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Exploring the Antimalarial Efficacy of *Globimetula oreophila* Leaf Fractions in *Plasmodium berghei*-Infected Mice: *In Vivo* Approach

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Abstract: The development of parasite resistance to first-line antimalarial medicines, especially the Artemisinin-based combination therapies (ACTs), has made the research and development of novel antimalarial medications vital. *Globimetula oreophila*, a plant used in traditional medicine to treat malaria, is a natural product that may provide new antimalarial drugs with fewer side effects, greater efficacy and lower risk of resistance than synthetic drugs. This study aims to evaluate the antiplasmodial properties of *G. oreophila*'s fractions. The plant leaves were air-dried and reduced in size using a pestle and mortar. The pulverized plant was macerated in 70% ethanol and fractionated with solvent in increasing polarity of n-hexane, chloroform, ethyl acetate, and n-butanol to produce the various fractions. The antiplasmodial activity of the n-hexane, chloroform, ethyl acetate, and n-butanol fractions of *G. oreophila* leaf extract was assessed using an *in vivo* method in *Plasmodium berghei*-infected mice via prophylactic, suppressive, and curative test. The fractions' median lethal dose (LD50) was estimated to be greater than 5000 mg/kg in mice. The median effective dose (ED50) of the fractions at doses of 125, 250, and 500 mg/kg produced a significant (p<0.001) decrease in the level of parasitemia. The ethyl acetate fraction had the best antiplasmodium activity compared to other plant fractions. The fractions of G. oreophila showed significant in vivo antiplasmodial activity, which upholds the earlier *in vivo* findings for the crude extract and its folkloric use. Further study should be carried out to isolate active secondary metabolites responsible for this observed antimalarial activity in all four investigated fractions.

Introduction

Malaria is a major public health concern leading to morbidity and mortality, which occurs in all six World Health Organization regions (1). In 2021, an estimated 247 million cases and 619,000 fatalities occurred worldwide, with sub-Saharan Africa accounting for the vast majority of cases and deaths—roughly (1). According to the WHO, 26.6% of all malaria cases and 31.3% of deaths worldwide occurred in Nigeria in 2021 (1). The population of people most vulnerable to malaria are pregnant women and children under the age of five. In 2021, 76.8% of all the world's malaria deaths were of children under the age of five (1). In 2021, an estimated 1.3 million women were exposed to malaria during pregnancy, which resulted in 961,000 children with low birth weight (1). The development and reemergence of parasite resistance to first-line antimalarial drugs, particularly ACTs, as well as the disruption of humanitarian initiatives, have led to a sustained rise in malaria-related death and morbidity despite all efforts (1).

According to Sivakrishnan (2018), "a medicinal plant" is any plant in which one or more of its organs contain substances that can be used for therapeutic purposes or are precursors for the synthesis of effective drugs. Medicinal plants contain diverse phytochemicals with known or unknown biological activities. Plant-derived substances are believed to be

more accessible, in line with patients' preferences, and safer and more cost-effective treatments. Examples of these substances include tropane alkaloids (atropine, hyoscine, scopolamine, and hyoscyamine), opium alkaloids (paperverine, codeine, and morphine), flavonoids (stilbenes, chalcones, luteolin, quercetin, rutin, apigenin, and kaemferol), terpenoids (stigmasterol, sitosterol, betulinic acid, lupeol, and olenolic acid), and essential oils (from caraway and peppermint) (2, 4). High concentrations of phytochemical constituents with antiplasmodial properties found in medicinal plants aid in treating malaria. For example, quinine from the bark of the *Cinchona* tree, a member of the Rubiaceae family of plants, is well known for treating malaria (5, 6). Alternatively, *Artemisia annua*, another ancient medicinal plant, is a significant source of the antimalarial drug artemisinin, a sesquiterpene lactone (5, 7).

G. oreophila is a plant species in the family of Loranthaceae (8, 9, 10), a large family of about 75 genera and over 900 species (11). It is a hemiparasitic plant commonly called mistletoe, found growing on several dicotyledonous trees, using them as a host for its root-like structure called haustoria (10, 12). *G. oreophila* is widely distributed in tropical Africa, particularly in Nigeria, Cameroon, Gabon, Congo, and the Central African Republic (10, 12). Studies have revealed that the host plant can significantly affect the chemical composition and, thus, the biological activity of the hemiparasite mistletoe (13, 14). *G. oreophila* has been used to cure various ailments, including diarrhea, stomachache, headache, and fever. As a treatment for these ailments, the leaves and stems are boiled, and the resulting decoction is drunk (15). *G. oreophila* (Loranthaceae), growing on a host plant of *Azadirachta indica*, is mainly used in northern Nigeria to manage malaria. Previous phytochemical reports showed that the genus Globimetula contains several secondary metabolites, such as triterpenes, tannins, glycosides, alkaloids, flavonoids, and saponins (9, 16, 17), which have been reported to possess antimalarial activity (18, 19). Flavonols, which belong to the class of flavonoids, are the major biological markers of the genus Globimetula, which have been shown to have antimalarial activity. The antiplasmodial potential of the ethanol leaf extract of *G. oroephila* has been previously reported (9, 10). In previous studies, we reported the isolation of a prenylated quercetin from the ethyl acetate fraction of this plant (10). Subsequent in-silico analysis of this compound against seven *P. falciparum* enzymes indicates its potential to competitively inhibit enzyme activity by targeting co-factor binding sites, particularly in specific proteases. These findings highlight the promise of prenylated quercetin as a potent inhibitor for therapeutic applications in the prevention and treatment of malaria (20), elemental analysis of the crude ethanol extract (21), and qualitative and quantitative phytochemical profiling of this plant (22). In the present study, we now report on the antiplasmodial effect of fractions of *G. oreophila* growing on *A. indica* via *in vivo* models in mice, considering its wide acceptability as a material remedy in most of Nigeria.

Experimental Section

Materials

Collection, Identification, and Preparation of Plant Material

G. oreophila comprising the leaves and fruits was collected from Sokoto Metropolitan, Nigeria, in July 2019 and authenticated by a Taxonomist (Musa Muhammad) of the Herbarium Section, Department of Plant Biology, Ahmadu Bello University, Zaria-Nigeria by comparison with herbarium specimen having Voucher number ABU0886. The leaves were air dried under shade, reduced in size manually using mortar and pestle, and stored for further use.

Chemicals

All organic solvents employed for extracting and fractionating plant material were of laboratory grade (Merck Millipore). The solvents utilized for column chromatography, the crystallization of compounds, and the chemicals used to assess antimalarial properties were of analytical grade. The standard chloroquine powder was purchased from Sigma Aldrich, St. Louis, MO, USA.

Extraction and Fractionation

The powder plant material (900 g) was macerated with 8 L of 70% ethanol for 3 days with occasional shaking. The extract was then concentrated in vacuo,affording 74.55 g (8.28%) of a sticky dark green semi-solid mass called the crude ethanol extract (CEE). First, 74.55 g of CEE was partitioned successfully with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol in sequential order of increasing polarity to obtain the following yield of fractions: hexane fraction (HF; 21.78 g), chloroform fraction (CF; 9.83 g), ethyl acetate fraction (EF; 6.96 g); and *n*-butanol fraction (nBF; 17.19 g), which were subjected to further studies (23).

Source and Maintenance of Experiment Animals

Locally bred adult Swiss Albino mice of either sex (19–30 g body weight) were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. The animals were fed with standard rodent commercial feed and water ad libitum and maintained under standard laboratory conditions in a polypropylene cage at room temperature throughout the study. All experimental protocols were approved by Ahmadu Bello University Zaria, Committee on Animal Use and

Care (ABUCAUC) with an approval number ABUCAUC/2023/061 and the "principle of laboratory animal care" (NIH publication No. 85-23, 1985) guidelines and procedures were followed.

Rodent Malaria Parasite

A mouse-infected chloroquine-sensitive strain of *P. berghei* NK-65 was obtained from the National Institute of Medical Research, Lagos (NIMR). The parasites were kept alive by continuous intra-peritoneal passage in mice at the Department of Pharmacology and Therapeutics Ahmadu Bello University Zaria, Kaduna State of Nigeria.

Acute Oral Toxicity Study

The hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), and butanol fraction were evaluated for their acute oral toxicity in non-infected Swiss albino mice of 6–8 weeks old and weighing 27–32 g according to Organization for Economic Cooperation and Development (OECD) guidelines in mice (OECD Test Guideline 425, 2008) (24). The mice were fasted overnight and weighed before the test. Limit tests of 2000 and 5000 mg/kg were used. Limit test of 2000 mg/kg: Six mice were used; two mice were dosed orally with the fractions' 2000 mg/kg body weight. After administration of the fractions, food was withheld for a further 2 h period. Death was not observed in the first 24 h. Then, four more mice were given the same extract dose (2000 mg/kg).

Observation included changes in skin and fur, eyes and mucous membranes, and respiratory and behavior patterns. The mice were then observed for signs and symptoms of toxicity and mortality over 14 days. The 5000 mg/kg limit test was also employed using the same procedure stated above.

Preparation of Parasite Inoculation

A carrier mouse with about 30% parasitemia was euthanized with diethyl ether, and the blood was collected using a cardiac puncture into a heparinized vacutainer tube containing 0.5% trisodium citrate. The blood was then diluted with physiological saline (0.9%) until an inoculum containing approximately 10^7 infected erythrocytes was obtained. The inoculum was appropriately preserved (through a refrigerator) for further study.

In Vivo Antimalarial Activity

Animal Grouping and Dosing

This study used thirty mice of both sexes for the grouping. The prophylactic study used pyrimethamine (1.2 mg/kg) as the positive control. For the suppressive and curative studies, the positive (Group IV) and negative (Group V) controls were administered with distilled water (10 mL/kg) and chloroquine (5 mg/kg), respectively, through the oralroute.

Prophylactic Test

The method described by Ryley and Peters (1970) was used to assess the repository effect of the fractions (25). Five groups of six mice each were respectively administered orally with 125, 250, and 500 mg/kg of the test fractions (test groups), 1.2 mg/kg pyrimethamine (positive control), and 10 mL/kg distilled water (negative control) once daily for 4 consecutive days. On the 5th day, the mice were intraperitoneally inoculated with 0.2 mL standard inoculum containing approximately 1×10^7 *P. berghei* infected erythrocytes. After 72 h of infection, the parasitemia level was assessed by studying the slides with thin blood smears under a microscope.

Suppressive Test

To establish the suppressive activity of *G. oreophila* fractions against chloroquine-sensitive *P. berghei* infection in mice, a method previously described by Peter et al. (1975) was adopted (26). Thirty mice were grouped into five groups, each containing six. An inoculum of 0.2 mL containing approximately 1×10^7 *P. berghei*-infected erythrocytes was given to each mouse via the intraperitoneal route. 3 h post-infection, doses of the fractions at 125, 250, and 500 mg/kg were orally administered to groups 1, 2, and 3, respectively, which served as the treatment groups once daily for 4 days (0 to 3). A parallel test was conducted with 5 mg/kg standard chloroquine and 10 mL/kg distilled water as vehicles in groups 4 and 5, respectively. On day 4, thin smears were made from the tail blood. The slides were then fixed with methanol, stained with 10% Giemsa solution for 30 min, and examined in 10 fields under a microscope. Average suppression was calculated using **Equation 1**.

$$\%Suppression = \frac{A_C - A_G}{A_C} \times 100 \quad \textbf{Equation 1}$$

Where A_C is average parasitemia in control group and A_G is average parasitemia in tested group.

Curative Test (Test on Established Infection)

The schizontocidal effect in established infection was assessed using the Rane test, as described by Ryley and Peters (1970) (25). Thirty mice were inoculated with 0.2 mL standard inoculum via the i.p. route. Seventy-two hours post-infection, the mice were randomly divided into five groups of six each. Graded doses of 125, 250, and 500 mg/kg of the fraction were administered to treatment groups 1, 2, and 3, respectively. Positive and negative control groups 4 and 5 received 5 mg/kg standard chloroquine and 10 mL/kg distilled water. The fractions were given once daily for four days, and on day seven, the schizontocidal effect was evaluated through a microscopic examination of Giemsa-stained thin blood smears across 10 fields of each slide.

Statistical Analysis

Data from the antiplasmodial study were analyzed using Statistical Package for Social Sciences (SPSS), IBM version 20. The result was presented as mean ± standard error of the mean (SEM) and percentages. Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons. Values of $p<0.05$ were considered significant.

Table 1. Prophylactic activity of fractions of *Globimetula oreophila* leaf extract in repository infection in mice.

Fractions	Treatment (mg/kg)	Average Parasitaemia ± SEM	% Suppression
Distilled water	10 mL/kg	25.20 ± 0.86	-
Hexane (HF)	125	17.04 ± 0.50 *	32.38
	250	15.88 ± 0.26 *	36.98
	500	14.52 ± 0.53 *	42.38
Chloroform (CF)	125	13.48 ± 0.46 *	46.51
	250	12.84 ± 0.71 *	49.05
	500	13.12 ± 0.70 *	47.94
Ethyl acetate (EF)	125	15.08 ± 0.90 *	40.16
	250	15.32 ± 0.81 *	39.21
	500	8.22 ± 0.55 *	67.38
Butanol (BF)	125	18.15 ± 0.24 *	27.98
	250	17.34 ± 0.94 *	31.19
	500	15.88 ± 0.47 *	36.98
Pyrimethamine	1.2	6.00 ± 0.86 *	76.19

Note: values are presented as mean ± SEM; Data analyzed by one-way ANOVA followed by Dunnett's post hoc test; n = 6, (*) shows a significant difference to the distilled water group ($p<0.001$).

Results

Acute Toxicity Test of *Globimetula oreophila* Leaf Fractions

Using the oral route and the OECD technique, the acute toxicity (LD_{50}) of the *G. oreophila* leaf fractions, such as the hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), and butanol fraction (BF), were assessed. The results indicated that the LD_{50} for each fraction was more than 5000 mg/kg. Following treatment, the animals showed no changes in their food or water intake, nor did they exhibit any changes in their behavior or autonomic functions (pupillary size, lacrimation, salivation, defecation, or urination). The animals did not show any signs of poisoning or mortality.

In Vivo Antimalarial Studies

Prophylactic Activity of Fractions of *Globimetula oreophila* Leaf Extract in *Plasmodium Berghei*-Infected Mice

At all the studied doses, the administration of the hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), and butanol fraction (BF) resulted in a significant decrease in parasitemia levels. However, the positive control (pyrimethamine) produced a higher reduction in parasitemia level with 76.19% chemosuppression (**Table 1**).

Suppressive Activity of the Fractions of *G. oreophila* Leaf Extract in *Plasmodium Berghei*-Infected Mice

In comparison to the negative control, the fractions (HF, CF, EF, and BF) exhibited statistically significant ($p<0.001$) chemosuppression at the tested doses. The HE and EF of the extract at a dose of 500 mg/kg afforded 59.39% and 60.15% chemosuppression, respectively. The highest percentage of inhibition activity was seen with EF compared to HF, CF, and BF in a dose-dependent manner at $p<0.001$ (**Table 2**).

Table 2. Suppressive effect of fraction of *Globimetula oreophila* in early malarial infection in mice.

Fractions	Treatment (mg/kg)	Average Parasitaemia ± SEM	% Suppression
Distilled water	10 mL/kg	26.40 ± 0.60	-
Hexane	125	16.4 ± 0.46 *	37.89
(HF)	250	11.4 ± 0.71 *	56.82
	500	10.72 ± 0.55 *	59.39
Chloroform	125	16.12 ± 0.31 *	38.94
(CF)	250	14.12 ± 0.19 *	46.52
	500	11.60 ± 0.54 *	56.06
Ethyl acetate	125	15.12 ± 0.83 *	42.73
(EF)	250	12.88 ± 0.58 *	51.21
	500	10.52 ± 0.78 *	60.15
Butanol	125	16.42 ± 0.30 *	37.80
(BF)	250	11.74 ± 0.51 *	55.53
	500	14.76 ± 0.85 *	44.09
Chloroquine	5	6.72 ± 0.69 *	74.55

Note: values are presented as mean ± SEM; Data analyzed by one-way ANOVA followed by Dunnett's post-hoc test; n = 6, (*) shows a significant difference to the distilled water group ($p<0.001$).

Curative Activity of the Fractions of *G. oreophila* Leaf Extract in *Plasmodium Berghei*-Infected Mice

All tested doses of the fractions significantly ($p<0.001$) reduced parasitemia levels compared to the negative control. The greatest reduction was observed in the group receiving 500 mg/kg, achieving approximately 65.97% suppression. Chloroquine, used as the standard drug, achieved 83.40% suppression. At a 500 mg/kg dose, both the CF and EF fractions

demonstrated a stronger curative effect than the other extracts, although similar activity levels were observed at the highest doses tested across the groups (**Table 3**). This indicates that CF and EF may have enhanced efficacy at higher concentrations.

Table 3. Curative effect of *Globimetula oreophila* leaf extract fractions in established infection in mice.

Fractions	Treatment (mg/kg)	Average Parasitaemia ± SEM	% Suppression Effect
Distilled water	10 mL/kg	28.80 ± 1.93	-
Hexane (HF)	125	16.80 ± 0.76 *	41.67
	250	12.68 ± 0.66 *	55.97
	500	12.72 ± 0.94 *	55.83
Chloroform (CF)	125	16.38 ± 0.56 *	43.13
	250	17.12 ± 0.67 *	40.56
	500	11.08 ± 0.89 *	61.53
Ethyl acetate (EF)	125	16.0 ± 0.11 *	44.44
	250	10.80 ± 0.62 *	62.50
	500	9.80 ± 0.49 *	65.97
Butanol (BF)	125	16.48 ± 0.42 *	42.78
	250	15.72± 0.16 *	45.42
	500	11.60± 0.60 *	59.72
Chloroquine	5	4.78 ± 0.41 *	83.40

Note: values are presented as mean ± SEM; Data analyzed by one-way ANOVA followed by Dunnett's post-hoc test; n = 6, (*) shows a significant difference to the distilled water group (p<0.001).

Discussions

The LD_{50} is a metric utilized to assess the difference between a safe, effective dose and a potentially harmful or lethal dose (9). In our study, the oral LD_{50} for all fractions of G. oreophila was determined to be over 5000 mg/kg in mice, suggesting that these fractions are practically non-toxic. Additionally, we observed no signs of toxicity in the animals, including changes in skin, eyes, and mucous membranes, as well as no alterations in behavior, trembling, diarrhea, or fur loss.

In vivo models are usually employed in antimalarial studies because they consider the possibility of the prodrug effect and the probable involvement of the immune system in eradicating the parasite (27). The most accurate metric in antimalarial investigations is typically the evaluation of the percentage suppression of parasitemia (27). It has been suggested that lowering parasitemia levels is essential for an organism to recover from symptomatic malaria (28). The ability to lower the parasitemia levels of the target organism has been shown by medicinal plant extracts with high chemosuppressive action (29). Using prophylactic, suppressive, and curative tests in *P. berghei*-infected mice at graded dosages of 125, 250, and 500 mg/kg, the *in vivo* antiplasmodial potential of HF, CF, EF, and BF was assessed.

Prophylactic antimalarials act at the pre-erythrocytic stage (initial phase of the parasite) of the parasite's life cycle (30). This also shows that the substance possibly acts as tissue schizontocides. Secondary metabolites in the fractions of the *G. oreophila* plant may be acting singly or in synergy in inhibiting enzymes at the pre-erythrocytic stage, such as *P. falciparum* dihydrofolate reductase-thymidylate synthase (DHFR-TS), *P. falciparum* cysteine proteases (falcipain-2 and falcipain-3), and glutathione S-transferase (GST) (31).

In the suppressive study, except for the butanol fraction at a dose of 500 mg/kg (p< 0.001), all the fractions showed a significant dose-dependent reduction in parasitemia levels when compared to the negative control (distilled-water-treated group). This further confirms the claim's validity about using plants to manage malaria. Dauda *et al*. studied the crude ethanol extract of the plant (9). Since secondary metabolites have been known to have antimalarial activity, it is possible that the presence of phytochemicals that are present in each of the fractions, such as carbohydrates, steroids, triterpenes, glycosides, saponins, tannins, flavonoids, and alkaloids, may have brought about the antimalarial activity observed (9). Thus, the antimalarial activity of *G. oreophila* fractions in *P. berghei*-infected mice might be due to the presence of one or a combination of these phytochemical constituents acting singly or in synergy in inhibiting enzymes such as heme oxygenase-1 (HO-1), serine repeat antigen (SERA), calcium-dependent protein kinase (CDPK), dihydrofolate reductase (DHFR), and aspartic and cysteine proteases, which have been studied for their potential to act on suppressive *in vivo* models of malaria (32, 33).

In the curative study, all the fractions exhibited a statistically significant inhibition in a dose-dependent manner, except for hexane and chloroform fractions, in which a non-does dependent parasitemia chemosuppression (p<0.001 in all cases) was seen when compared with the negative control (distilled-water-treated group), probably due to non-selectivity of the fractions to the proliferation process of the parasite. The observed inhibition of plasmodial growth could be attributed to the fraction's ability to inhibit malaria protease enzymes required for parasite survival. These enzymes, such as aspartic protease, dihydrofolate reductase (DHFR), cysteine protease enzymes, and proteasome, which are potential targets for antimalarial drug discovery, act on the erythrocytic stage of the parasite life cycle (31). Suppressive and

curative antimalarials act at the erythrocytic stage (the parasite's life cycle that causes the clinical symptoms), of which chloroquine is a potent suppressive and curative antiplasmodial agent (34). The mechanism of action of chloroquine is through the formation of a heme-chloroquine complex that caps hemozoin molecules and prevents further bio-crystallization of toxic heme produced within the parasite's digestive vacuole (35). Although the mechanism of action of antimalarial activities of flavonoids has not been fully established, inhibition of the fatty acid biosynthesis (FAS II) of the parasites and inhibition of the influx of L-glutamine and myoinositol into the infected erythrocytes are some proposed mechanisms (36).

The acidic character of flavonoids (due to phenolic -OH groups) prevents them from entering the acidic food vacuole of parasites, unlike basic quinoline-based antimalarials (37, 38). As a result, flavonoid-based compounds do not interfere with degrading hemoglobin, which is the only site of action for most current medications against which malaria parasites have developed resistance (37, 38). Hence, the exhibited chemosuppressive and chemo-prophylactic effects may be due to these secondary metabolites in the plant, which have been reported to have antimalarial activity (39, 40). When an extract exhibits a percentage chemosuppression equal to or more than 50% at dosages of 250, 500, and 1000 mg/kg body weight per day, *in vivo* antiplasmodial activity can be categorized as moderate, good, and very good (41, 42). According to this classification, at doses of 250 and 500 mg/kg, the hexane, chloroform, ethyl acetate, and n-butanol fractions displayed a good suppressive and curative effect.

Conclusion

In this study, the ethyl acetate fraction had the highest activity in all three models compared to other fractions against *P. berghei*-infected mice. The order of increasing antimalarial activity is n-butanol<n-hexane<chloroform<ethyl acetate fraction. The fractions of *G. oreophila* showed significant *in vivo* antiplasmodial activity, which is consistent with the earlier *in vivo* findings of the crude extract, as well as its traditional use, which further supports the in silico analysis of the prenylated quercetin as an inhibitor for *P. falciparum* enzymes. Further study would be carried out to isolate active secondary metabolites responsible for this observed antimalarial activity in all four investigated fractions and subject the isolated compounds to in silico analysis against *P. falciparum* proteases using computational studies.

Abbreviations

OECD = Organization for Economic Cooperation and Development; ED_{50} = effective dose; LD_{50} = Lethal Dose 50; ACTs = Artemisinin-based combination therapies.

Declarations

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Conflict of Interest
The authors declare no conflicting interest.

Data Availability
The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [ethical concerns about plagiarism].

Ethics Statement
This study was conducted by the Declaration of Helsinki and approved by Ahmadu Bello University Zaria, Committee on Animal Use and Care (ABUCAUC) with an approval number: ABUCAUC/2023/061.

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