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IN VITRO EVALUATION OF ANTIPLASMODIAL ACTIVITY OF EXTRACTS OF *ACANTHOSPERMUM HISPIDUM* DC (ASTERACEAE) AND *FICUS THONNINGII* BLUME (MORACEAE), TWO PLANTS USED IN TRADITIONAL MEDICINE IN THE REPUBLIC OF CONGO.

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Abstract

The aim of this study was to evaluate extracts from two medicinal plants, *Acanthospermum hispidum* and *Ficus thonningii*, used in traditional medicine in Congo Brazzaville, for *in vitro* antiplasmodial activities against two laboratory strains of *Plasmodium falciparum:* the chloroquine sensitive 3D7 and the chloroquine resistant Dd2. ELISA HRP2 assay was used to evaluate the *in vitro* inhibitory activity of the extracts alone or in combination with chloroquine. Cytotoxicity was assessed on human HeLa cell line and reflected by the selectivity index. Methanolic extract of *Acanthospermum hispidum* exhibited a strong and a moderate inhibitory activity on the growth of Dd2 and 3D7 at 2.8 µg/ml and 9.2 µg/ml concentrations respectively with a selectivity index >10. The combination of the most active extract (methanolic extract of *Acanthospermum hispidum*) with chloroquine showed a synergistic interaction on both strains. The good selectivity index of *Acanthospermum hispidum* on HeLa cells reflects the safety of this plant. Extracts from *Ficus thonningii* did not show any promising antiplasmodial activity on both 3D7 and Dd2. Except the methanolic extract which exhibited a slight antiplasmodial activity with inhibitory concentration and selectivity index corresponding to 9.61 µg/ml and 11.16 respectively. Methanolic extract of *Acanthospermum hispidum* exhibited moderate to high inhibitory activity on 3D7 and Dd2 laboratory strains and a synergistic antimalarial effect when combined with chloroquine. *Ficus thonningii* seems to have no antimalarial activity. Phytochemical analysis, *in vivo* investigations using animal models and later clinical trials in collaboration with traditional practitioners are necessary to clarify the potential antimalarial activity of both plants.

Key Words: Medicinal plants, malaria, *in vitro* activity, *P. falciparum*, cytotoxicity

Abbreviations: ACT – Artemisinin based-combination therapy; DMSO – Dimethyl sulphoxide; HRP2 – Histidin rich protein-2; ELISA – Enzyme-linked immunosorbent assay; nm – Nanometer; OD – Optical density; IC_{50} – 50% inhibitory concentration; FBS – Fetal bovine serum; SI - Selectivity index

Introduction

The development of new tools like artemisinin-based combination therapy (ACT) for the treatment of uncomplicated malaria, the long lasting insecticide treated nets, the intermittent preventive treatment for pregnant women and children have led to reduction of the burden of malaria in some endemic countries (Mmbando et al., 2010; WHO, 2008). Elimination and eradication of malaria have been set as new goals that may be achieved if new tools are elaborated and widely deployed, including efficacious malaria vaccines (Snow et al., 2005; Leading, 2010).

Despite the aforementioned efforts, malaria remains the number one killer disease of public health importance in many sub-Saharan countries as it is still responsible for 800,000 death cases per year with 90% occurring in sub-Saharan Africa (WHO, 2008). In the Republic of Congo, there is no recent data on the burden of malaria. Nonetheless, it is conceived to be the main cause of hospital admission (49 to 51%) and represents about 35% of children death in hospitals in Brazzaville and Pointe-Noire, the two main cities (National Malaria Control Program, 2007: unpublished data). Interventions that have been implemented by the National Malaria Control Programme may have had some impact on malaria transmission, which was reported to be perennial and high in Brazzaville (Trape and

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Zoulani, 1987). Congo Brazzaville adopted a policy of first line treatment of malaria with ACTs in 2006. Unfortunately, the cost of ACT treatment is not affordable by all the population. Therefore for economic, social and cultural reasons, the use of herbal medicines to treat malaria has increased over the past few years; particularly by people living in rural areas.

Most traditional healers use medicinal plants through knowledge acquired by inheritance. The development of traditional medicine in various countries including Congo-Brazzaville has evolved without clear guidelines or policies on evaluation of efficacy/effectiveness and subsequent usage. However, it is acknowledged that the most well known antimalarials including quinine and artemisinin are derived from plants. Therefore, it is not surprising that screening of plant extracts is currently one of the first steps in drug discovery for the identification of potential new therapeutics.

In the present study, two plants *Acanthospermum hispidum* and *Ficus thonningii* were selected for screening based on the results of a pilot study conducted in Owando, a city in the Northern region of Congo, in collaboration with local traditional healers. Traditionally, *Acanthospermum hispidum* has been used for the treatment of uncomplicated malaria and other infections like sterility, vaginal and parasitic infections. This plant is known for its anti-inflammatory, anti-oxydant and hepato-protective properties (Kpemissi et al., 2008). Also, the plant has shown inhibitory effects on *Plasmodium falciparum* W2 strain *in vitro* (Bero et al., 2009; Sanon et al., 2003$_{a,b}$). *Ficus thonningii* is widely used by herbalists and it has been reported to possess analgesic and anti-inflammatory properties (Otimenyin et al., 2004). *In vivo* animal studies have shown that ethanolic extract of *Ficus thonningii* leaves possesses positive effects on cardiovascular and kidney functions (Musabayane et al., 2007).

Since these two plants are believed to treat uncomplicated malaria in Congolese children and adults, we decided to: (1) evaluate the antiplasmodial activity of different extracts of both plants *Acanthospermum hispidum* and *Ficus thonningii* on the *in vitro* growth of laboratory established chloroquine-sensitive 3D7 and chloroquine-resistant Dd2 strains of *Plasmodium falciparum*; (2) assess the *in vitro* combination effects of potential active extracts and chloroquine on both 3D7 and Dd2; (3) evaluate the cytotoxic activity of the extracts in order to determine their influence on human cell viability using cell culture systems that provide good models for assessing cytotoxicity of various compounds.

Materials and Methods

Selection of Plants

The use of two plant species in this study was based on the ethnobotanical survey conducted in the city of Owando in close collaboration with traditional healers and on literature reporting plant distribution throughout the country. The ethnobotanical survey was conducted during the second week of January 2009 using a well-structured questionnaire to collect information including malaria diagnosis, type of plants used, type of symptoms treated and how herbal medicine was prepared and administered. A total of eight herbalists were interviewed and they mention nine plant species. Of these, four (*Acanthospermum hispidum*, *Annona senegalensis*, *Ficus thonningii* and *Rauvolfia obscura*) were repeatedly strongly and recommended by almost all herbalists. In the present study, *Acanthospermum hispidum* and *Ficus thonningii* were selected because information on their antiplasmodial activity was limited and these plants are widely distributed in Congo.

Collection of Plant Materials

Fresh leave samples of *Acanthospermum hispidum* and *Ficus thonningii* were collected in the Southern area of Brazzaville (Mfilou) between March and May 2010. The samples were authenticated at the Centre d'Etudes sur les Resources Végétales (CERVE) of Brazzaville, Republic of Congo, by comparison with the reference specimens of the Herbarium: voucher numbers "Makany 172" and "Liben 1256" for *Acanthospermum hispidum* and *Ficus thonningii* respectively.

For treating patient, traditional healers collected plant leaves that were prepared as follows: about 246 g of fresh leaves (corresponding approximately to 48 g of dried leaves) were boiled with 1.5 l of water for 1 hour. And a volume of 250 ml of decoction for adult or about 125 ml for children is administrated orally two to three times per day, usually for a period of one week. Upon request, the traditional healers confirmed that for study purpose, leaves could be used later after collection, air-dried in the shade and well conserved in a box protected against the sun light. For this reason, air-dried leaves were used in the present study. They were finely powdered before extraction with appropriate solvents.

Preparation of aqueous extract

The powder (50g) was suspended in distilled water (500ml) maintained at 100°C for 1 hour. Then the mixture

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was filtered and the filtrate product concentrated and dried under reduced pressure in a rotary evaporator. The resulting crude extract was kept in a – 20°C freezer utile use.

Preparation of methanolic and ethanolic extracts

The powder (50g) was each suspended in absolute ethanol (500ml) and methanol (500ml) and macerated for 24 hours with shaking at room temperature, after which, the suspensions were filtered. Methanol or ethanol was removed using a rotary vacuum evaporator and the dried extract kept in the freezer at -20°C until use.

***In vitro P. falciparum* culture**

Two different *Plasmodium falciparum* strains were used: chloroquine-sensitive 3D7 and chloroquine-resistant Dd2. Parasites were maintained in continuous culture as described by Trager and Jensen (1976), but without addition of serum. Briefly, *Plasmodium falciparum* parasites were maintained in continuous culture on human erythrocytes (blood group 0^+), provided by the Blood Bank of Tübingen (Germany), in RPMI 1640 medium (Sigma), supplemented with L-Glutamin (200µM), Gentamycin (50µg/ml), Hepes and Albumax. The medium was changed every 24 hours and parasitemia maintained between 1 and 3%, with 5% hematocrit. Incubation conditions were as follows: 37°C, 5%CO_2, 5%O_2 and 90%N_2. Parasites were synchronized (using 5% D-Sorbitol) before being used for assays.

Growth inhibition assays

Assays were performed in triplicates using serial dilutions of crude extracts with the culture medium in 96-well microplates. Stock solutions of extracts were prepared in dimethyl sulpfoxide (DMSO) or deionised water at 10 mg/ml, and further diluted in medium to have 1 mg/ml working solutions. At the initial concentration of 200 µg/ml, extracts were tested in triplicate in eight serial twofold dilutions. Chloroquine disphosphate (AppliChem GmbH, Germany) was used as standard antiplasmodial product with an initial concentration of 100 ng/ml. The sensitivity of laboratory established *Plasmodium falciparum* strains to *Acanthospermum hispidum* and *Ficus thonningii* extracts was measured using histidin rich protein-2 (HRP2) double site sandwich enzyme-linked immunosorbent assay (ELISA) as described by Noedl et al. (2002). Briefly, *Plasmodium falciparum* cultures (0.05% parasitemia and 1.5% hematocrit) were incubated for 72h in the presence of different concentrations of plant extracts and the control drug. After incubation, the plates were frozen and thawed twice for total erythrocyte lysis and 100µl/well of haemolysed culture were placed in an ELISA plate previously coated with primary antibody anti-HRP2 (MPFM-45A, ICL, Inc, Newberg, OR, USA), followed by 1 hour room temperature incubation before incubation with the second antibody (MPFG-45A, ICL, Inc, Newberg, OR, USA). Final reaction was initiated by incubating with TMB chromogen for 10 minutes. The reaction was stopped by adding 50µl of 0.2M sulfuric acid and the absorbance read at 450 nanometer (nm).

The HRP2 assay generates the optical density (OD) value for each well. OD values were proportional to the quantity of produced HRP2. OD values from control wells represent the maximum amount of HRP2 that is produced by parasites and OD values from blank wells represent background of HRP2. A 100% growth value, which corresponds to maximum amount of HRP2 produced, was obtained by subtracting the mean OD value of blank wells from that of the control wells. Therefore, the growth inhibition value at each concentration of the plant extract or chloroquine was obtained by adjusting OD values from plant extract treated wells for HRP2 background activity. These values were then expressed as a percentage of growth inhibition value and plotted against corresponding concentrations of the plant extract using R Statistical software (www.carn.r-project.org) to generate dose-response curves from which concentrations inhibiting 50% of parasite growth (IC_{50}) were calculated (Kaddouri et al., 2006).

Drug interaction assay

Based on the methodology of Azas et al. (2002), the standard isobolar analysis was used to evaluate the combination effects of the methanolic extract of *Acanthospermum hispidum* (being the more active extract) and chloroquine on both 3D7 and Dd2 strains. Following the procedure described by Berenbaum (1978), various combination mixtures of the extract and chloroquine (different ratio: 5:0, 4:1, 3:2, 2.5:2.5, 2:3, 1:4 and 0:5 of their respective IC_{50}s determined beforehand) were tested using 96-well microtiter plates. Each combined solution was tested in duplicate in two independent experiments. Incubation and subsequent determination of IC_{50}s were done as already described in the previous section.

Cytotoxicity assay and selectivity index

Cytotoxicity of the plant extracts was evaluated on tumoral human HeLa cells using the Neutral red assay according to Borenfreund and Puerner (1984) procedure, with slight modifications. Cells were cultured in DMEM medium (PAA laboratories GmbH) supplemented with L-Glutamin (200µM), 5% fetal bovine serum (FBS), plasmocin and penicillin-streptomycin solution, then incubated at 37°C and 5% CO_2. To determine the *in vitro* toxicicity, cells in growth were dissociated with 0.05% Trypsin 0.02% EDTA, suspended into completed medium, counted and distributed into 96-well

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plates at 2 x 10^4 cells per well in 100 µl, and then incubated under the same condition as described above. After 24 hours, , the medium was removed and replaced by 100 µl of culture medium containing extracts at various concentrations (from 16 to 500 µg/ml), except 8 wells per plate in which 100 µl of the medium free of extract was added and considered as controls. The plates were incubated for additional 24h, after which, the products were removed and replaced with 100 µl of completed medium containing 50 µg/ml of neutral red and incubated for 3hours. Three of drug free control wells were incubated with the medium and served to evaluate the background. Later, the medium was removed, the plates washed with PBS and 100µl/well of acetic acid-ethanol added, kept for 10 minutes at room temperature on a shaker, and then the absorbance read at 550 nm in a spectrophotometer (Asys Expert 96, Biochrom). Each plant extract was tested in triplicate and the mean IC_{50} value was determined using R Software.

The selectivity index (SI = IC_{50} on HeLa cells/IC_{50} on *P. falciparum*) was calculated to evaluate the safety of the tested extracts.

Results

In vitro antiplasmodial activity

Among the six tested extracts of *Acanthospermum hispidum* and *Ficus thonningii*, the methanolic extract of *Acanthospermum hispidum* showed the highest antiplasmodial activity with IC_{50} values of 9.02 and 2.82 µg/ml on 3D7 and Dd2 respectively (Table 1). The aqueous extracts of both plants presented no or the worst antiplasmodial activities.

Table 1: The mean IC_{50} values for plant extracts (µg/ml) and chloroquine (ng/ml), and respective mean selectivity index values

Plant	Extract	IC_{50}±SD on *P. falciparum*		IC_{50}±SD on HeLa cells	SI	
		3D7	Dd2		HC/3D7	HC/Dd2
A. hispidum	Methanolic	9.02±1.84	2.82±2.08	110.48±0.76	12.00	39.17
	Ethanolic	26.8±2.50	12.73±3.12	123.38±1.15	4.60	9.69
	Aqueous	72.3±0.16	75.46±1.53	206.4±2.17	2.85	2.73
F. thonnigii	Methanolic	16.05±3.83	9.61±1.34	107.32±0.78	6.68	11.16
	Ethanolic	18.85±4.33	11.57±1.13	93.66±0.01	4.96	8.09
	Aqueous	35.22±4.10	83.28±4.77	363.02±0.44	10.30	4.37
Chloroquine		4.84±5.95	69.15±4.26	>500	-	-

HC: HeLa cells, SD: Standard deviation, SI: Selectivity index

Drug interaction assay

IC_{50} values of the methanolic extract of *Acanthospermum hispidum* and chloroquine when tested in combination on both 3D7 and Dd2 strains are presented in Table 2. Results of the isobolar analysis of interactions of the two drugs are shown in Figure 1.The isobolograms show a synergistic interaction between the plant extract and chloroquine on the 3D7 strain (**a**). The same effect was obtained on the Dd2 strain (**b**).

Table 2: In vitro antiplasmodial activity of the methanolic extract of *Acanthospermum hispidum* combined with chloroquine

Strains	Drugs	Mean IC_{50}±SD values at extract:chloroquine ratio of:				
		4:1	3:2	2.5:2.5	2:3	1:4
3D7	Extract (µg/ml)	5.41 ± 0.4	3.69 ± 0.7	2.7 ± 0.2	2.7 ± 0.1	1.26 ± 0.4
	Chloroquine (ng/ml)	0.72 ± 0.2	1.11 ± 0.6	1.88 ± 0.9	2.03 ± 0.1	2.75 ± 1.1
Dd2	Extract (µg/ml)	1.74 ± 0.7	1.29 ± 0.3	0.9 ± 0.8	0.76 ± 0.5	0.42 ± 0.3
	Chloroquine (ng/ml)	12.44 ± 1.3	20.74 ± 2.1	28.35 ± 3.3	38.69 ± 0.8	46.33 ± 2.8

SD: Standard deviation

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Cytotoxicity assays and selectivity index

The results of the cytotoxicity on HeLa cells and the selectivity index (SI) of the tested extracts are also shown in Table 1. The methanol extract of *Ficus thonningii* had the highest cytotoxic effect (IC_{50}=93.66 µg/ml) while the aqueous extract of the same plant had the lowest (IC_{50}=363.02 µg/ml). Extracts of *Acanthospermum hispidum* showed low level of toxicity with IC_{50}s superior to 100 µg/ml. The impact of the toxicity was assessed by analysing the SI, defined as the ratio of the cytotoxicity to antiplasmodial activity and determined by dividing the IC_{50} value for HeLa cells by the IC_{50} value for *Plasmodium falciparum*. When the selectivity index was > 10 the extract was considered to have a selective antiplasmodial activity and to be non toxic. Therefore, the methanolic extract of *Acanthospermum hispidum* had the most interesting SIs. The SI of ethanolic extracts for both *Acanthospermum hispidum* and *Ficus thonningii* was comparable.

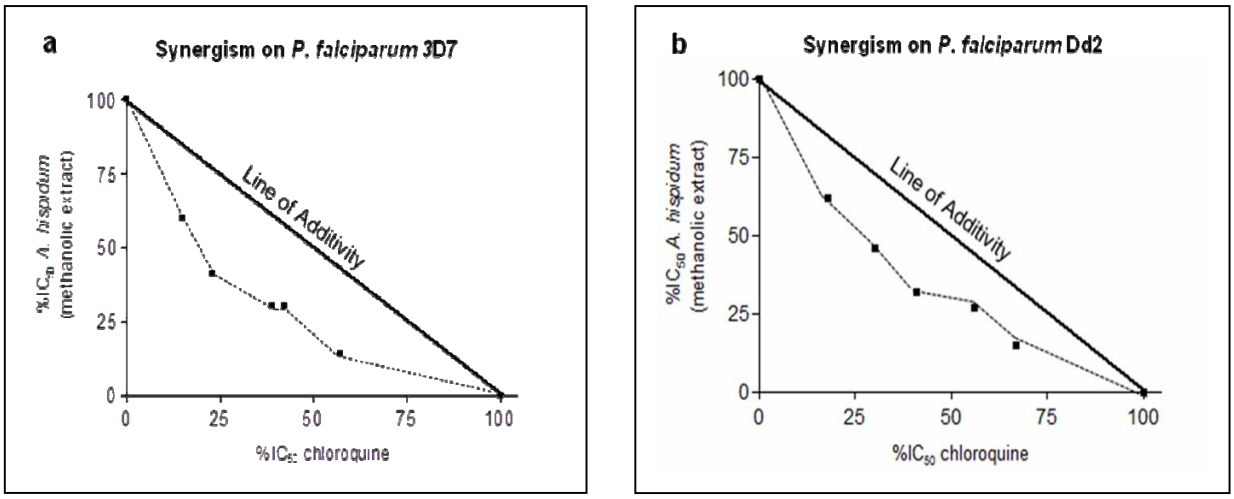

Figure 1: Interaction between methanolic extract of *Acanthospermum hispidum* and chloroquine on the 3D7 strain (**a**) and on the Dd2 strain (**b**). Results were plotted using an isobologram analysis based on the IC_{50} of each drug. The x and y axes are the percentages of the IC_{50} of the plant extract and chloroquine (in mixtures) respectively, with 100% representing the effective IC_{50} of each drug tested alone. Synergism is indicated by the concave lines drawn from data points below the straight line of the theorical additivity.

Discussion

The spread of parasite resistance to antimalarial drugs is a major reason for research and discovery of new effective agents. Screening of plants used in traditional medicine for antiplasmodial activity is one way to discover promising drugs/compounds. The decision to screen two plant species used in this study was based on previous ethnobotanical survey, literature and on the wide geographic distribution of the plants in the country.

I*n vitro* inhibitory activity of aqueous, methanolic and ethanolic extracts of *Acanthospermum hispidum* and *Ficus thonningii* leaves on chloroquine sensitive and chloroquine resistant laboratory strains were tested and scored according to the classification established by Deharo et al. (2001), whereby an extract is considered active if its IC_{50} <5 µg/ml, moderately active if IC_{50} <10 µg/ml and inactive if IC_{50} >10 µg/ml.

Based on this classification, our findings indicate that methanolic extract of *Acanthospermum hispidum* leaves has a strong and moderate antiplasmodial activity on Dd2 (IC_{50} = 2.82 µg/ml) and 3D7 (IC_{50} = 9.02 µg/ml) laboratory strains respectively. In contrast, the ethanolic extract of the same plant was inactive on both strains. The aqueous extract was also inactive, confirming previous studies which demonstrated the lack of antimalarial effect of the aqueous extract of this plant. (Bero et al., 2009; Sanon et al., 2003b). In these studies, the dichloromethane extract was

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shown to have the strongest inhibitory activity. However, the Dd2 strain was not used. Aerial parts of *Acanthospermum hispidum* have been shown to contain antioxidants, glycosides, coumarins and flavonoids (Araùjo et al. 2008). Thereby, the antiplasmodial activity observed in this study might be attributed to one of these compounds since antioxidants, glycosides, coumarins and flavonoids have been reported to possess antimalarial activity (Sriwilaijroen et al., 2010). The IC_{50} of *Acanthospermum hispidum* methanolic extract on the strain 3D7 in our study is lower than that obtained by Bero et al. (2009). A possible explanation could be that environmental parameters such as geographic area and nature of the soil may have an influence on the presence and/or quantities of bioactive compounds in plants.

Our study showed poor antiplasmodial activity of *Ficus thonningii* irrespective of the solvent used and corroborating findings of a recent study showing the lack of significant antiplasmodial activity of leaves of this plant on the chloroquine sensitive strain 3D7 (Olivia et al., 2010). Similarly, aqueous extract which mimics the traditional preparation did not present any antimalarial activity. These results confirm the importance of solvent selection for medicinal plant extraction in testing antiplasmodial activity and raise issues on the validity of the *in vitro* system. Indeed, traditional healers use water to mix the leaves and treat malaria. Nevertheless, the potential of *Ficus thonningii* in the treatment of uncomplicated malaria cannot be excluded since certain plant extracts containing pro-drugs may only be active after particular metabolic process *in vivo*.

In the presence of chloroquine, the methanolic extract of *Acanthospermum hispidum* exhibited a promising synergistic effect on 3D7 and Dd2 strains. This suggests that the mode of action of this extract is different from that of chloroquine. This is an important finding in the context of malaria endemic regions where resistance to chloroquine is high and traditional medicine is highly practiced as it is the case in Congo-Brazzaville (Ndounga et al., 2007; Mbatchi et al., 2006). However, as organic extracts are not used by traditional healers to treat malaria, further *in vitro* antiplasmodial studies of drug combinations should be carried out using chloroquine or an artemisinin derivative and active compounds or fractions resulting from the active extract. If this synergism can be confirmed and also reproduced *in vivo*, these compounds might be used in combination with chloroquine against resistant strains of *Plasmodium falciparum*.

Regarding cytotoxicity assays, extracts of both *Acanthospermum hispidum* and *Ficus thonningii* showed low levels of toxicity on HeLa cells. However, correlating the cytotoxicity with the antiplasmodial activity, only the methanolic extract of *Acanthospermum hispidum* had a good selectivity index (SI >10), suggesting a selective activity of this extract against the parasite strains.

Though bioactive compounds have not been isolated in this study, further work need to be carried out in isolating the compounds which may be responsible for these actions on *Acanthospermum hispidum*. Also, while only the leaves have been investigated in this work based on their traditional use in the Republic of Congo, it would be of interest to screen other parts of the plant such as roots.

In conclusion, the *in vitro* antiplasmodial activies of methanolic, ethanolic and aqueous extracts of *Acanthospermum hispidum* and *Ficus thonningii* that were investigated towards the chloroquine sensitive strain 3D7 and the chloroquine resistant strain Dd2, revealed that only the methanolic extract of *Acanthospermum hispidum* showed moderate to high inhibitory activity, and a strong synergistic antimalarial effects on both strains when combined with chloroquine. All extracts have a relatively low cytotoxic level on HeLa cells. However, it is important to note that methanol is toxic and the *in vitro* data provides a partial view on the antimalarial potential of these plants. Thus, next steps should include *in vivo* studies with animals, isolation and characterization of inhibitory molecules, and investigation of the mechanism of their action.

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