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Antiprotozoal properties of Indonesian medicinal plant extracts

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Abstract

Tithonia diversifolia, *Cyclea barbata*, *Tinospora crispa*, *Arcangelisia flava*, *Pycnarrhena cauliflora* are plants used in Indonesia for the traditional treatment of malaria. In the search for new antiparasitic drugs, the parts traditionally used of these 5 plants were extracted with various solvents and evaluated *in vitro* against *Plasmodium falciparum* but also against *Babesia divergens* and *Leishmania infantum*. Seven crude plant extracts out of 25 tested displayed high antimalarial activities with $IC_{50} < 5 \mu\text{g/ml}$ and in the case of some of them an interesting selectivity regarding their cytotoxicity against mammalian cells. *A. flava* appeared to be the most promising antiplasmodial plant with the highest antiplasmodial activity (IC_{50} values less than $3 \mu\text{g/ml}$) and the weakest cytotoxicity. By contrast, only *P. cauliflora* radix, through its dichloromethane and methanol extracts also demonstrated a high activity against *L. infantum*, with IC_{50} values around $3 \mu\text{g/ml}$; their high selectivity index, especially on VERO cells, hypothesises a specific parasitocidal action. Moreover, for all the extracts showing antiplasmodial activity, a positive correlation was demonstrated with antibabesial activity, suggesting that these antiplasmodial extracts could be a potential source of antibabesial compounds. These preliminary results confirm the antiplasmodial interest of some of these plants used in traditional medicine but also their effects on leishmaniasis and babesiosis. Ongoing phytochemical investigations should allow identification of the chemical series responsible for these activities.

Keywords: *Plasmodium falciparum*; *Leishmania infantum*; *Babesia divergens*; traditional medicine ; Indonesia ; antiparasitic drugs

1. Introduction

Malaria is the most prevalent parasitic disease in the world and continues to be one of the largest public health problems, especially in the developing countries. There is an estimated 3.2 billion people at risk of being infected and leading to an estimated 584 000 malaria deaths 78 % of which concern children under 5 years (WHO, 2014c). *Plasmodium falciparum*, the most deadly species of malaria parasite already shows resistance to all antimalarial drugs, including artemisinin and its derivatives, which are the latest and the most effective treatments of malaria (Straimer et al., 2015; WHO, 2014c; Witkowski et al., 2009; Wongsrichanalai et al., 2002). Unfortunately the use of artemisinin-based combination therapies (ACTs) advocated by the World Health Organization (WHO) did not allow for the emergence and the spread of multi-resistance in the Greater Mekong region (Cambodia, Vietnam, Thailand, Myanmar and Laos) threatening the strategies of malaria eradication in the other endemic areas (WHO, 2014b). Based on these reasons there is an urgent need to discover new antimalarial agents, and crude extracts of natural products appear to be a promising avenue as they are more affordable for people in developing countries (Benoit-Vical, 2005; Benoit-Vical et al., 2008; Benoit-Vical et al., 2003).

Indonesia is rich in medicinal plants which the population use traditionally for curing diseases including malaria. This study, based on ethnobotanical data, was carried out on 5 medicinal plants selected as potential sources of antimalarial agents: *Tithonia diversifolia*, *Cyclea barbata*, *Tinospora crispa*, *Arcangelisia flava*, *Pycnarrhena cauliflora*. Except *T. diversifolia*, the 4 other plants are from the Menispermaceae family studied largely for their use in traditional medicine and principally for malaria treatment (Verpoorte et al., 1982b). Some of these plants have already been investigated for their antiplasmodial activities and pharmacological properties but no standardised studies have yet been performed with them. The infusion of *T. diversifolia* leaves is traditionally used for lowering blood glucose (Miura

et al., 2005), flatulence, malaria fever (Njoroge and Bussmann, 2006), and also the healing of wounds. An ethanol extract of *T. diversifolia* exhibited anti-malarial activity by its leaf extracts (Oyewole et al., 2008). *C. barbata* leaves have been used by the Javanese for stomach medicine and as a prophylactic against malaria fever (Manilal and Sabu, 1985; Saxena et al., 2003). *T. crispa* is traditionally used for the treatment of fever, rheumatic arthritis, hepatitis, anti-hyperglycaemia, and also malaria by using stem infusions (Pathak et al., 1995; Pushpangadan and Atal, 1984). *T. crispa*, which is abundant in the Philippines, is used freely by the natives under the name of *makabuhay* (meaning "You may live"), as a panacea, especially valuable in malarial fevers (The Southwest School of Botanical Medicine, 1918). Supporting the traditional uses, *T. crispa* scientifically proved as anti-hyperglycaemia (Noor and Ashcroft, 1998) and also both *in vitro* and *in vivo* anti-malarial activity (Najib Nik et al., 1999; Rungruang and Boonmars, 2009). *A. flava* is traditionally used as a stem decoction for typhoid fever (Mandia, 1999), hepatitis, stomach disturbance and malaria (Subeki et al., 2005). The anti-hepatotoxicity (Wongbutdee et al., 2003) and anti-malarial activity has been profiled by several studies (Lovin et al., 2012; Nguyen-Pouplin et al., 2007; Vennerstrom and Klayman, 1988; Verpoorte et al., 1982a). *P. cauliflora* is a Dayak tribe folk medicine used for flavouring, flatulence (by placing the soaked leaves on the stomach), fever and malaria (by drinking the leaf infusion). Only local accounts published in Indonesian can be cited for these uses. One study of *P. cauliflora* has just revealed its cytotoxic activities related to apoptosis and cell cycle arrest (Masriani and Adnyana, 2011), but its anti-parasitic properties have not yet been established. Indeed, despite widespread traditional use of *P. cauliflora* reported on malaria treatment in Indonesia, to the best of our knowledge, no studies have been conducted on this plant. Other *Pycnarrhena* species have been studied for their biologically active principles (Siwon et al., 1981b; Verpoorte et al., 1978).

Furthermore, because *Plasmodium* and *Babesia* belong to the Apicomplexa phylum they share many common biochemical pathways, we can therefore hypothesise the possibility of finding common active compounds for both pathogens. *Babesia divergens* babesiosis is a widespread illness transmitted by livestock ticks. This pathogen is the main agent of bovine babesiosis in Europe but can also affect splenectomized humans (Ceci et al., 2014; L'Hostis et al., 1995; Zintl et al., 2014). Infections can occur without producing symptoms, but babesiosis may also be severe and sometimes fatal due to the intraerythrocytic development of the parasite (Melhorn and Schein, 1984). There are effective babesiacides, but imidocarb dipropionate is practically the only drug available on the market and therefore the most widely used. More specific new fast-acting treatments for babesiosis should now be developed (Vial and Gorenflot, 2006).

Leishmaniasis, identified as a “Neglected Tropical Disease” by WHO, is another parasitic disease. The pathogen is endemic in 98 countries and territories, with the number of new cases estimated at 1.3 million per year (WHO, 2014a). Nowadays, malaria and leishmaniasis are responsible for over a million deaths a year and threaten more than 350 million people worldwide, mostly, but not only, in tropical and subtropical countries.

Here we offer a standardised study that focuses on the search for new antiparasitic drugs targeting *Plasmodium*, *Leishmania* and *Babesia*. Indeed, even though the five selected plants are traditionally used for the treatment of malaria in Indonesia, the antiplasmodial activities of their different extracts were not totally investigated. The extracts showing the best antiplasmodial activity were also tested on *Babesia divergens* cultures to investigate another Apicomplexa parasite. Moreover, in some tropical zones, malaria and leishmaniasis infections are largely co-endemic, the extracts were thus tested on both *P. falciparum* and *L. infantum* to evaluate their anti-parasite activities as potential sources of new active

compounds. The cytotoxicity of these extracts was assessed in parallel in order to evaluate the specificity of their anti-parasitic activities.

2. Materials and Methods

2.1. Plant material

The plants were identified in the biology laboratory of the Department of Pharmacy, Islamic University of Indonesia, Yogyakarta-Indonesia (Herbarium of Laboratory of Biology, Department of Pharmacy, Faculty of Science and Mathematics, UII, Yogyakarta, Indonesia). Samples were air-dried and powdered.

Tithonia diversifolia (Hemsl.) A. Gray. (Asteraceae; voucher specimen 007-02/L-PB/UII/2013) was collected in the Sleman district of Yogyakarta. *T. diversifolia* is a shrub, locally called “kembang bulan”.

Cyclea barbata Miers (Menispermaceae; voucher specimen 008-03/L.S-PB/UII/2013) was collected in the Bantul district of Yogyakarta and locally known as “Cincau rambat” (Javanese). *C. barbata* can be easily distinguished by its deltoidovate, hispid leaves with acuminate apex, finely mucronate acumen and long male and female inflorescences with dense capitate flowers.

Tinospora crispa (L.) (Menispermaceae; voucher specimen 009-03/S-PB/UII/2013) known by the vernacular name of “brotowali”, was collected from Yogyakarta. *T. crispa* is a woody tropical liana with shiny green leaves.

Arcangelisia flava (L.) Merr. (Menispermaceae; voucher specimen 010-04/S-PB/UII/2013), collected in South Borneo, is a large, woody, glabrous and dioecious liana, up to 20 m long; with a stem of up to 5 cm in diameter and with yellow wood exuding a yellow sap when cut.

Pycnarrhena cauliflora (Miers.) Diels (Menispermaceae; voucher specimen 011-01/S.R-PB/UII/2013) was collected in Sintang district, West Borneo. This plant can be found in

primary or secondary forests and tend to grow in clusters. From 8 species in the genus *Pycnarrhena*, only 4 (*P. australiana*, *P. ozantha*, *P. manilensis*, *P. longifolia*) have been investigated in any detail.

2.2. Preparation of plant extract

For *T. diversifolia* (leaves), *C. barbata* (stem and leaves), *T. crispa* (stem), and *A. flava* (stem), powdered samples were extracted by maceration in 70 % ethanol for 24 hours, 300 g of plant powder with 1.5 L of solvent (Figure 1). This process was repeated for two days consecutively using fresh 70% ethanol. Each ethanol extract was filtered and filtrates were evaporated to dryness using a rotary vacuum evaporator. Dried ethanol extracts were solubilized in ethanol to be fractionated by n-hexane/ by liquid-liquid separation method which created two separate layers (n-hexane and first ethanol layer). The n-hexane layer gave the n-hexane fraction after evaporation. The fractionation process was continued by fractionation of the first ethanol layer using ethyl acetate (liquid-liquid separation) which produced ethyl acetate and second ethanol layers giving respectively after evaporation, the ethyl acetate fraction and ethanol fraction residue.

For *P. cauliflora* powdered radix part (Figure 2) was directly treated by n-hexane to extract lipophilic compounds. After filtration the n-hexane extract was evaporated to dryness. The precipitate resulting of n-hexane extraction was further put back in suspension/solubilized using dichloromethane to obtain dichloromethane extract after filtration. The new precipitate was submitted to a methanol extraction followed by a filtration and an evaporation of the filtrate.

This extraction method was employed to investigate the possible active compounds responsible for plant activities. Particularly for *P. cauliflora*, dichloromethane was used to extract active compounds with semi-polar characteristics in order to explore new compounds

other than those already published. For each part of leaves of *T. diversifolia*, stem and leaves of *C. barbata*, and stem of *T. crispa* 3 different extracts were obtained: ethanol, n-hexane and ethyl acetate fractions, and 1 fraction residue. For stem of *A. flava* only the ethanol, n-hexane and ethyl acetate fractions were obtained. For each part of stem and radix of *P. cauliflora* it was produced n-hexane, dichloromethane and methanol extracts. Finally, it yielded 25 extracts ready for antiparasitic activity investigations.

To evaluate the anti-parasite (against *Plasmodium*, *Babesia* and *Leishmania*) activities, stock solutions of the 25 crude and semi-purified extracts (1 mg/mL) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and diluted for use in RPMI 1640 (Biowest, France). The dilutions were checked in order to confirm that the extracts did not precipitate under these conditions. The extracts were tested immediately after their dissolution in DMSO and RPMI, then kept at 4°C for 3-8 days, and tested again to validate the conservation of mother solutions. The maximum percentage of residual DMSO in the assay was 2 % and was evaluated alone on parasite cultures to check its absence of activity.

2.3. *In vitro* culture of *Plasmodium falciparum* and antiplasmodial activity

The antiplasmodial activity was assessed on the FcM29-Cameroon chloroquine-resistant strain of *Plasmodium falciparum*, cultured continuously according to the Trager and Jensen method (Trager and Jensen, 1976), in a 5 % CO₂ atmosphere at 37°C, taking into account the modifications previously described (Benoit-Vical et al., 2007). Briefly, the parasites were maintained *in vitro* in human red blood cells and diluted in RPMI 1640 medium, supplemented with 25 mM HEPES, L-glutamine (Biowest, France) and completed with 5 % human serum (French Blood Bank, EFS; Toulouse, France). The antiplasmodial activity was assessed as previously reported by Desjardins et al. (Desjardins et al., 1979) and modified as follows. Extract dilutions and reference compounds were tested in triplicate at least 3 times

independently, in 96-well plates with cultures at a parasitaemia of 1 % and a haematocrit of 1 %. For each test, the plates of parasite culture were incubated with extracts or reference compounds for 48 h and [³H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation (Benoit-Vical et al., 2007). Parasite growth was estimated by [³H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2 % DMSO) was referred to as 100 % growth. The IC₅₀ values (50 % inhibitory concentration) were graphically determined as concentrations *versus* percentage of parasite growth.

2.4. *In vitro* anti-*Babesia divergens* activity

Babesia divergens (Rouen 1986 strain) was cultured continuously according to Précigout *et al* (Precigout et al., 1991). The parasites were maintained *in vitro* in human red blood cells (O⁺; EFS; Toulouse, France), diluted to 6 % haematocrit in RPMI 1640 medium (Biowest, France) supplemented with 30 nM NaHCO₃ and complemented with 10 % human AB⁺ serum (EFS).

The antibabesial activity of extracts was evaluated by the same radioactive micromethod as for *Plasmodium* in 96-well culture plates except for the incubation time, which was reduced to 24 h. The drug-free control parasite cultures were considered as 100 % growth, and an imidocarb sensitivity control was included in every plate. IC₅₀ were determined graphically from concentration *versus* percent inhibition curves.

2.5. Antileishmanial activity on promastigote stages

The effect of the compounds tested on the growth of *Leishmania infantum* (line MHOM/MA/67/ITMAP-263) promastigotes was assessed by luciferase assay. Briefly, promastigotes in log-phase in RPMI 1640 medium (Biowest, France) supplemented with

10 % fetal calf serum (Hyclone, ThermoFischer, France), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL geneticin (Sigma, France)) were incubated at an average density of 10^6 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO or MeOH (final concentration less than 0.5 % v/v), in duplicate. Appropriate controls treated by DMSO, MeOH and amphotericin B (reference drug purchased from Sigma Aldrich) were added to each set of experiments. After a 72 h incubation period at 24°C, each plate well was then microscope-examined for any possible precipitate formation. To estimate the luciferase activity of promastigotes, 80 µl of each well were transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates were incubated for 2 min. Luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50 % (IC₅₀) was defined as the concentration of drug required to inhibit 50 % of the metabolic activity of *Leishmania infantum* promastigotes compared to the control. IC₅₀ were calculated by non-linear regression analysis processed on dose–response curves, using TableCurve 2D V5® software. IC₅₀ values are the geometric mean value calculated from three independent experiments.

2.6. Cytotoxic evaluation on macrophages and VERO cell lines

The cytotoxicity tests were performed on macrophages and VERO cell lines for compounds showing the best antileishmanial and antiplasmodial activities, respectively. The evaluation of cytotoxicity by MTT assay on the J774A.1 cell line (mouse macrophage cell line, Sigma-Aldrich) was evaluated according to Mosmann (Mosmann, 1983) with slight modifications, and cytotoxicity on VERO cells (monkey epithelial cell line, Sigma-Aldrich) was assessed using [³H]-hypoxanthine incorporation. Briefly, cells ($5 \cdot 10^4$ cells/mL) in 100 µL of complete medium were seeded into each well of 96-well plates and incubated at 37°C in a humidified

5 % CO₂ with 95 % air atmosphere. The complete medium consisted of RPMI 1640 supplemented with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) for J774A.1 cell line and MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), NEAA 1X for VERO cell line. After 24 h incubation, 100 µL of medium with various product concentrations and appropriate controls (DMSO and doxorubicin) were added and the plates incubated for 72 h at 37°C, 5 % CO₂. For cytotoxic evaluation on macrophages, each plate well was then microscope-examined to detect any precipitate formation before the medium was aspirated from the wells. One hundred µL of MTT solution (0.5 mg/mL in RPMI) was then added to each well and the cells incubated for 2 h at 37°C. Then the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. The plates were shaken vigorously for 5 min and absorbance was measured at 570 nm with a microplate spectrophotometer (EON).

For cytotoxic evaluation on VERO cells, the plates were incubated with the test compounds for 48 h and [³H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of the incubation (Benoit-Vical et al., 2007) to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2 % DMSO) was referred to as 100 % of growth. The IC₅₀ values (50 % inhibitory concentration) were graphically determined as concentrations *versus* percentage of parasite growth.

3. Results and discussion

Antiplasmodial activities of *T. diversifolia*, *C. barbata*, *T. crispa*, *A. flava* and *P. cauliflora* extracts collected from Indonesia are reported in Table 1. Seven out of the 25 crude extracts from these 5 plants showed very interesting activities against *P. falciparum* with IC₅₀ values less than the threshold of 5 µg/ml (Willcox et al., 2011). These best antiplasmodial extracts

were also tested on the VERO cell line in order to evaluate the specificity of their activity. *T. diversifolia* ethanol and ethyl acetate extracts were active against *P. falciparum* but their cytotoxic values were equivalent (selectivity index less than 1). The ether extract of aerial parts of this plant had already demonstrated *in vitro* antiplasmodial activity (IC₅₀: 0.75 µg/ml on the chloroquine-sensitive strain FCA). Those findings led to the extraction and identification of the active compound: taginin C, a sesquiterpene lactone with an IC₅₀ of 0.33 µg/ml against FCA strain (Goffin et al., 2002). The ethanol extract of *T. diversifolia* was active at 200 mg/kg *in vivo* in a mouse malaria model (Elufioye and Agbedahunsi, 2004). However, because of the low selectivity index of *T. diversifolia*, this plant presents a limited interest for further pharmacological investigations. *A. flava* ethanol, N-hexane and ethyl acetate (Figure 3) extracts demonstrated a good antiplasmodial activity (IC₅₀ around 3 µg/ml) and interestingly a high selectivity regarding their activities against the VERO cell line (> 50 µg/ml). *A. flava* possesses alkaloid compounds having antioxidant and cytotoxic activities (Keawpradub et al., 2015). Other findings revealed that *A. flava* extracts have IC₅₀ ranging from 0.4 to 8.6 µg/mL against *P. falciparum* (Nguyen-Pouplin et al., 2007) and one of its active compounds, berberine, inhibits telomerase activity in *P. falciparum* (Sriwilaijareon et al., 2002). *P. cauliflora* showed the best antiplasmodial IC₅₀ values with dichloromethane and methanol extractions particularly of the radix part (3.3 µg/ml with both solvents) and the weakest cytotoxicity for the radix dichloromethane extract (> 50 µg/ml). Bisbenzylisoquinoline alkaloids of *P. cauliflora*, *i.e.* obaberine and pycnarrhine (Siwon et al., 1981a) have been reported to be responsible for various activities involving antioxidant and cytotoxic properties (Masriani and Adnyana, 2011) but no studies to date have revealed its antiplasmodial activity. This novel plant thus appears to be particularly promising and deserves further study.

Surprisingly and despite their traditional use for malaria treatment, no extracts from *C. barbata* (stem and leaf) or *T. crispa* (stem) showed antiplasmodial activity. Previous studies have reported alkaloid extraction from the root of *C. barbata* with both cytotoxic and antimalarial activities. Five bisbenzylisoquinoline alkaloids were isolated as active principles: tetrandrine, limacine, thalrugosine, homoaromaline, cycleapeltine (Lin et al., 1993). Some of these bisbenzylisoquinoline alkaloids were reported elsewhere for their high antiplasmodial activity (Fang and Jiang, 1986; Marshall et al., 1994). However, *T. crispa* extracts showed *in vivo* antiplasmodial activity of stems against *P. berghei* (Marthianti, 2006) and also *in vitro* but the IC₅₀ value of 0.8 mg/mL reported for methanol stem extract (Najib Nik et al., 1999) is far too high to have a pharmacological interest and is thus in accordance with our results. Because *Babesia* is another protozoan apicomplexan parasite that infects erythrocytes and also causes haemolytic disease, it was interesting to also determine the activity of these plant extracts against *Babesia in vitro*. The 8 extracts showing the best antiplasmodial activity were tested on *Babesia divergens* culture (Table 2). With the exception of *T. diversifolia* ethanol extract, for which the standard deviation of the results was too large to conclude, all the extracts tested showed IC₅₀ values ranging from 1.2 to 14.1 µg/ml. These data are particularly interesting because they concern crude extracts and are not so far from the IC₅₀ of the control drug imidocarb (0.5 µg/ml) and better than those already reported with other Indonesian medicinal plant extracts (Murnigsih et al., 2005; Subeki et al., 2007). Our results suggested a strong correlation between antiplasmodial and antibabesial activities and so the potential use of antibabesial compounds from the antiplasmodial drugs portfolio. Moreover, because *Babesia* is an erythrocyte parasite sharing numerous biochemical pathways with *Plasmodium* with the notable exception of the haemoglobin degradation pathways, we could hypothesise that these extracts act on parasitic pathways that do not involve hemozoin polymerisation.

The same 25 extracts tested against *P. falciparum* were also evaluated against *L. infantum* and in parallel on its macrophage host cell (Table 3). Only 3 extracts out of 25 showed IC₅₀ values of less than 50 µg/ml. Dichloromethane (Figure 4) and methanol extracts of *P. cauliflora* radix showed a good activity on *L. infantum* with IC₅₀ values of 1.7 and 4.4 µg/ml, respectively. However the selectivity indexes obtained on macrophages are relatively low (around 3). The analysis of these data shows that both best extracts (*P. cauliflora*) on *Leishmania* were also active, at similar concentrations, on *Plasmodium*. It is particularly interesting since the anti-parasite activity of this plant (whether on *Plasmodium*, *Babesia*, or *Leishmania*) has thus far never been reported. Moreover Malaria and Leishmaniasis are co-endemic in Indonesia and the same plant extracts locally present could fight both pathogens.

4. Conclusions

Based on an ethnopharmacology approach, 7 plant extracts out of 25 tested displayed good antimalarial activities with IC₅₀ < 5 µg/ml. Activities observed with ethanolic crude extracts, the solvent of extraction here studied closest the aqueous traditional extraction, could justify the traditional use of both plants *T. diversifolia* and *A. flava* for malaria treatment. However regarding their cytotoxicity against mammalian cells, *A. flava* appeared to be the most promising antiplasmodial plant of this study with the highest antiplasmodial activity (IC₅₀ values less than 3 µg/ml) and the lowest cytotoxicity. Moreover, for all extracts showing antiplasmodial activity a positive correlation was found with antibabesial activity, suggesting that antiplasmodial extracts can be a potential source of antibabesial compounds. In contrast, *A. flava* had no activity on *L. infantum*. Furthermore, only *P. cauliflora* radix, through its dichloromethane and methanol extracts also demonstrated a good activity against *L. infantum*, with IC₅₀ values equivalent for the 3 pathogens; their selectivity index, especially on VERO cells, suggests a specific parasitocidal action more than a global cytotoxic effect.

These preliminary results are sufficiently interesting to pursue phytochemical investigations in order to identify the chemical series responsible for these activities and obtain standardized preparations. This is an absolute necessity before considering any therapeutic applications to malaria, leishmaniasis and/or babesiosis for either herbal preparations or pure molecules.

Conflict of interest

The authors declare no conflict of interest.

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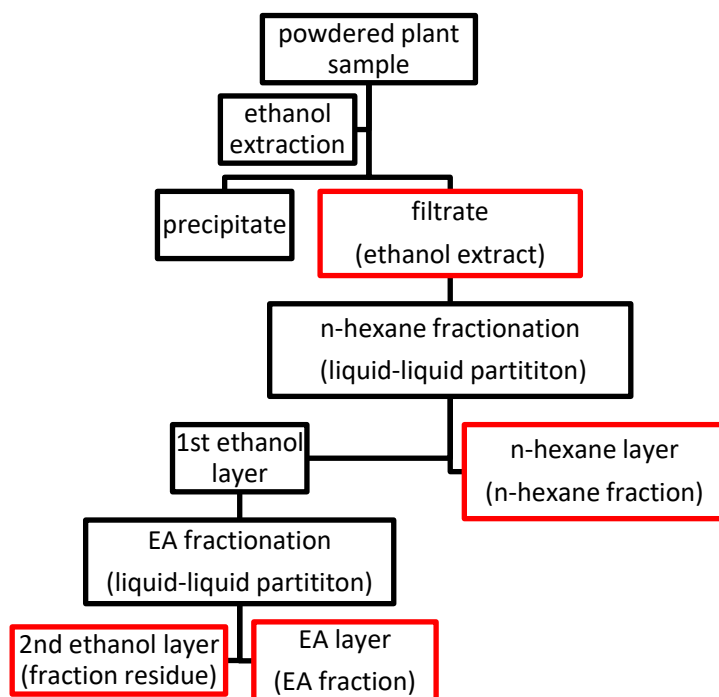
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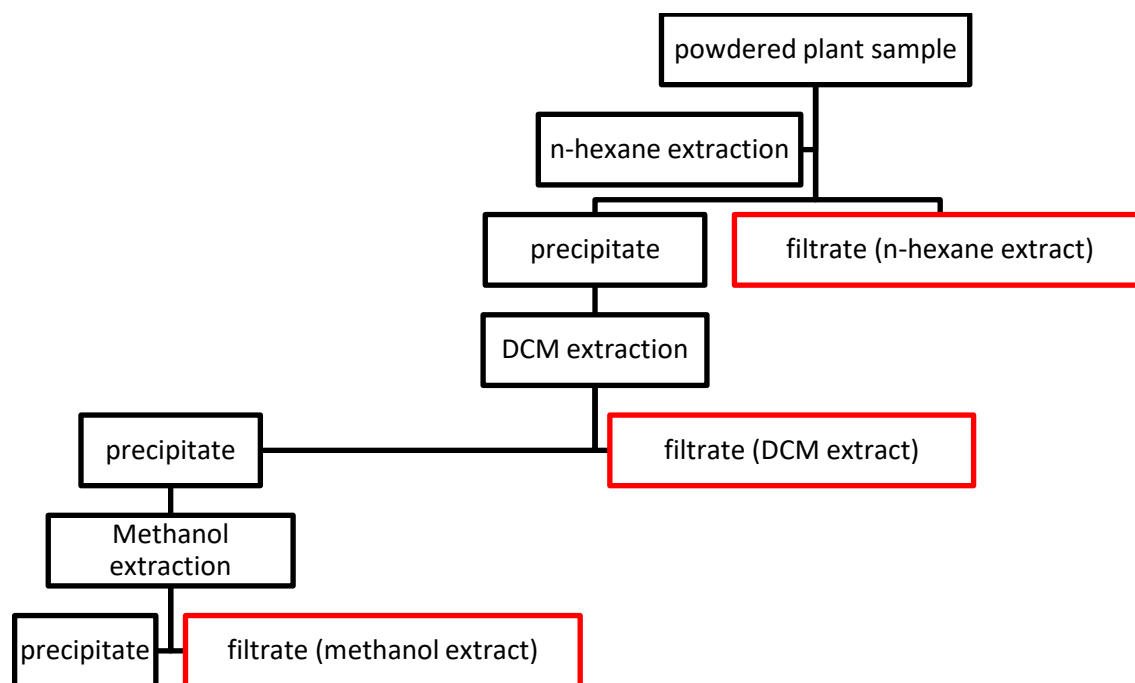
Figure 1: Representative scheme of *T. diversifolia*, *C. barbata*, *T. crispa*, and *A. flava* extraction



EA : ethyl acetate

Red boxes correspond to extracts used for antiparasitic activity investigations

Figure 2: Representative scheme of *P. cauliflora* extraction



DCM: dichloromethane

Red boxes correspond to extracts used for antiparasitic activity investigations

Figure 3: Representative curve of percentage inhibition of *Plasmodium falciparum* growth *in vitro* by *A. flava* ethyl acetate extract

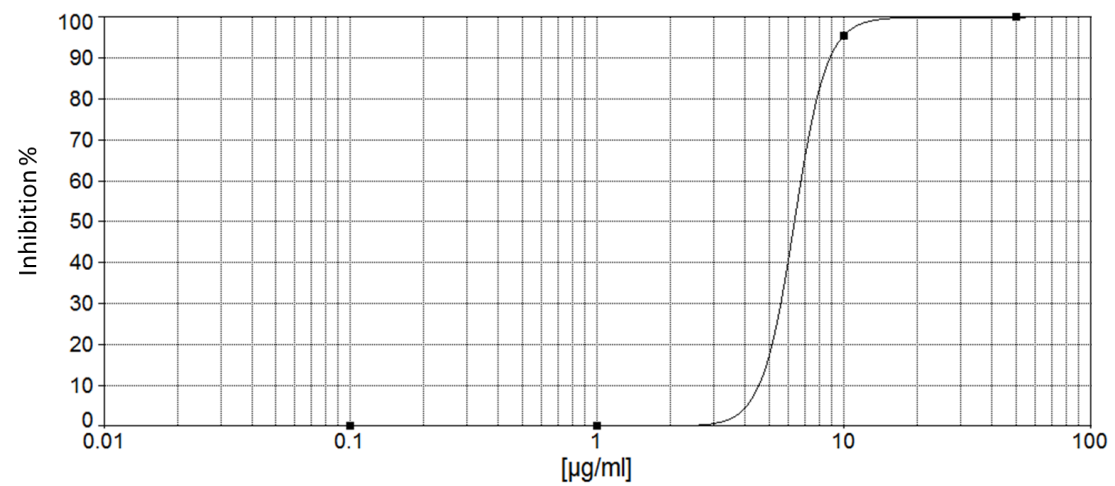


Figure 4: Representative curve of percentage inhibition of *Leishmania infantum* promastigote growth *in vitro* by *P. cauliflora* dichloromethane extract (radix part)

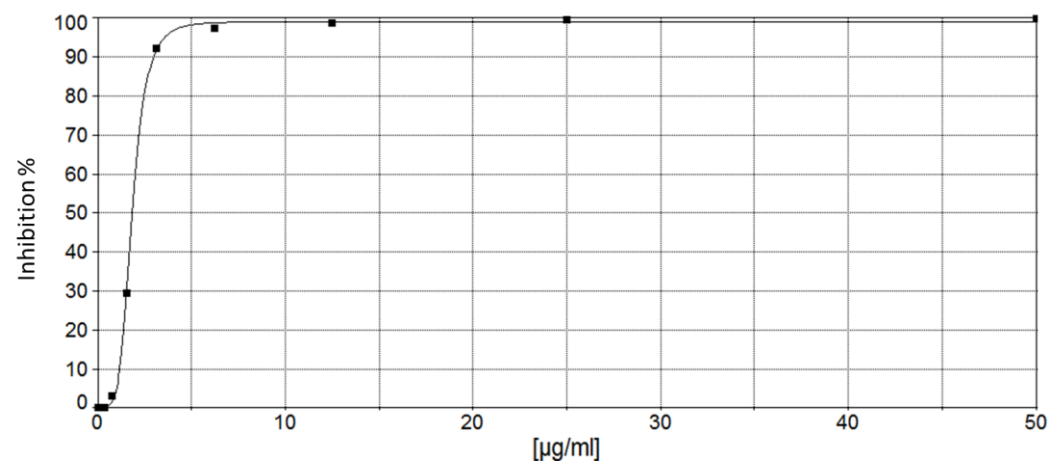


Table 1. Antiplasmodial activity against *P. falciparum*, chloroquine-resistant strain (FCM29-Cameroon strain), and cytotoxicity on VERO cell line

Extract	IC ₅₀ , µg/mL; Mean ^{&} ± SD Antiplasmodial activity / Cytotoxicity (Selectivity Index) [†]						
	<i>T. diversifolia</i> (leaf)	<i>C. barbata</i> (stem)	<i>C. barbata</i> (leaf)	<i>T. crispa</i> (stem)	<i>A. flava</i> (stem)	<i>P. cauliflora</i> (stem)	<i>P. cauliflora</i> (radix)
EtOH	3.9 ± 2 / 3.5	> 50 / ND [‡]	> 50 / ND	50 / ND	2.8 ± 0.9 / >50	- [#]	-
	(0.9)				(>18)		
N-hexane	16.7 ± 6 / ND	13.7 ± 7 / ND	41 ± 8 / ND	18.7 ± 5 / ND	2.8 ± 0.8 / >50	> 50 / ND	> 50 / ND
					(>18)		
Ethyl acetate	3.3 ± 0.5 / 3.1 ± 1.7	27 ± 5 / ND	33 ± 13 / ND	> 50 / ND	3 ± 0.7 / >50	-	-
	(0.9)				(>18)		
Residue	> 50 / ND	> 50 / ND	> 50 / ND	> 50 / ND	-	-	-
Dichloromethane	-	-	-	-	-	10.5 ± 8 / 17.5 ± 2	3.3 ± 0.1 / >50
						(1.7)	(>15)
Methanol	-	-	-	-	-	> 50 / ND	3.3 ± 0.3 / 6.5
							(2)
Chloroquine (drug control)	0.28 ± 0.03 [§] / 69.3 ± 21 (245)						

[&] Mean of at least 3 independent experiments; [†] Selectivity Index: Cytotoxicity/activity ratio; [‡] Not done; [§] Corresponding to the IC₅₀ value of 0.51 ± 0.04 µM;

[#] No corresponding extract

Table 2. Anti-*Babesia* activity against *B. divergens*

Extract	IC ₅₀ , µg/mL; Mean ^{&} ± SD
<i>T. diversifolia</i> ethanol extract	36.2 ± 23
<i>T. diversifolia</i> ethyl acetate extract	1.7 ± 0.7
<i>A. flava</i> ethanol extract	6.4 ± 2.8
<i>A. flava</i> N-hexane extract	4.8 ± 2.9
<i>A. flava</i> ethyl acetate extract	6.3 ± 3.2
<i>P. cauliflora</i> dichloromethane extract (stem)	1.5 ± 1
<i>P. cauliflora</i> dichloromethane extract (radix part)	14.1 ± 9
<i>P. cauliflora</i> methanol extract (radix part)	1.2 ± 0.7
Imidocarb (drug control)	0.5 ± 0.5 [§]

[&] Mean of at least 3 independent experiments; [§] Corresponding to the IC₅₀ value of 1.4 µM

Table 3. Antileishmanial activity against *L. infantum* promastigote and cytotoxicity on murine J774A.1 macrophage line

Extract [§]	IC ₅₀ , µg/mL; Mean ^{&} ± SD
	Antileishmanial activity / Cytotoxicity (Selectivity Index)
<i>T. diversifolia</i> ethyl acetate extract	21.9 ± 1.2 / 3.3 ± 1.4 (0.03)
<i>P. cauliflora</i> dichloromethane extract (radix part)	1.7 ± 0.5 / 5.8 ± 0.9 (3.4)
<i>P. cauliflora</i> methanol extract (radix part)	4.4 ± 0.9 / 12.4 ± 1.5 (2.8)
Amphotericin B (drug control)	0.06 ± 0.01 / 3.3±1.4 (55)

[&] Mean of at least 3 independent experiments; [§] All extracts tested against *P. falciparum* (see Table 1) were also evaluated against *L. infantum* promastigote. With the exception of those reported here in Table 3, all the other extracts showed IC₅₀ greater than 50 µg/ml against *Leishmania* (data not shown) and were not tested for their cytotoxicity.