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Research Article

In-Vivo Antimalarial Activity of Aqueous and Ethanolic Extracts of *Vernonia amygdalina* on *Plasmodium berghei* Infected Mice

Mary Aigbiremo Oboh[1]*, Emmanuel Taiwo Idowu[2], Olubunmi Adetoro Otubanjo[2] and Olugbenga O Aina[3]

[1]Parasitology Unit, Department of Zoology, University of Lagos, Akoka, Yaba, Lagos, Nigeria and Mycology Laboratory, University of Cheikh Anta Diop, Dakar, Senegal

[2]Parasitology Unit, Department of Zoology, University of Lagos, Akoka, Yaba, Lagos, Nigeria

[3]Biochemistry Division, Nigeria Institute of Medical Research, Yaba, Lagos, Nigeria

***Corresponding Author**: Mary Aigbiremo Oboh, Parasitology and Mycology Laboratory, University of Cheikh Anta Diop, Dakar, Senegal.

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Abstract

Background: Development and promotion of phytomedicines may be the sustainable solution to malaria treatment.

Objective: To evaluate the anti-plasmodial activity of extracts of *Vernonia amygdalina* on *Plasmodium berghei.*

Methods: Hundred inbred mice weighting 12 - 23g were infected with a standard inoculum of 2 x 10^6 parasitized erythrocytes in PBS (0.2 ml). The mice were divided into two groups (suppressive and curative groups). the group for suppressive test were further divided into two (aqueous and ethanolic treatment), and each randomized into five groups (A-E). Animals in group A of all the groups received no form of treatment, B were administered 5 mg/kg suppressive dose of chloroquine. In group C, D E, animals were administered 400, 800 and 1600 mg/kg of aqueous extract and same was repeated for the ethanolic extract sub-groups. On day 5, thin blood films were made and examined. The second group of 50 mice followed same experimental design but with a CQ curative dose of 10 mg/kg.

Results: The highest suppressive effect was seen in the 1600 mg/kg aqueous extract (60.6%) and the 800 mg/kg ethanolic extract (43.3%).

Conclusion: This extract has demonstrated a strong potential for suppressing malaria and the efficacy could be enhanced by combining with other herbal plants in achieving parasite clearance.

Keywords: *Anti-Plasmodial; Plasmodium berghei; Vernonia amygdalina; Suppressive; Curative; Drug; Resistance; Alternative-Drug*

Introduction

Malaria is a life threatening disease that is caused by species of the genus *Plasmodium* and transmitted by the female *Anopheles* mosquito [1]. It is the most prevalent tropical disease resulting in high morbidity with its consequent economic and social loss. Malaria accounted for an estimated 207 million cases in 2012 with 627,000 deaths occurring in same year, 90% of these deaths occurred in sub-Saharan African [1].

Malaria is widespread in many part of the world mainly in tropical and subtropical regions but extending into some of the temperate areas. Most malaria endemic regions are characterize by warm temperature and rainfall both suitable for mosquito breeding. Malaria infection particularly in sub-Saharan Africa is caused by *Plasmodium falciparum* [2].

Evidence on Nigeria shows that malaria incidence throughout the country had been on the increase over the years ranging between 1.12 million at the beginning of 1990 and 2.25 million by the turn of the millennium 2000 and 2.61 million in 2003 [Alaba and Alaba, unpublished].

Four major problems are associated with the management of malaria, the most important is that the parasite are resistance to the most widely, affordable and safest drugs which were used as the first line treatment such as chloroquine and fansidar and even the most recently introduced artemisinin combination therapy [3-5]. Secondly, the overall control of the mosquitoes which transmit malaria is made difficult by their resistance to a wide range of insecticides. The third is a new and rapidly developing problem which is the widespread production of fake antimalarial drugs [6]. Lastly many countries in Africa lack the infrastructures and resources to manage and control malaria [2].

A number of chemotherapeutic drugs in treating malaria and vector control measures have been developed. Some of these measures include the use of Long-Lasting Insecticide-treated Bed Nets (LLINs), which incorporates insecticide directly into net fibers and in which production tripled from 30 million in 2004 to 110 million in 2007 [7], Indoor Residual Spraying (IRS), Intermittent Preventive Treatment (IPT) of children and pregnant women.

Currently, antimalarial experts are focusing on therapies that combine artemisinin derivative with other companion drugs, these combinations are referred to as Artemisinin-based combination therapy (ACT), and such include atovaquone-proguanil (Malaron™), Artemether-lumefantrine (Coartem™). Some of the most potent antimalarial agents developed from plants relying on traditional knowledge leads include quinolines and endoperoxides/artemisinin derivative [8,9]. Within the context of growing antimalarials resistance, development and promotion of phytomedicines may be the sustainable solution to malaria treatment [10]. Enhanced antimalarial effect of chloroquine by aqueous *Vernonia amygdalina* leaf extract in mice infected with chloroquine resistant and sensitive *Plasmodium berghei* strains has been demonstrated with a dose-dependent efficacy being observed [11].

This study thus sought to evaluate the *in vivo* antimalarial activity of ethanolic and aqueous leaf extracts of *Vernonia amygdalina* on *Plasmodium berghei* infected mice.

Materials and Methods

Animals

Hundred inbred male and female (non-pregnant) albino mice (were purchased from the animal house of Nigerian Institute of Medical Research, Lagos, Nigerian) weighting 12 - 23g. The animals were observed under 12 hours light and dark cycles in aerated cages inside a well ventilated rodent cubicle and handled according to the Institutional ethics and guidelines for the protection of human and animal welfare. They were fed with pellet diet and water ad libitum.

Plant Material

Fresh, green leaves of *Vernonia amygdalina* were harvested from a garden within the premises of University of Lagos (UNILAG) Yaba, Lagos. The leaves were identified to be *Vernonia amygdalina* by Mr. Kolade, E.A, a taxonomist in the Department of Botany, Faculty of Science, UNILAG.

The leaves were then air-dried for six weeks in the glass house located inside the Botanical and Zoological garden of UNILAG. The air-dried leaves were then taken to the Biochemistry Division of the Nigerian Institute of Medical Research, (NIMR), Yaba, Lagos, and grounded into powdery form. The plant was extracted as shown below following a previously described protocol [11].

Aqueous Extraction of *Vernonia amygdalina*

Three hundred and fifty gram of powdered *Vernonia amygdalina* was steeped in distill water (1,000 ml) at 26°C for 7 days under sterile condition. On day 7, the concoction was filtered, sterilized by passing it through a 45m Millipore membrane filter (St Quentine, France). The filtrate was then freeze dried under low pressure, the concentrated samples were then removed and stored in a sterile bottle at 4°C until it was used.

Ethanolic extraction of *Vernonia amygdalina*

Extraction of *Vernonia amygdalina* with ethanol was also carried out in the Biochemistry Division of the Nigerian Institute of Medical Research, Yaba, Lagos. 350g of dried *Vernonia amygdalina* leaves was placed inside a thimble which was loaded into a soxhlet extractor. The soxhlet extractor was then placed onto a round bottom flask containing ethanol (70%, 1700 ml), a condenser was fixed to the thimble with the aid of a retort stand and the whole set-up placed inside a Bunsen burner and plugged to electrical energy. After extraction, the extract was filtered and concentrated under low pressure for 7 days.

Drugs

Tablets of chloroquine (Emzor Pharmaceuticals Limited, Nigeria) were dissolved in phosphate buffered saline (PBS) to final doses of 5 mg/kg and 10 mg/kg body weight.

Parasite inoculum preparation and density determination

The *Plasmodium berghei* clone (CQ sensitive) used was generously provided by the Biochemistry division of the Nigerian Institute of Medical Research NIMR, Lagos, Nigeria.

A standard inoculum was prepared from a donor mouse with *Plasmodium berghei* parasitized erythrocytes. Infected blood from the donor mouse was obtained by ocular plexus method with the aid of a microhaematocrit after anesthesia with chloroform.

Parasitaemia was established with the aid of a thin tail blood film under oil immersion at X 100 magnification and measured as a percentage of infected erythrocytes in fields of 500 erythrocytes. Each mouse was infected with a standard inoculum of 2 x 10^6 parasitized erythrocytes suspension in phosphate buffered saline (0.2 ml) from a donor mouse that was prepared based on percentage parasitaemia and number of erythrocytes counted per microlitre of blood using an improved Neubauer haematocytometer. The mean percentage parasitaemia for each dose is determined arithmetically by counting the number of parasites in the red blood cells in relation to the total number of cells.

Suppressive treatment test using aqueous and ethanolic extracts of *Vernonia amygdalina*

A 4-day suppressive test according to Peter [12] was used. The animals were randomized into 5 groups of 5 mice each denoted by A-E. Animals in group A were passaged with 2 x 10^6 parasitized erythrocytes in 0.2 ml Phosphate Buffered saline (PBS, pH 7.2) suspension and served as the negative control.

Animals in group B (positive control) were administered 5 mg/kg body weight suppressive dose of chloroquine. In group C, D and E, the experimental animals were administered selected doses of 400, 800 and 1600 mg/kg body weight respectively of the aqueous *Vernonia amygdalina* extract. All treatments commenced immediately after inoculation (Day 0) and continued on day 1, 2, 3 via an oral route using a canula. On day 5, a thin blood film from the tail of each infected mouse was prepared, fixed with methanol, stained with 3% Giemsa stain for 30 minutes and examined microscopically under oil immersion and used to calculate parasitaemia reduction using the formula below.

The same process was carried out for the alcoholic extract on another set of mice.

$$\text{AV \% suppression} = \frac{\text{Av \% parasitaemia in control- Av \% parasitaemia in test}}{\text{Av \% parasitaemia in control}} \times 100$$

Curative treatment using aqueous and ethanolic extracts of *Vernonia amygdalina*

Another set of 25 mice was randomized into 5 groups of 5 animals each labeled A-E. Animals in group A (negative control) were inoculated with 2 x 10^6 parasitized erythrocytes in 0.2 ml of PBS. Animals in group B (positive control) were given a curative dose (10 mg/kg) of chloroquine daily for 3 days.

The test groups (C, D and E) were administered the selected doses of 400, 800 and 1600 mg/kg body weight of the aqueous extract of *Vernonia amygdalina*. All treatments commenced on day 3 post inoculation (The rationale for 3 days post-treatment is to allow for establishment of infection since it is not a prophylactic assay assessment).

Thin tail blood films were prepared starting from day 3 post inoculation until day 7. The parasitaemic level was determined as previously described. Same protocol was followed for another 25 mice using alcoholic extract.

Result and Observation

4-day suppressive test with aqueous and ethanolic extracts of *Vernonia amygdalin*a in mice infected with *Plasmodium berghei*

A dose-dependent suppressive effect was seen in the aqueous extract as the highest aqueous dose (1600 mg/kg) displayed the highest suppressive effect (60.6%) which is followed by the 400 mg/kg dose.

In contrast to this, the highest suppressive effect of the ethanolic extract was produced by the 800 mg/kg dosage (49.6%) followed by the 1600 mg/kg which gave a 43.3% parasitaemic reduction (Table 1 and 2).

Table 1: *Suppressive test of aqueous and ethanolic leaf extracts of Vernonia amygdalina in Plasmodium berghei infected mice.*

-ve control: Negative Control Group; +ve control: Positive Control; S.D: Standard Deviation.

Extract	Group	Dose(mg/kg)	Parasitaemia (% ± S.D)	Average reduction in Parasitaemia (%)
Aqueous	A	-ve control	8.85 ± 1.2	0
	B	+ve control	-	100
	C	400	4.54 ± 3.6	48.7
	D	800	5.13 ± 5.5	42.0
	E	1600	3.49 ± 1.9	60.6
Ethanolic	A	-ve control	8.85 ± 1.2	0
	B	+ve control	-	100
	C	400	6.19 ± 1.5	30.9
	D	800	4.46 ± 1.0	49.6
	E	1600	5.02 ± 7.6	43.3

Table 2: *Response of Plasmodium berghei (in thousand) infection to treatment with Vernonia amygdalina aqueous and ethanolic extracts (400, 1600 mg/kg) in mice.*

-ve control: Negative control, values in parenthesis represent initial days after treatment

Extract	Group	Dose (mg/kg)	Post infection (Treatment days)				
			3[0]	4[1]	5[2]	6[3]	7[4]
Aqueous	A	-ve control	43.4 ± 19.9	38.6 ± 22.3	41.4 ± 12.7	34.0 ± 18.3	29.4 ± 9.9
	B	+ve control	70.2 ± 17.3	6 ± 3.5	0	0	0
	C	400	105.4 ± 17.1	76.1 ± 45.4	94.7 ± 50.7	47.3 ± 33.6	44.9 ± 27.7
	D	800	91.3 ± 20.9	59.9 ± 33.5	114.0 ± 47.0	50.0 ± 17.3	32.6 ± 17.6
	E	1600	32.4 ± 14.7	81.9 ± 34.4	72.0 ± 27.7	38.3 ± 6.5	22.8 ± 12.9
Ethanolic	A	-ve control	43.4 ± 19.9	38.6 ± 22.3	41.4 ± 12.7	34.0 ± 18.3	29.4 ± 9.9
	B	+ve control	70.2 ± 17.3	6 ± 3.5	0	0	0
	C	400	40.8 ± 47.9	82.2 ± 18.0	106.9 ± 40.0	40.7 ± 14.1	18.5 ± 8.2
	D	800	54.6 ± 39.3	65.3 ± 12.9	58.3 ± 21.7	94.3 ± 106.1	41.4 ± 7.1
	E	1600	24.2 ± 12.2	89.1 ± 37.4	101.0 ± 0.0	44.5 ± 0.0	55.4 ± 0.0

7-day curative test with aqueous and ethanolic extracts of *Vernonia amygdalina* in mice infected with *Plasmodium berghei*

Unlike chloroquine at curative dose of 10 mg/kg (Figure 1) which produced clearance of parasite on the fifth day, 0% cure rate was not achieved following 7 day post treatment with aqueous and ethanolic extracts at 400 mg/kg, 800 mg/kg and 1600 mg/kg respectively.

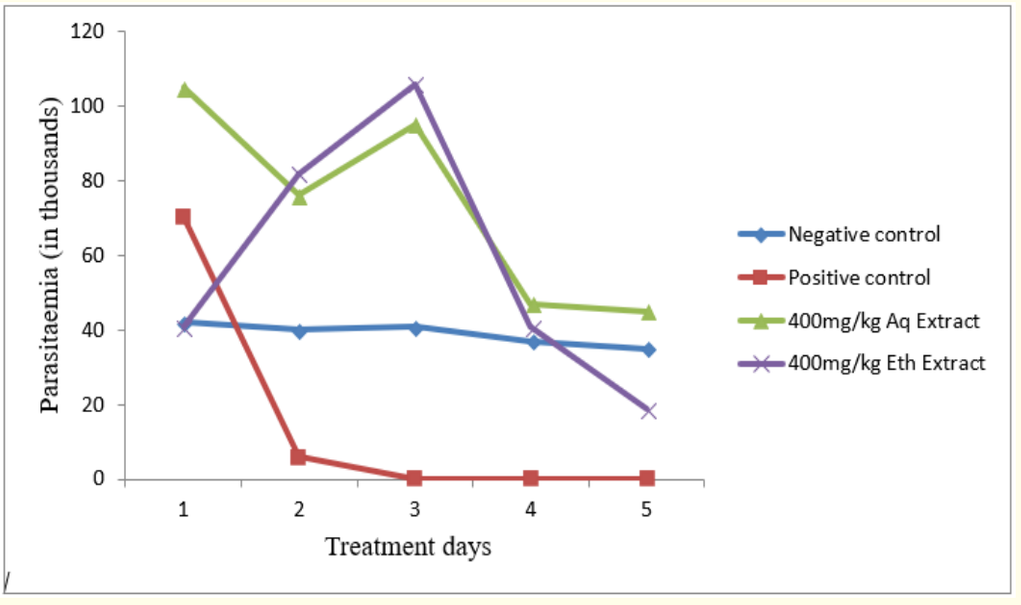

Figure 1: *Comparative effect of aqueous and ethanolic extracts of Vernonia amygdalina at 400 mg/kg body weight.*

400 mg/kg Aq extract = 400 mg/kg body weight aqueous extract of Vernonia amygdalina, 400 mg/kg Eth extract = 400 mg/kg body weight ethanolic extract of Vernonia amygdalina.

Nevertheless, at 400 mg/kg dose, there was an observed difference in the reduction of parasitaemia with the aqueous and ethanolic extracts (Figure 1), but there was no observed difference in the reduction of parasitaemia for the 800 mg/kg in both the aqueous and ethanolic extracts (Figure 2). However, at 1600 mg/kg body weight there was an observed difference in the reduction of parasitaemia with the aqueous and ethanolic extracts (Figure 3).

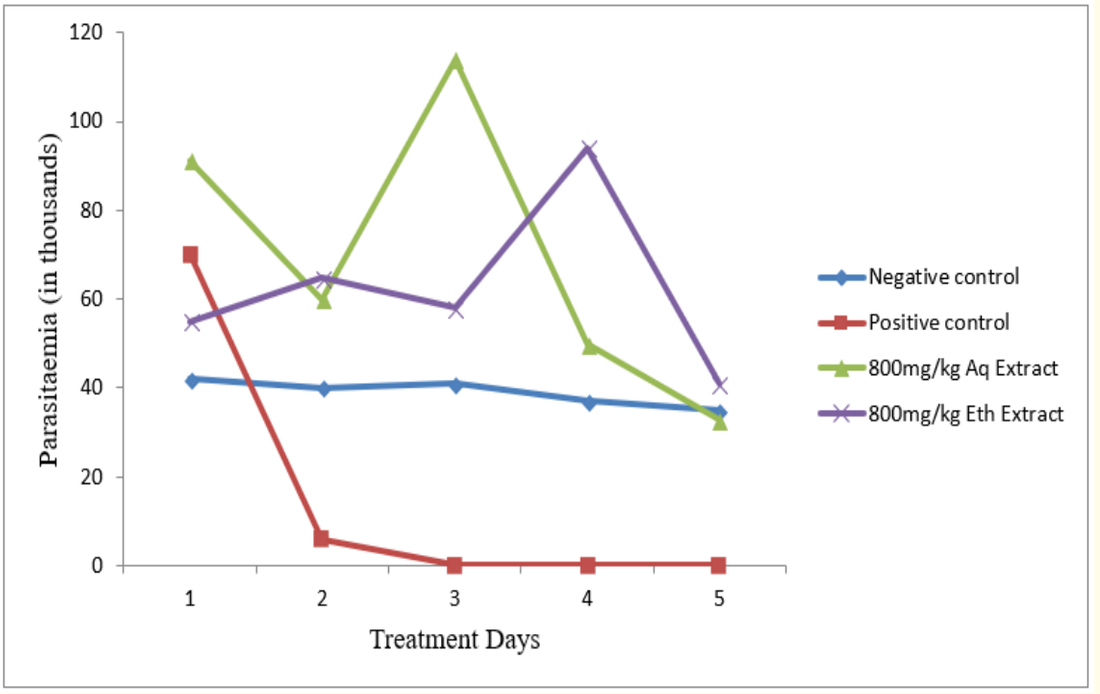

Figure 2: *Comparative effect of aqueous and ethanolic extracts of Vernonia amygdalina at 800 mg/kg body weight.*

800 mg/kg Aq extract = 800 mg/kg body weight aqueous extract of Vernonia amygdalina, 800 mg/kg Eth extract = 800 mg/kg body weight ethanolic extract of Vernonia amygdalina.

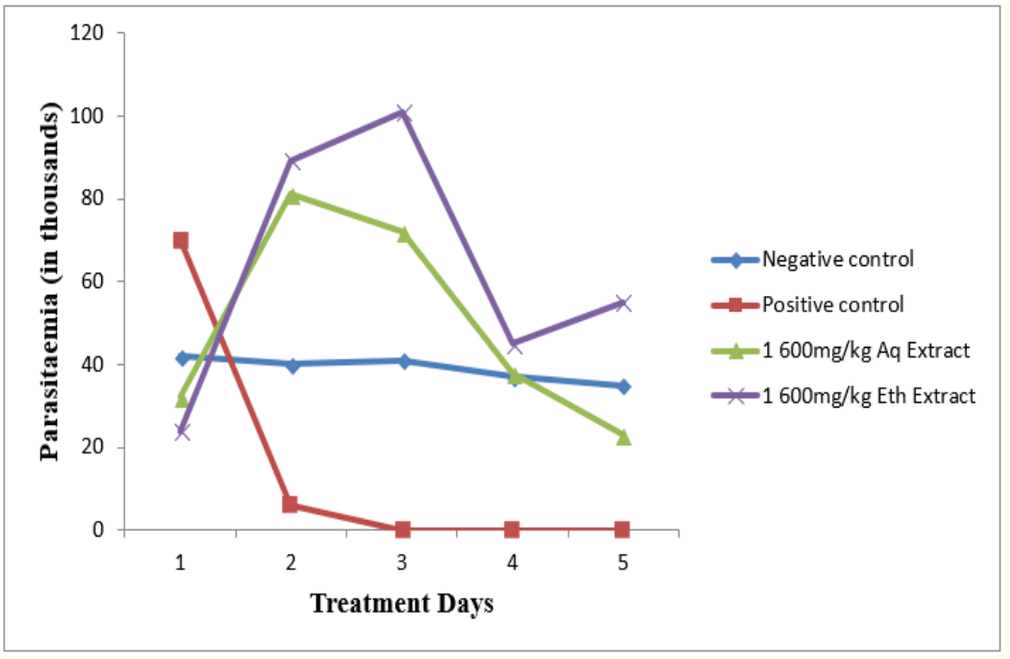

Figure 3: *Comparative effect of aqueous and ethanolic extracts of Vernonia amygdalina at 1600 mg/kg body weight.*

1600 mg/kg Aq extract 1600 mg/kg body weight aqueous extract of Vernonia amygdalina, 1600 mg/kg Eth extract = 1600 mg/kg body weight ethanolic extract of Vernonia amygdalina.

Discussion and Conclusion

The increasing failure of antimalarials including chloroquine and sulphadoxine-pyrimethamine in many endemic countries of Africa has generated tremendous public health concern necessitating further exploration of plants for newer antimalarial agents and optimization of those with existing antimalarial property when used singly, as composite medicine or in combination with orthodox medicine [13].

The present study has demonstrated the antiplasmodial activity of *Vernonia amygdalina*. The 4-day suppressive test carried out revealed that *Vernonia amygdalina* extract resulted in a suppression of *Plasmodium berghei* growth in infected mice at all dosage. This finding confirms the work of Jegede (2009, unpublished) which similarly observed the antimalarial activity of *Vernonia amygdalina* at same dosage and also that of Masaba [14] although at a much lower doses (10 µg/mL, 50 µg/mL, 100 µg/rnL and 200 µg/mL).

The suppression observed could be due to the active ingredients present in *Vernonia amygdalina* such as saponins, sesquiterpene lactone, steroid glycosides, alkaloids, tannins and flavonoids [15]. Other phyto-constituents that have been shown to have antiplasmodial activity include quinoline alkaloids such as cepharanthine, cryptolepine, isocyptolepine and neocryptolepine, coumarins and terpenoids [16,17]. Coumarins and terpenoids phyto-constituents are abundantly present in *Vernonia amygdalina* and may thus contribute to the observed antiplasmodial activity [16].

Decoction of *Vernonia amygdalina* have been used in most Nigeria herbal homes in the control of tick and treatment of constipation, cough, hypertension and feverish condition [18,19]. Methanolic and chloroform extracts of *Vernonia amygdalina* have been observed to have anti-leishmanial activity *in vitro* [20].

Meanwhile, both the aqueous and ethanolic extracts were not able to clear parasitaemia at all doses. This substantiate the work of Iwalokun [11] which shows that *Vernonia amygdalina* used alone although at lower doses (31.75 mg/kg, 62.5 mg/kg and 125 mg/kg body weight) was observed not to give a 0% cure rate. Although the aqueous extract at the highest dose (1600 mg/kg) showed more activity than the ethanolic extract. Taken together, this extract and extracts of other plants can be used in combination with each other or with other antimalarial drugs to produce a more efficacious result as has been shown by Hilou., et al. [21] where *Amaranthus spinosus* and *Boerhavia erecta* were used in combination at different dosage to suppress *Plasmodium falciparum.*

There is therefore need to continue research on *Vernonia amygdalina* since it would no doubt provide cost effective strategy of malarial control in combination with other plants especially in developing countries where affordability and accessibility to orthodox drugs are barriers to health care.

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Conflict of Interest

All authors have declared that no conflicting interest exist.

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