In-vivo antimalarial activity of aqueous leaf and bark extracts of *Trema orientalis* against *Plasmodium berghei* in mice

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Abstract The control of malaria relies on the use of chemical antimalarial, but the development of resistance necessitates research into alternatives. *Trema orientalis* (L.) Blume is used in Nigerian folklore medicine for the treatment of malaria. This study investigates the in vivo antiplasmodial activity of aqueous leaf and bark extracts of *T. orientalis* in rodent model. Swiss Albino mice weighing 23–27 g were intraperitoneally infected with *Plasmodium berghei*. The lethal dose in mice was estimated to be greater than 5000 mg/kg based on toxicity signs and death. Aqueous crude leaf and bark extracts were administered at 400, 800 and 1600 mg/kg. Chloroquine was used as positive control while the negative control was treated with distilled water. Parasitemia, parasite inhibition, body weight and packed cell volume (PCV) were determined. The parasite inhibition of the leaf extracts at 400, 800 and 1600 mg/kg was 51.55, 62.78 and 76.08 % while that of the bark extract was 44.3, 65.82 and 74.23 % respectively. The percentage parasitemia of the leaf extract at 400, 800 and 1600 mg/kg decreased by 45.0, 70.3 and 74.7 % while that of the bark decreased by 37.4, 53.0 and 52.0 % respectively. The PCV of mice treated with 400, 800 and 1600 mg/kg leaf extract were 48.85, 49.88 and 50.99 % while that of the bark extract was 49.38, 48.88 and 51.94 % respectively. The results indicate that the plant has a promising antiplasmodial activity against *P. berghei*, which validates its folkloric use.

Keywords *Plasmodium berghei* · *Trema orientalis* ·
Phytomedicine · Alternative antimalarial

Introduction

Malaria is the most important tropical disease known to man. Malaria is spreading as a result of environmental changes, including global warming, civil disturbances, increasing travel and drug resistance (Greenwood 1997). According to Ibrahim et al. (2012) malaria is a disease of poverty in underdeveloped countries and it remains an important health problem globally. Each year 300–500 million new cases are diagnosed and approximately 1.5 million people die of the disease; the majority of them are children (Greenwood et al. 2005). The reemerging of malaria in many parts of the world is due to the rapid increase of resistance to most of the available antimalarial drugs, as well as resistance of vectors to insecticides (Ridley 2002; Zirihi et al. 2005). Drug resistant strains of *P. falciparum* have been found in many endemic areas of the world and many conventional antimalarial drugs have been associated with treatment failure. However, WHO's Artemisinin—Based Combination Therapies (ACT) now has a longer duration of action providing sustained activity to eliminate any remaining parasite (Onasanya and Ademowo 2013). Furthermore, the difficulty of creating efficient vaccines and also adverse side-effects of the existing antimalarial drugs highlight the urgent need for novel, well-tolerated antimalarial drugs (Ridley 2002) for both prophylaxis and treatment of malaria.

There is broad consensus on the need for development of new agents, owing to the increasing resistance of the parasite to available agents. Antimalarial drug development can follow several strategies, ranging from minor modifications

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of existing agents to the design of novel agents that act against new targets (Rosenthal 2003). Natural products are the source of the two most important drugs currently available to treat severe falciparum malaria, quinine and artemisinin derivatives. In the case of artemisinin, relatively simple chemical modifications of the natural parent compound have led to the synthesis of a series of highly potent antimalarials (Wright 2005; Batista et al. 2009). The development of these two important drugs from natural sources and the utilization of many plants traditionally in various parts of the world triggered the search for new antimalarial drugs of natural origin using in vitro and in vivo studies.

In malaria-endemic countries, several plants are utilized in traditional medicine for the treatment of malaria and/or fever. Furthermore, several studies have been undertaken to evaluate not only the inhibitory effects of various plant extracts on *P. falciparum* (Tran et al. 2003; Wanyoike et al. 2004) using in vitro culture, but also in vivo antimalarial properties on *P. berghei*-infected mice (Andrade-Neto et al. 2003; Sudhanshu et al. 2003).

Trema orientalis (L.) Blume (Ulmaceae) is an evergreen tree. Both stem bark and leaf decoctions of *T. orientalis* are used to treat malaria, manage pain in tired muscles and aching bones as well as venereal disease (Ayensu 1978). The hexane extract of stem bark of *T. orientalis* showed antiplasmodial activity, in an in vitro study done by (Abiodun et al. 2011). The leaves of *T. orientalis* contain tannins, saponins, flavanoids, triterpenoid (simiarenol, simiarenone, trematol). Octacosanoic acid, 1-octacosanyl acetate, simiarenone, simiarenol, episimiarenol, and triterpene alcohol, trematol has been isolated from stem bark (Rastogi and Mehrotra 1993). Other studies have also reported the presence of methylswertianin, decussatin, glycosides of decussatin, sweroside, scopoletin, (–)-epicatechin, lupeol, *p*-hydroxybenzoic acid, adian-5-en-3-one, 2 α , 3 β -dihydroxyurs-12-en-28-oic acid in the stem bark (Tchamo et al. 2000; Ogunkoya et al. 1972, 1973, 1977). The stem bark also contains (–)-ampelopsinF, (–)-epicatechin, (+)-catechin, (+)-syringaresinol, *N*-(*trans-p*-coumaroyl) tyramine, *N*-(*trans-p*-coumaroyl) octopamin, *trans*-4-hydroxycinnamic acid, and 3,5-dimethoxy-4-hydroxyphenyl-1-O- β -D-glucoside.

Experimental

Plant material

Fresh leaves and barks of *T. orientalis* were collected in February 2013 from residential quarters in Ado, Ekiti State, South Western Nigeria. The fresh leaves and barks were wrapped with plastic sheets during transportation. The

plant was identified as *T. orientalis* by taxonomists (Ugbogu O.A and Shosanya O.S) and voucher specimen was deposited at the Forestry Research Institute of Nigeria, Ibadan, with voucher specimen number FHI No. 109670.

Extraction

The leaves and bark of *T. orientalis* were air dried at room temperature under shade and reduced to appropriate size by grinding with an electric mill. A total of 415 g dried leaves were extracted in 1000 ml of water for 24 h. While a total of 900 g of bark were extracted in 1000 ml of water for 24 h. The aqueous extracts were concentrated in a rotary evaporator under reduced pressure at 40 °C. All the extracts were kept in airtight containers and were stored at 4 °C for use in antiplasmodial bioassay and toxicity tests. The yield of aqueous leaf extract of *T. orientalis* is 6 % w/w while that of bark is 6.44 % w/w.

Acute toxicity testing

Thirteen Swiss albino mice were randomly divided into three groups. After being fasted for 2 h, mice in the first group (3 mice) were given water, the second and third group (5 mice each) were orally given 0.05–5 g/kg of the aqueous leaf and bark extract respectively and observed for any signs of toxicity in the first 4 hours and subsequently daily for 10 days to assess safety of the extract. Animals were observed for gross changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea and mortality (OECD 2001). Blood samples were collected to determine white blood cell count, red blood cell count, Hemoglobin Concentration (Hb) and Packed Cell Volume (PCV) as described by Jain (1986).

Animals and parasite

Eighty albino mice (40 males and 40 nulliparous female) (25 $\pm$ 2 g) bred at the animal house at IAMRAT, University College Hospital, Ibadan were used. They were provided with food and water ad libitum in the animal house at the Department of Zoology, University of Ibadan. Animals were acclimatized for 1 week to the experimental environment. The care and handling was according to international guidelines for the use and maintenance of experimental animals (OECD guidelines 2001) and the University of Ibadan Ethics committee approved the protocol. Chloroquine sensitive strain of *P. berghei* (NK-65) obtained from the Institute of Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria was used. The parasites were maintained by serial passage of blood from infected mice to non-infected ones on weekly basis.

In vivo antimalarial tests

Parasite inoculation

Albino mice previously infected with *P. berghei* and having parasitemia level of 20–30 % were used as donor. The donor mice were then sacrificed by decapitation and blood was collected by cardiac puncture into heparinized vacutainer. The blood was then diluted with physiological saline based on parasitemia level of the donor mice and the red blood cell (RBC) count of normal mice (Waako et al. 2005) in such a way that 1 ml blood contains 5×10^7 infected RBCs. Each mouse was then given 0.2 ml of this diluted blood intraperitoneally, which contained 1×10^7 *P. berghei* infected RBCs.

Experimental design

For evaluating the crude extract, infected mice were randomly divided into eight groups of 10 mice per group. Group I is the positive control, treated with chloroquine 25 mg/kg

Parasitemia measurement

Thin smears of blood were made from the tail of each mouse on days 10, 11, 12, 13, 17 and 23 post-infection. The slides were fixed in absolute methanol for 15 min and stained with 10 % Geimsa's stain at pH 7.2 for 15 min. The stained slides were then washed gently using distilled water and air dried at room temperature. Two stained slides from each mouse were examined under Olympus microscope (Japan) with an oil immersion eyepiece of 100× magnification. Three different fields on each slide were examined to calculate the average parasitaemia as described by Hilou et al. (2006).

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

Finally, percent parasitemia inhibition of the extracts was compared with respect to the controls and parasitemia suppression was calculated using the following formula (Kalra et al. 2006).

$$\% \text{ Inhibition} = \frac{\text{Mean parasitemia of negative control} - \text{mean parasitemia of treated group}}{\text{Mean parasitemia of negative control}} \times 100$$

(CQ25), group II was negative control given distilled water (CON) (2 ml), group III–V were treated with aqueous leaf extract of *T. orientalis* at 400 mg/kg (TOL400), 800 mg/kg (TOL800) and 1600 mg/kg (TOL1600) respectively. While group VI–VIII were treated with aqueous bark extract of *T. orientalis* at 400 mg/kg (TOB400), 800 mg/kg (TOB800) and 1600 mg/kg (TOB1600) respectively. Doses were selected based on acute toxicity studies. Volume administered to the mice was 0.2 ml and gavage was used for oral administration.

The 4 day inhibition test

This test was used to evaluate the schizontocidal activity of the extracts against *P. berghei* infected mice according to the method described by Peter et al. (1995). Infected mice were randomly divided into their respective group as described above. The mice were treated with extracts 10 days post-infection for 4 days. Blood was collected from the tail of the mice on days 10, 11, 12, 13, 17 and 23 post-infection and Geimsa's stained thin blood film was then prepared to determine parasitaemia and percentage inhibition. In addition, each mouse was observed daily for determination of survival time (Shakya 2012).

Measurement of blood parameters

Packed cell volume (PCV) was measured to predict the effectiveness of the test extract in preventing hemolysis resulting from increasing parasitemia associated with malaria. Heparinized capillary tubes were used for collection of blood from tail of each mouse. The capillary tubes were filled with blood up to 3/4th of their volume and sealed at the dry end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge (Hawksley, United Kingdom) with the sealed end outwards and centrifuged for 5 min at 11,000 rpm. The tubes were then taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader (Hawksley, United Kingdom).

Monitoring of body weight changes

The body weight of each mouse was measured before and after infection and treatment using a sensitive digital weighing balance (Suofei, China). In order to rule out the effect of the extract on body weight and PCV; the crude extract was administered to healthy mice at the doses used for 4 days. The extract was found to have no effect on PCV and weight.

Data analysis

Data are expressed as mean $\pm$ standard error of mean (SEM). Data were analyzed using Prism 5 GraphPad. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine statistical significance for comparison of parasitemia inhibition, weight and PCV among groups. *P* value of less than 0.05 was considered statistically significant.

Results

Acute toxicity study

The acute toxicity study indicated that the leaf and bark extracts caused no mortality in all doses (0.05–5 g/kg) within the first 24 h as well as for the following 14 days. Physical and behavioral observations of the experimental mice also revealed no visible signs of overt toxicity like lacrimation, loss of appetite, tremors, hair erection, salivation and diarrhea. This suggests that lethal dose of the extract is greater than 5 g/kg. Table 1 shows the haematological parameters of the acute toxicity test group.

Effect on 4 day inhibition test

The results of the study showed that the aqueous both leaf and bark extracts *T. orientalis* displayed chemosuppressive activity against *P. berghei* (Table 2).

Percentage parasite inhibition analysis indicated that the both leaf and bark extract produced a dose-dependent decrease in level of parasitemia compared to negative control mice (Table 3). Inhibition in leaf extract was first observed on day 2 post treatment. TOL1600 exhibited a higher parasite inhibition (76.08 %) compared to other doses of the extracts and the activity was almost comparable to that of chloroquine on day 23 post treatment (Table 2). However, inhibition was observed in bark extract on day 1 post treatment. TOB1600 exhibited a significant parasite inhibition (74.23 %) compared to other doses of the extracts and the activity was almost comparable to that of CQ25 on day 23 post treatment, although, TOB400 and TOB800 reduced on day 23 compared to day 16 (Table 2).

Percentage parasite inhibition increased with time in both leaf and bark extracts. The leaf and bark extracts averted loss of weight associated with infection at all dose levels compared to negative control mice. However, the increase in body weight was not found to be dose-dependent, as the highest percentage change was observed with TOL400 (41.15 %) and a slight weight decrease with TOL1600 (−0.85 %) in the leaf extracts and the highest percentage change was observed with TOB1600 (18.30 %) and minimum with TOB800 (12.15 %) in the bark extracts. There were no detectable differences in preventing weight reduction associated with parasitemia between the doses as well as with chloroquine (Table 4).

All the extract doses and the chloroquine significantly prevented reduction of PCV when compared to negative control animals (Table 5).

Table 1 Haematological parameters of acute toxicity effect of *Trema orientalis* on mice

Haematological parameters									
Groups	PCV	HB	RBC	WBC	Platelet	Lymphocyte	Neutrophil	Monocyte	Eosinophil
Control (A)									
Control 1	36	11.7	6.13	9400	140,000	70	26	3	1
Control 2	40	13.6	6.45	5450	94,000	66	30	2	2
Control 3	34	11.6	5.38	4500	62,000	72	24	2	2
Bark (B)									
50 mg/kg	38	12.5	6.33	5700	100,000	69	32	0	1
300 mg/kg	35	11.6	5.42	5350	94,000	66	30	2	2
1000 mg/kg	33	10.8	5.31	5500	102,000	75	24	1	0
2000 mg/kg	32	10.4	5.23	10,600	172,000	65	31	3	1
5000 mg/kg	26	8.5	4.43	5000	84,000	65	30	3	2
Leaf (C)									
50 mg/kg	42	13.6	7.11	6700	110,000	74	22	3	1
300 mg/kg	40	13.5	6.36	5650	90,000	68	29	2	1
1000 mg/kg	37	12.4	6.46	8950	154,000	68	29	3	0
2000 mg/kg	34	11.4	5.37	6800	103,000	70	26	2	2
5000 mg/kg	32	10.4	5.23	4100	64,000	79	17	1	3

PCV Packed cell volume, HB haemoglobin, RBC red blood cell count, WBC white blood cell count, B bark extract, C leaf extract

Table 2 Percentage parasite inhibition of infected mice treated with crude aqueous extract of *Trema orientalis* in the 4 days inhibition test

Extract	Animal group	% Inhibition					
		D10	D11	D12	D13	D17	D24
Leaf	Control	–	–	–	–	–	–
	Chloroquine	12.74	–28.31	34.54	66.61	84.82	100
	TOL400	–85.31	–12.06	14.29	31.84	45.41	51.55
	TOL800	–162.63	–43.38	11.65	31.84	49.18	62.78
	TOL1600	–98.06	22.28	23.30	44.16	60.24	76.08
Bark	TOB400	–73.87	3.52	35.926	53.27	65.53	44.3
	TOB800	–23.97	15.41	39.39	53.27	66.12	65.82
	TOB1600	–12.96	38.19	49.72	53.53	71.41	74.23

Table 3 Percentage parasitemia of infected mice treated with crude aqueous extract of *Trema orientalis* in 4 days inhibition test

Extract	Animal group	% Parasitaemia					
		D10	D11	D12	D13	D17	D24
Leaf	Control	4.63 ± 0.32	5.97 ± 0.33	7.21 ± 0.42	7.79 ± 0.30	8.5 ± 0.30	9.7 ± 0.29
	Chloroquine	4.04 ± 0.88	7.66 ± 1.00	4.72 ± 0.75	2.6 ± 0.32	1.29 ± 0.17	0 ± 0
	TOL400	8.58 ± 0.47	6.69 ± 0.25	6.18 ± 0.28	5.31 ± 0.31	4.64 ± 0.45	4.7 ± 0.42
	TOL800	12.16 ± 1.2	8.56 ± 0.78	6.37 ± 0.55	5.31 ± 0.54	4.32 ± 0.49	3.61 ± 0.54
	TOL1600	9.17 ± 0.79	7.3 ± 0.74	5.53 ± 0.65	4.35 ± 0.64	3.38 ± 0.60	2.32 ± 0.39
Bark	TOB400	8.05 ± 1.11	5.76 ± 0.90	4.62 ± 0.87	3.64 ± 0.71	2.93 ± 0.48	5.4 ± 0.86
	TOB800	5.74 ± 0.40	5.05 ± 0.37	4.37 ± 0.42	3.64 ± 0.42	2.88 ± 0.43	2.7 ± 1.61
	TOB1600	5.23 ± 2.27	3.69 ± 2.08	3.62 ± 1.94	3.62 ± 2.11	2.43 ± 1.31	2.5 ± 0.49

Table 4 Weight of infected mice treated with crude aqueous extract of *Trema orientalis* in the 4 days inhibition test

Extract	Animal group	Weight						% Change
		D10	D11	D12	D13	D17	D24	
Leaf	Negative control	27.6 ± 0.58	26.8 ± 0.52	23.4 ± 0.51	18.5 ± 0.67	12.3 ± 0.48	4.7 ± 0	–82.97
	Chloroquine	27.1 ± 0.41	27.6 ± 0.18	28.5 ± 0.29	29.4 ± 0.32	30.4 ± 0.32	31.5 ± 0.24	16.24
	TOL400	22.6 ± 1.09	23.4 ± 0.79	24.8 ± 0.77	26.8 ± 0.70	29.1 ± 0.48	31.9 ± 0.18	41.15
	TOL800	22.3 ± 0.82	22.6 ± 0.62	23.5 ± 0.58	25.0 ± 0.57	27.4 ± 0.40	30.2 ± 0.32	35.43
	TOL1600	23.5 ± 0.92	22.5 ± 1.18	22.5 ± 1.18	22.6 ± 1.16	22.8 ± 1.13	23.3 ± 1.11	–0.85
Bark	TOB400	23.2 ± 1.18	23.2 ± 1.07	23.5 ± 1.09	24.3 ± 1.05	25.5 ± 0.96	27.0 ± 0.80	16.38
	TOB800	24.7 ± 1.18	25.0 ± 0.98	25.3 ± 1.12	26.0 ± 1.14	26.8 ± 1.10	27.7 ± 0.63	12.15
	TOB1600	23.5 ± 1.34	24.5 ± 1.27	24.7 ± 1.23	25.5 ± 1.15	26.6 ± 1.06	27.8 ± 0.95	18.30

Discussion

Developing new compounds from natural products could be an important source of new antimalarials in the long term; it is also possible to develop standardized and validated phytomedicines more quickly and cheaply.

The 4 day parasite inhibition test, which mainly evaluates the antimalarial activity of candidates on early infections, which evaluates the curative capability of candidate extracts on established infections, are commonly used for antimalarial drug screening. Determination of percentage inhibition of parasitemia is the most reliable parameter. It

Table 5 Packed cell volume of infected animals treated with aqueous leaf and bark extract and of *Trema orientalis* in the 4 day suppressive test

Extract	Animal group	PCV				
		D10	D11	D12	D13	D17
Leaf	Control	49.22 ± 3.21	46.5 ± 12.84	39.49 ± 8.75	35.92 ± 5.13	27 ± 6.24
	Chloroquine	56.18 ± 2.00	56.91 ± 2.28	57.49 ± 2.39	57.84 ± 2.73	58.61 ± 2.83
	400	47.81 ± 9.17	48.16 ± 7.89	49.24 ± 7.38	50.46 ± 6.43	51.45 ± 6.20
	800	48.63 ± 6.78	49.38 ± 6.80	51.10 ± 6.87	51.03 ± 6.51	51.64 ± 6.49
	1600	49.79 ± 5.20	50.34 ± 5.10	51.22 ± 5.16	52.12 ± 4.86	53.4 ± 4.50
Bark	400	48.88 ± 3.68	49.29 ± 3.71	49.53 ± 3.56	50.04 ± 3.53	49.97 ± 3.32
	800	47.5 ± 3.61	49.20 ± 3.17	49.37 ± 3.09	49.83 ± 3.24	50.4 ± 3.41
	1600	50.63 ± 4.96	52.22 ± 5.98	52.42 ± 5.96	53.14 ± 6.10	53.93 ± 6.43

can be clearly seen from the results that percentage parasitemia measured in the test was reduced by the aqueous leaf and bark extracts of *T. orientalis* in *P. berghei* infected mice, pointing to the fact that the plant is a potential antimalarial. The leaf and the bark aqueous extracts were found to possess comparable blood schizontocidal activity at the concentrations tested. This was evident from the chemosuppression obtained during the 4 day suppressive test, suggesting the possible localization of the active ingredients in these two parts of the plant. Most precisely, aqueous extract of *T. orientalis* has not been evaluated using aqueous extract in vivo. *T. orientalis* had been shown to exhibit various pharmacological activities including anti-microbial properties and antiplasmodial (Abiodun et al. 2011). These effects may be mainly due to the fact that it contains important biologically active compounds such as tannins, saponins, flavanoids, triterpenoid (simiarenol, simiarenone, trematol) in the plant. Octacosanoic acid, 1-octacosanyl acetate, simiarenone, simiarenol, episimiarenol, and a new triterpene alcohol, trematol has been isolated from stem bark (Rastogi and Mehrotra 1993). Other studies have also reported the presence of methylswertianin, decussatin, glycosides of decussatin, sweroside, scopoletin, (–)-epicatechin, lupeol, *p*-hydroxybenzoic acid, adian-5-en-3-one, 2 α , 3 β -dihydroxyurs-12-en-28-oic acid in the stem bark bark (Tchamo et al. 2000; Ogunkoya et al. 1972, 1973, 1977).

Anemia and body weight loss reduction are the general features of malaria-infected mice (Langhorne et al. 2002). So, an ideal antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to the rise in parasitemia. Despite the fact that the increase in weight was not consistent with increase in dose, the aqueous extract of *T. orientalis* significantly prevented weight loss associated with increase in parasitemia level. The preventive effect increase at the highest dose of the bark extract (TOB1600) and the lowest dose of the leaf extract (TOL400) which is consistent with the results of the

parallel sub-acute toxicity study carried out to rule out the effect of the extract on the measured parameters. The fact that the preventive effect increased might indicate that the plant could have appetite stimulating effect.

PCV was measured to evaluate the effectiveness of the crude extract in preventing hemolysis due to parasitemia level. The underlying cause of anemia includes the following mechanisms; the clearance and/or destruction of infected RBCs, the clearance of uninfected RBCs, and erythropoietic suppression and dyserythropoiesis. Each of these mechanisms has been implicated in both human and mouse malarial anemia (Lamikanra et al. 2007).

As observed in the results, all doses of the leaf and bark extracts reduced parasitemia after second dose. This delay of activity may be indication that the extract might have a delayed onset of action. The curative effect of leaf and bark extracts occurred in a dose-dependent manner, with the highest suppression observed with its higher dose, this is similar to the previous reports of Okokon et al. (2011, 2012) on the effect of *Carpolobi alutea* and *Melanthra scadens* on *P. bergei* respectively.

Conclusions

The present study indicates that the aqueous leaf and bark extracts of *T. orientalis* have good antiplasmodial activity, with varying degree and/or differential effect on the measured parameters. The extract was effective in suppressing parasitemia and in protecting infected animals from parasite-induced PCV reduction. These findings suggest that the phytochemicals responsible for antimalarial activity of the plant are polar in nature and future studies on the plant regarding antimalarial activity should be conducted using the nonpolar solvent to determine the solvent extraction with the highest activity. In addition, this study provides evidence of the claims by the traditional healers of the efficacy of aqueous extracts of *T. orientalis*.

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