	250	3.23 ± 0.14^{b}	69.84
Leaves	500	2.88 ± 0.23^{ab}	73.11
Stem bark	250	4.76 ± 0.90^{b}	55.55
Stem bark	500	5.39 ± 0.17^{b}	49.67

Values are expressed as mean ± SEM, $n = 7$/group. Where: Negative control = infected without treatment (untreated) and CqT = chloroquine treated. Values bearing different letter are significantly ($p < 0.05$) different, while those with the same letters are not significantly different

Inhibitory Potential of Methanol Extracts of *T. didymostemon* Leaves and Stem Bark on *P. berghei* Established Infection (Curative or Rane Test)

In the curative test, the methanol leaves extract of *T. didymostemon* showed a dose dependent reduction in parasitaemia level while the reduction in the stem bark extract was not dose dependent (Table 3). The parasitaemia levels of the extracts treated groups were significantly lower ($p < 0.05$) than that of the negative control (untreated group). This implies that the extracts had antiplasmodial activity against the established malaria parasite infection. Study conducted by Abdillah *et al.* (2013) showed similar observation. Furthermore, the mice treated with the leaves extracts showed significantly higher ability to reduce the level of parasitaemia when compared with the stem bark extract treated mice (Table 3). The highest percentage clearance of established malaria parasite infection was seen in the mice treated with chloroquine and this was followed closely by the 500 mg/kg of the leaves extract treated mice. The group treated with 250 mg/kg of the stem bark extract showed the least percentage parasite clearance (Table 3).

The mean survival time (MST) of the experimental mice in the extracts treated groups were prolonged when compared with the negative control group (Table 3). This indicates that the extracts caused an overall decrease in the pathological effect of the parasites within the mice. MST of chloroquine treated group was beyond 28 days and it was significantly higher than the extracts treated groups. This is expected as chloroquine being a pure compound with well-known antiplasmodial potential thereby resulting in an increased MST. The increased MST in the extract treated groups may also be attributed to the presence of some phyto-constituents which may not only have been parasiticidal but may have antipyretic, analgesic, immunomodulatory and antioxidant properties. The increased survival time of the mice treated with the extracts suggest that the extracts reduced the overall pathologic effect induced by the *P. berghei* parasite. The methanol leaves extract of *T. didymostemon* had a better curative potential than the methanol leaves extract of *Vernonia amygdalina* (Madaki, 2015). However, the curative potential of *T. didymostemon* leaves was lower than ethanol root extract of *Olea europaea* (Osheke *et al.,* 2014).

A review conducted by Lawal *et al.* (2015), identified alkaloids, terpenoids, flavonoids, quinones, phenolics, xanthones, lignans etc as antiplasmodial agents and these phytochemicals have been identified in *T. didymostemon* extracts (Ebohon *et al.* 2020b). Active phytochemical components of natural products have been suggested to be responsible for their antiplasmodial effect (Ayoola *et al*., 2008; Omoregie and Okugbo, 2014). Some plants exert their antiplasmodial effect by inhibiting protein synthesis (Kirby *et al.,* 1989) or causing the oxidation of erythrocytes (Etkin, 1997) depending on the phyto-constituents present. Also, the immune strengthening property of these phytochemicals might play a role in their antimalarial efficacy (Adetutu *et al.,* 2016). A good example is flavonoid, which has been reported to have potential immune-modulatory effects (Siqueira *et al.,* 2011). Phenolics which are good antioxidant may help in the management of oxidative stress which is implicated in malaria infection. The activity of these compounds may be as a result of a single or synergistic action.

In vivo antiplasmodial activity can be classified as moderate, good and very good if a plant extract displayed percentage parasite suppression ≥ 50% at concentrations of 500, 250 and 100 mg/kg body weight per day (Deharo *et al*., 2001). In standard screening test, 30% suppressive effect of parasitaemia by anti-malarial test compounds (Silva *et al.,* 2011) or its ability to prolong the mean survival time of treated groups compared to control group are often considered effective (Trigg and Kondrachine, 1998). Based on these postulations, *T. didymostemon* extracts may be considered moderately effective since its highest percentage parasite suppression was observed at 500 mg/kg body weight.

Table 3: Inhibitory potential of methanol leaves and stem bark extracts of *T. didymostemon* on *P. berghei* established infection in mice (Curative or Rane test)

Extracts/Drug	**Extracts/Drug concentration** (mg/kg body weight)	**Percentage parasitaemia**	**Percentage inhibition**	**Mean survival time (day)**
Negative Control	-	9.83 ± 0.35^{d}	0.00	17
CqT	10	0.44 ± 0.02^{a}	95.52	28
Leaves	250	4.25 ± 0.09^{c}	56.72	19.4
Leaves	500	3.15 ± 0.12^{b}	67.92	20.4
Stem bark	250	5.18 ± 0.20^{c}	47.25	18.3
Stem bark	500	5.07 ± 0.33^{c}	48.37	18

Values are expressed as mean ± SEM, n = 7/group. Where: Negative control = infected without treatment (untreated) and CqT = chloroquine treated. Values bearing different letter are significantly ($p < 0.05$) different, while those with the same letters are not significantly different.

CONCLUSION

The findings from the study have shown that methanol extracts of *T. didymostemon* leaves contains bioactive compounds with antiplasmodial activity as evidenced by its ability to reduce parasitaemia in *P. berghei*-infected mice. The study has provided scientific validation on the ethnomedicinal uses of *T. didymostemon* in the management of malaria infection. Further studies are suggested in other to identify, isolate and characterize the exact bioactive compound(s) responsible for this observed activity.

AUTHOR'S CONTRIBUTION

EO: Contributed in conceptualization of the study, data curation, formal analysis, investigation, resources, validation and writing the original draft of the manuscript. IF: Contributed in conceptualization of the study, validation, investigation and resources. OES: Contributed in conceptualization of the study, review and editing of the manuscript, supervision and project administration. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

None declared.

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