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Research Article

Antiplasmodial activity and acute oral toxicity of *Rauvolfia vomitoria* leaves extracts

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Abstract

Background: Malaria is one of the most important diseases in Republic of Congo with 47.9% of outpatients visit for 64.8% admissions. The rise of antimalarial resistant strains requires the active search of new active compounds and medicinal plants can be an alternative. From an ethnobotanical survey a list of plants with antimalarial reputations was drawn up, including *Rauvolfia vomitoria*.
Aim: The objective of this study is to see if we can validate the supposed activity of *Rauvolfia vomitoria* and its toxicity.
Material and Methods: *Rauvolfia vomitoria*'s leaves have been collected, dried and sprayed. Leaves powder were macerated in distilled water for 24 hours. Another part of leaves powder were macerated for 24 hours successively in hexane, dichloromethane, mix of diclhoromethane: methanol (v:v) and methanol. The crude extracts were prepared and tested for antiplasmodial activity on NF54 strains and field isolates with the SYBR Green I-based in vitro assay technique. The extracts with the best antiplasmodial activities were used on rats for acute toxicity.
Results: All *Rauvolfia vomitoria*'s leaves extracts have shown a very good antiplasmodial activity ($0.63 \leq CI_{50} \leq 20.19$µg/ml) and no toxicity up to 2000mg/kg.
Conclusion: *Rauvolfia vomitoria*'s leaves have an antiplasmodial activity. This study confirms the use of the plant by the traditional healers. We will pursue the work to find the active compounds of the plant.
Keywords: Antiplasmodial activity; Sybr Green assay; acute oral toxicity; field isolates; reference strain.

1. Introduction

Since centuries, plants have always been used as medicines in developing countries in general and particularly in Sub-Saharan Africa [1]. The Republic of Congo is not making exception on this ancestral habit. However, this massive use has not often a scientific basis. Indeed, until nowadays, there are few data available to inform the Congolese population about the efficacy and/or toxicity of the plants used.

Due to its geographical position, the Republic of Congo is endowed with rich and diverse flora but is also prone to several diseases, including malaria. Malaria, in

particular, is one of the most dreadful diseases in the country. The disease alone accounts for 47.9% of outpatients visit and mainly affects children under five and pregnant women [2,3].

Although World Health Organization (WHO) has made considerable efforts in the control of the disease with a decrease of cases between 2000 and 2015 [4], access to antimalarials is not always obvious. Those issues lead the populations to rely on illegal drugs sold on streets or traditional healing with plants.

To date, there is not a vaccine to solve this problem yet as it is still on a clinical trial [5,6]. To this is added the progressive installation of parasite resistance against the artemisinin-based combination therapies (ACTs) placed on market like recently reported in South-East Asia [7-9].

If we think about the fact that Quinine comes from a plant, the *Cinchona*, the alternative of medicinal plants is still relevant. Studies have itemized some Congolese plants with antimalarial reputations [10,11]. From this, with another ethnobotanical survey of 26 health practitioners, a wider list of 30 plants was drawn up, including *Rauvolfia vomitoria* (**unpublished data**).

Thus, this study aimed to evaluate the antiplasmodial activity of *Rauvolfia vomitoriain vitro* and its toxicity *in vivo* in mice.

2. Material and Methods

2.1 Plant collection and preparation of extracts

Rauvolfia vomitoria Afzel. (Apocynaceae) is a tree up to 8 m tall native to tropical Africa. The leaves grow in whorls of three and are elliptic and pointed at the end, 5-12 cm long and 3-6 cm wide. Flowers are tiny, sweet-scented, pale greenish-white and somewhat hairy inside. The orange fruits are shaped like small balls, each containing a single seed [12]. Certain species of *Rauvolfia* are a source of medicinal alkaloids, especially reserpine. The generic name Rauvolfia is named after a 16th century German physician, Leonhart Rauvolf, who travelled the world over collecting and documenting medicinal plants [13].

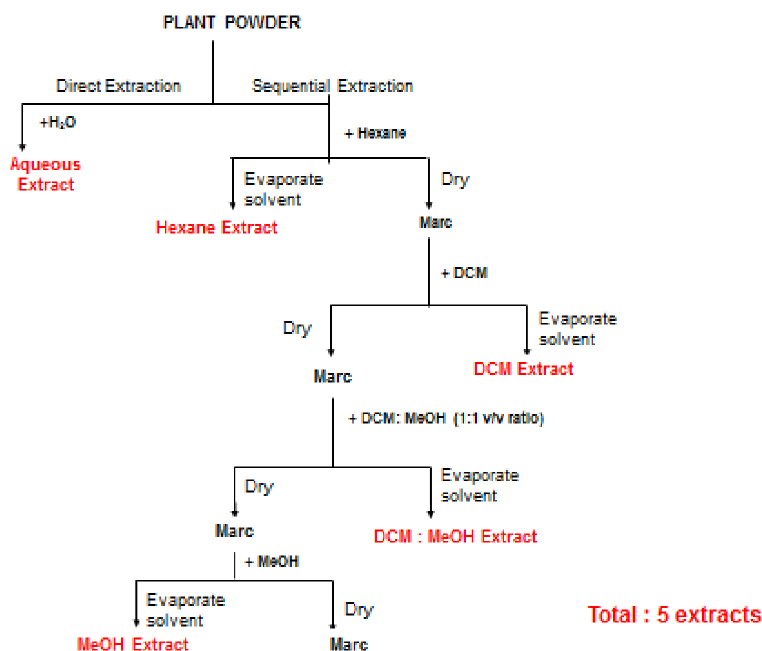
Rauvolfia vomitoria's leaves have been collected in Pool area, south of Brazzaville, Republic of Congo in November 2015. A sample was taken to the National Herbarium for recognition and confirmation by a taxonomist, Professor Moutsabote.

The leaves were dried under the shade at room temperature then sprayed with a blender. 50 g of leaves powdered were macerated in 500 ml of distilled water for 24 hours at room temperature. Another 50 g were macerated at room temperature for 24 hours successively in 500 ml of hexane, dichloromethane (DCM), mix of DCM: MeOH (v:v) and methanol (MeOH) as shown in figure 1.

Then, the mixtures were filtered with a Whatmann filter paper. The filtrate was concentrated and dried under reduced pressure in a BuchiRotavapor R-114 (From Buchi ® Switzerland). The resulting crude extracts were kept at 4 - 8°C until use.

The initial stocks solutions of 1mg/ml of the crude extracts were prepared by dissolution in dimethyl sulfoxide (DMSO) for organic extracts and in deionized water for aqueous extract. Then, there were filtered (millipores filter 0.45µm) and diluted in RPMI 1640 ("Roswell Park Memorial Institute" 1640) culture medium to achieve a final stock solution of 250µg/ml.

Figure 1: Protocol of *Rauvolfia vomitoria* extraction



2.2 Samples collection, cultivation of malaria parasites and antiplasmodial activity assay

All the experiments were carried out under sterile conditions. *In vitro* activity tests on chloroquine-sensitive strain (NF54) strains as well as on field isolates were carried out according to the Rieckmann microtest recommended by WHO [14].

2.2 Field isolates

Parasited blood was collected from patients at the health facilities selected for the study (Formation sanitaire urbaine à base communautaire; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [FSU-COM] of Yopougon Toit Rouge and EL RAPHA of Abobo, Abidjan Côte d'Ivoire) and showing clinical signs of uncomplicated malaria as describe by WHO [15].

After an explanation of the study and obtaining written informed consent and/or assent, a thick and thin blood films were made, stained with a 10% GIEMSA solution and examined under a microscope to assess parasite density which should be at least of 0.025 Patients were treated with amodiaquin eartesunate or artemether-lumefantrine according to the recommended national therapeutic regimens.

Thus, for the positive samples, about 4 ml of blood was collected in a tube with Ethylenediaminetetraacetic acid (EDTA, From BD Vacutainer®). For each sample, demographic, clinical and biological (parasitemia) data were recorded.

Collected samples were transported at 4°C to Center of Diagnosis and Research on acquired immunodeficiency syndrome (AIDS) and other infectious disease, Abidjan, within 6 h.

Parasitized erythrocytes were washed three times in RPMI 1640 medium (Invitrogen, UK). Giemsa stained thin blood smears were examined under the microscope to determine the parasite density and confirm *Plasmodium* species (*P. falciparum* monoinfections). Samples with parasitemia ranging from 0.1% to 0.25% were used directly to test drug susceptibility. If parasitemia exceeded 0.25%, infected erythrocytes were diluted to this parasitemia range with uninfected erythrocytes.

Washed infected red blood cells were used for antiplasmodial testing by adding a 2% hematocrit suspension of O+ human erythrocytes in complete medium constituted by RPMI 1640 with L-Glutamin and 25mM HEPES (GIBCO™) supplemented with 25mg Gentamicin, NaHCO3 and 20% Albumax (GIBCO™).

From the final stocks solutions of 250µg/ml of crude extracts, dilutions from 25 to 0.78µg/mlwere made while distributing in the plates.

Chloroquine dihydrophosphate (CQ) from SIGMA®, used as control, was dissolved in deionized water and dilutions were made from 200 to 6.25 nM.

In each well of plates, 50 µl of the concentration ranges of drugs to be tested as well as 200 µl of the mixture complete medium + infected red blood cells were distributed in duplicate. The plates were covered with a sterile lid; cultures were fed with 5% CO_2 and incubated at 37◦C during 72 hours.

2.4 Reference strain

The CQ-sensitive strain NF54 was donated by the Centre Suisse, Abidjan. After thawing, parasites were synchronized at the ring stage by treatment with 5% D-sorbitol.

Parasites were grown and maintained in culture using themethod of Trager and Jensen with some modifications [16,17]. The parasites were cultured in a complete medium constituted by RPMI 1640 medium with L-glutamine and 25mM HEPES buffer supplemented with 25mg Gentamycin, 5% $NaHCO_3$, 10 mg hypoxanthine, 10 ml sterile human serum and 2.5 mg Albumax II. Cultures were fed with 5% CO_2 and incubated at 37◦C. Every day, infected red blood cells were transferred into fresh complete medium to propagate the culture. The haematocrit was kept at 1.5-2%.

For antiplasmodial testing, in each well of plates, 50 µl of the concentration ranges of drugs to be tested as well as 200 µl of the mixture complete medium with infected red blood cells were distributed in duplicate. The plates were covered with a sterile lid; cultures were incubated at 37◦C with 5% CO_2 during 72 hours. This assay was executed twice.

For both field isolates and reference strains, a blood smear was made from the control wells at the end of the incubation to validate the tests according to the growth of the parasites. The plates were then stored at -20 °C until the day of measurement of the antiplasmodial activity of the extracts by Sybr Green assay.

2.5 Sybr Green assay (parasite viability)

The day of analysis, plates were removed from the -20°C. Parasite viability/activity was measured by using the SYBR® GREEN I nucleic acid gel stain (Life Technologies™) and the spectra Max Gemini-XPS GEMINI® XPS-05153 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The assay was processed as described in (WorldWide Antimalarial Resistance Network network) WWARN protocol [18].

2.6 Acute oral toxicity

Toxicity assay on rats was carried out according to Organization for Economic Co-operation and Development (OECD) Protocol 420: Determination of Acute Oral Toxicity - Predetermined Dose Method [19].

The assay was carried out on Albino Wistar rats with the most antiplasmodial active extracts of *R. vomitoria*.

For each active extract tested, there were 5 groups of 5 rats (females nulliparous and non-gravid, 8-12 weeks, 140-200 g) administered with doses of 5, 50, 300, 1000 and 2000mg / kg. The 6th group served as control. Each animal was weighed and marked for an individual identification.

The rats came from the laboratory of pharmacology, UFR Sciences Pharmaceutiques and Biologiques, Felix Houphouet-Boigny University, Abidjan (Côte d'Ivoire). The assay was performed in the same lab.

Animal's acclimatization was spread over a week with a standard meal consisting of granules with water. We observed a 12-hours cycle of light / darkness at room temperature. The animals were fasted 24 hours before starting the test.

Each animal individual weight was determined shortly before the administration and then at least once a week. Weight changes were recorded.

The animals were observed individually during the first 30 minutes, 4 hours after treatment and daily for 14 days. Particular attention has been made to the observation of various manifestations such as breathing, restlessness, convulsions, salivation, diarrhea, lethargy, sleep, skin's changes, eyes, mucous membranes and coma.

At the end of the assay (day 15), rats were sacrificed after being placed under a jar with cotton soaked in ether. Then, we carried out a macroscopically observation.

2.7 Phytochemical Screening

Phytochemical screening of the different extracts was carried out to detect sterols, polyterpens, polyphenols, flavonoids, tannins, quinones, alkaloids and saponins by using standard procedures.

2.7.1 Sterols and polyterpens

5 ml of each solution was evaporated to dryness, without charring the residue, in a capsule in a water bath. The residue was dissolved in 1 ml of hot anhydrous acetic acid. The solution was then poured into a test tube. 0.5 ml concentrated sulfuric acid was added carefully along the tube wall.

The appearance, at the interphase, of a purple ring, turning blue then green, indicates a positive reaction.

2.7.2 Polyphenols

To 2 ml of each solution we added a drop of 2% ferric chloride alcohol solution. In the presence of polyphenolic derivatives, ferric chloride causes the appearance of a blackish-blue or green color.

2.7.3 Flavonoids

5 ml of each solution were evaporated to dryness in a capsule and then cooled. The residue was taken up in 5 ml of hydrochloric alcohol half. The solution was then poured into a test tube. We then added 2 to 3 magnesium chips (paying attention to the heat release). A pink-orange or purplish color appears. The addition of 3 drops of isoamyl alcohol which intensifies this coloration and confirms the presence of flavonoids.

2.7.4 Tannins

2.7.4.1 Catechic tannins with STIASNY reagent (30% formalin, 1 / 0.5 concentrated HCL)

5 ml of each solution was evaporated to dryness in a capsule and then 15 ml of STIASNY reagent was added to the residue. The mixture is kept in a water bath at 80 °C for 30 minutes. After this time, the mixture is set aside until cool. The observation of precipitation in large flakes characterizes catechic tannins.

2.7.4.2 Gallic tannins

We filtered the previous solution. The filtrate is collected and saturated with sodium acetate. The addition of 3 drops of 2% $FeCl_3$ causes the appearance of an intense blue-black color denoting the presence of gallic tannins.

2.7.5 Quinonic substances (with the Borntraegen reagent)

2 ml of each solution were evaporated to dryness in a capsule. The residue was triturated in 5 ml of hydrochloric acid (1:5). In a test tube, the solution was heated for half an hour in a boiling water bath. After cooling, the hydrolysate was extracted with 20 ml of chloroform in a test tube. Then, we collected the chloroform phase in another test tube and added 0.5 ml of ammonia diluted to half (½). The appearance of a color ranging from red to purple indicates the presence of quinones.

2.7.6 Alkaloids (with Dragendorf and Bouchardat reagents)

4ml of each solution was evaporated to dryness in a capsule. The residue was collected with 4 ml of alcohol60°. These 4 ml were then divided into 2 different test tubes of 2 ml.

- In the first test tube, we added 2 drops of Dragendorf reagent (potassium iodobismutate reagent). The appearance of a precipitate or an orange color indicates the presence of alkaloids.
- In the second test tube, we added 2 drops of Bouchardat reagent (iodine-iodide reagent). The appearance of a reddish-brown precipitate indicates a positive reaction.

2.7.7 Saponins (foam index)

0.1 g of aqueous extract is dissolved in a test tube containing 10 ml of distilled water. The tube is shaken vigorously for 30-45 seconds lengthwise and then left standing for 15 minutes. The height of the foam is measured. The persistence of foam more than 1 cm high indicates the presence of saponins.

2.8 Ethical consideration

The study protocol was first approved by the National Ethical Committee of Côte d'Ivoire. This approval was critical for the study start. For each patient who met inclusion criteria, the protocol was read and explained to him/her or the legal guardian (for children) who in case of acceptance had to sign the written informed sheet afterward to authorize recruitment of the child in the study. The patient or guardian was given a copy of the informed consent and patient information sheet.

2.9 Statistical analysis

Data were analyzed by applying Student's t-test and One-way ANOVA followed by Dunnett's with SPSS version 17 and XLSTAT version 6. Results were considered significant when p <0.05 and highly significant when p <0.01.

For the antiplasmodial testing results, the Inhibitory Concentrations (IC_{50}) were calculated from a dose response curve by non-linear regression analysis using the IC estimator program version1.2 [20,21]then IC50 values were expressed in Minimum (MINI), Maximum (MAXI) and Median (MED). The acute toxicity values were expressed as mean ± SD.

3. Results and Discussion

Through this study, we evaluate the *in vitro* antiplasmodial activity of *Rauvolfia vomitoria* leaves and its acute toxicity on rats.

In Republic of Congo, *Rauvolfia vomitoria* leaves are used in decoction in case of malaria. They are also used against fever, flu, gonorrhea.

We included 26 patients with positive parasitemia. Out of these 26 samples collected, 19 samples were kept. The 6 others were discarded because of the aberrations of their IC_{50} values.

As shown in table 1, among these field isolates (n=19), 3 (15.8%) were resistant to Chloroquine (CQ) and 16 (84.21%) were sensitive to Chloroquine. All the extracts have shown a good antiplasmodial activity.

Even if these results highlight the presence of strains still sensitive to chloroquine, it would be wise to have a larger sample before making any conclusion. In 2001, a previous study in Abidjan obtained on 34 field isolates a prevalence of 32.5% for resistant isolates and 67.5% for chloroquine-sensitive isolates [22].

The table 2 represents the antiplasmodial activity of the extracts on the reference chloroquine-sensitive strain NF54. The table 3 presents all the chemical coumpounds revealed in each extract.

The fact that the aqueous extract is very active as well on field isolates as on reference strain confirms the choice of traditional healers and urges us to carry out further work on this extract. The used of the plant is widely notified [23-26] but there are few studies relying on her antiplasmodial activity.

A previous study has been realised with ethanolic extracts of *Rauvolfia vomitoria's*sterm bark on chloroquino-resistant strain FcB1. The IC50 was 2.5 µg/ml [27].

Another study on Albino Wistar rats suggested that the ethanolic root extract of *Rauwolfia vomitoria* was more teratogenic than the leaf extract and had potential to cause cerebral tissue damage [28].

Table 1: Inhibitory Concentrations 50 of extracts (µg/ml) and Chloroquine (nM) on field isolates

Isolates	Extracts	Aqueous	Hexane	DCM	DCM: MeOH	MeOH	CQ
CQ Sensitive isolates	**Isolate 1**	0.77	1.43	0.78	5.74	1.56	22.26
	Isolate 4	2.87	6.77	5.28	2.06	3.36	12.245
	Isolate 6	5.86	8.99	6.79	6	5.94	41.8
	Isolate 7	2.91	5.12	0.78	1.73	11.17	51.53
	Isolate 8	3.77	6.16	4.71	0.87	0.92	3.02
	Isolate 9	1.15	10.93	11.77	3.12	11.95	12.43
	Isolate 10	0.76	20.79	17.98	0.76	6.22	12.05
	Isolate 11	18.61	0.72	1.09	2.33	0.63	23.19
	Isolate 12	0.64	1.22	1.72	0.74	0.69	8.3
	Isolate 14	0.67	1.46	2.9	0.77	0.66	15.905
	Isolate 16	2.47	6.24	5.58	2.87	2.26	6.21
	Isolate 17	1.07	2.99	2.63	2.89	3.24	11.57
	Isolate 21	15.11	1.52	5.05	0.74	2.27	6.76
	Isolate 22	7.57	8.17	13.9	5.15	6.04	18.325
	Isolate 23	0.77	12.2	2.49	1.98	2.57	8.95
	Isolate 24	0.66	0.66	0.73	0.69	3.01	22.46
CQ Resistant isolates	**Isolate 5**	0.83	7.61	8.9	6.17	6.21	113.78
	Isolate 15	1.25	0.71	9.48	12.09	2.15	122.29
	Isolate 26	0.71	6.19	2.44	3.1	5.73	103.03
	MINI IC50	**0.64**	**0.66**	**0.73**	**0.69**	**0.63**	**3.02**
	MAXI IC50	**18.61**	**20.79**	**17.98**	**12.09**	**11.95**	**122.29**
	MED IC50	**1.15**	**6.16**	**4.71**	**2.33**	**3.01**	**15.905**

Table 2: Inhibitory Concentrations 50 of extracts (µg/ml) and Chloroquine (nM) on NF54 strain.

Strain	Aqueous	Hexane	DCM	DCM: MeOH	MeOH	CQ
NF54	7.81	12.18	12.27	2.35	4.92	21.27
	4.08	2.56	6.21	1.54	0.77	10.52
MINI IC50	**4.08**	**2.56**	**6.21**	**1.54**	**0.77**	**10.52**
MAXI IC50	**7.81**	**12.18**	**12.27**	**2.35**	**4.92**	**21.27**
MED IC50	**5.945**	**7.37**	**9.24**	**1.945**	**2.845**	**15.895**

This is the first time where the aqueous extract of *Rauvolfia vomitoria* leaves is tested as well on field isolates as on reference strain. And also the first time where the antiplasmodial activities of this extract is demonstrated.

The difference on results between field isolates and reference strain on the one hand and between the extracts on the other hand can be explained. Indeed, there are different genetic fingerprints between the strains and the interaction with each organic extract can influence the results due to the specificity of each solvent used to extract specific chemical families.

Regarding the acute oral toxicity, no death was observed up to the dose of 2 g/kg body weight, indicating that the medium lethal dose (LD_{50}) could be greater than 2 g/kg body weight in rats. At the end of the assay, significant changes in the weight were observed in the 300mg/kg body weight aqueous group, 5mg/kg body weight MeOH group,5mg/kg body weight and 300mg/kg body weight DCM: MeOH group (Table 4). The macroscopic observation of organs didn't show a significant difference in comparison with the control group.

A subacute toxicity study will be necessary. However, all these results confirm the fact that *Rauvolfia vomitoria* is administrated to people without any toxicity as said the traditional healers.

Table 3: Phytochemical compounds of *Rauvolfia vomitoria* leave extracts

	Sterols Polyterpens	Polyphenols	Flavonoids	Tannins		Quinones	Alkaloids		Saponins
				Gallic	Catechic		D	B	
Aqueous	-	+	+	-	-	-	+	+	+
Hexane	+ +	-	-	-	-	-	-	+	
DCM	+ +	-	-	-	-	-	+	+	
DCM: MeOH	+	+ +	-	-	-	-	+ + +	+ + +	
MeOH	+	+ + +	+	-	+ +	-	+ +	+ +	

- : Absence+: Presence D: Dragendorff Reagent

Table 4: Body weight gain of female rat treated orally with *Rauvolfia vomitoria* leaves extracts

	Before treatment				After treatment			
	control	Aqueous	DCM :MeOH	MeOH	control	Aqueous	DCM :MeOH	MeOH
5mg/kg		153.80±10.26	153.60±8.14	153.20±6.98		167.60±12.18 ^{ns}	**169.20±9.04** $^{\neq}$	**166.60±10.45** $^{\neq}$
50mg/kg		153.60±8.74	158.80±10.96	153.80±13.76		160.40±8.44 ^{ns}	163.80±10.96 ^{ns}	160.40±13.94 ^{ns}
300mg/kg	156.20±17.68	154.00±10.37	153.80±9.04	158.00±20.14	156.80±18.45	**174.80±16.16** $^{\neq}$	**169.00±11.34** $^{\neq}$	167.80±15.90 ^{ns}
1000mg/kg		155.00±6.04	153.60±6.15	153.60±8.68		156.60±7.34 ^{ns}	157.40±6.88 ^{ns}	158.81±8.60 ^{ns}
2000mg/kg		153.80±5.12	153.60±8.68	154.00±9.08		156.205±8.30 ^{ns}	157.00±12.75 ^{ns}	158.20±5.17 ^{ns}

Values are expressed as mean ±SD (n=5) ns: non significative difference compared to control ≠: $p < 0.05$ compared before the treatment

4. Conclusion

All the extracts of *Rauvolfia vomitoria* used have shown a good antiplasmodial activity and no toxicity up to 2mg/kg of body weight in rats. This is the first time where the aqueous extract of the plant is demonstrated.

Due to the number of times this plant has been cited by traditional health practitioners and the scarcity of scientific data on its antimalarial activity, it was necessary to validate or not this supposed activity.

The fact that among the antimalarials used during these years there are active compounds derived from plants represent a real hope for the search for an effective remedy against malaria. It is true that the parasite can easily develop resistances as has been the case for chloroquine and even derivatives of artemisinin. To face it, it may be necessary to see the possibility instead of isolating a single active principle to use molecules regrouping a pool of active principles, which could slow down the installation of these resistances.

Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced the outcome.

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