In Vitro and In Vivo Activity of Anogeissus leiocarpa Bark Extract and Isolated Metabolites against Toxoplasma gondii

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In vitro and *in vivo* activity of *Anogeissus leiocarpa* bark extract and isolated metabolites against *Toxoplasma gondii*

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Abstract

Toxoplasma gondii, belonging to the Apicomplexa phylum, is a cosmopolitan protozoan parasite which affects at least 30% of the world's population. In West Africa, the leaves and bark of the tree species *Anogeissus leiocarpa* (DC.) Guill. & Perr. are used against zoonosis in traditional medicine and play a key role in controlling diseases induced by Apicomplexans such as malaria. In this study, extracts, fractions and pure compounds obtained from an ethanol extract of the bark of *A. leiocarpa* were evaluated against *T. gondii* infection *in vitro* and *in vivo*. The crude bark extract showed a significant activity on tachyzoites from the *T. gondii* RH strain (IC_{50} = 59.30 µg/mL). The crude bark extract without tannins and pure trachelosperogenin E purified by Centrifugal Partition Chromatography showed the highest activity (IC_{50}s = 12.83 µg/mL and 26.63 µg/mL, respectively) with satisfying selectivity indexes of 9.61 and 9.75, respectively. The crude bark extract without tannins and pure trachelosperogenin E were able to significantly inhibit host cell invasion by the parasite *in vitro*, while the crude bark extract without tannins was able to increase mice survival in our murine model of chronic toxoplasmosis. These results provide new biological data for natural compounds that could enhance the current panoply of treatments against toxoplasmosis.

Keywords

Toxoplasma gondii, *Anogeissus leiocarpa*, Combretaceae, centrifugal partition chromatography, natural products, chemosensitivity.

Abbreviations

CBEWT, Crude Bark Extract Without Tannin; CPC, Centrifugal Partition Chromatography; HRP, Horseradish Peroxidase; PBS, Phosphate Buffered Saline; SAG-1, Surface Antigen 1; SI, Selectivity Index.

Introduction

Toxoplasmosis is an important parasitic disease caused by the protozoan *Toxoplasma gondii*. Approximately one-third of the world population have been infected by this parasite [1]. Toxoplasmosis, a generally benign affection, can nevertheless cause

severe life-threatening disease in immunocompromised patients and in congenitally affected children [2]. The population structure of *T. gondii* consists in three main clonal lineages correlated with virulence expression in mice: type I (including RH, a highly virulent strain in mice), type II (including avirulent strain like ME-49) and type III (avirulent strains) [3].

Only a few treatments are currently available against toxoplasmosis. They mainly consist in a synergic combination of pyrimethamine and sulfonamide and act by blocking the folate biosynthesis pathway, which is essential for parasite survival and growth. However, treatment of toxoplasmic encephalitis and chorioretinitis by these drugs may fail due to host intolerance or bad absorption of the molecules [4,5]. Moreover, resistant strains against sulfadiazine [6,7] leads to the need for new active compounds against toxoplasmosis.

African birch (*Anogeissus leiocarpa* (DC.) Guill & Perr., Combretaceae), is a large tree that mainly grows from Senegal to Sudan. Its leaves and bark are traditionally used in West Africa to treat skin diseases, coughing, asthma, fever, rheumatisms [8] and bacterial infections [9]. Its leaves, roots and bark have an *in vitro* activity on *Plasmodium falciparum* [10,11]. In view of these previous results, we hypothesized that the ethanol bark extract of *A. leiocarpa* could inhibit the growth of *T. gondii,* which also belongs to the Apicomplexa phylum like *P. falciparum.*

From a chemical point of view, we showed previously that the commercially-provided bark of *A. leiocarpa* is mostly composed of highly abundant water-soluble tannins, together with a diversity of low molecular weight metabolites including ellagic acid derivatives, flavonoids, triterpenes and saponosides [12].

The aim of this study was thus to evaluate both the *in vitro* and *in vivo* activities of an *A. leiocarpa* bark extract and purified compounds on *Toxoplasma gondii* infection.

The *in vitro* antitoxoplasmic activity was assessed by incubating tachyzoites of the RH strain with the extract for 72 hours. The crude bark extract was fractionated by centrifugal partition chromatography (CPC). The obtained chemically simplified fractions or purified compounds were chemically profiled mainly by Nuclear Magnetic Resonance and tested again *in vitro* on the RH strain tachyzoites. The most active compounds were then assessed for their inhibition effect on cell invasion by tachyzoites, and finally evaluated *in vivo* on a model of infected mice.

Results and Discussion

The aim of this study was to evaluate the *in vitro* and *in vivo* antitoxoplasmic activities and cytotoxicity of the ethanol (99%) bark extract of the African tree *A. leiocarpa*. The fractionation of the crude extract (3 g injected) and the chemical profiling of the fractions and purified compounds were achieved by CPC and a dereplication process based on ^{13}C NMR [13], respectively. A three-phase solvent system composed of *n*-heptane, M*t*BE, CH_3CN and water was used in a sequential CPC elution mode in order to fractionate the different *A. leiocarpa* compounds over a large polarity range. Water-soluble tannins and sugars being the main compounds in the initial solid-liquid ethanolic crude bark extract, a CH_3CN and water-rich stationary phase was selected in order to trap these highly polar compounds in the CPC column over the whole fractionation experiment. At the end of the fractionation process, 25 chemically simplified fractions or purified compounds were obtained (Table I).

The most hydrophobic compounds were eluted with the *n*-heptane rich mobile phase during the first step of the fractionation experiment (from t_0 to 50 min), representing 10.7 % of the crude extract mass (305.5 mg, fractions F_1 to F_7).

During the second step of the CPC fractionation process (from 51 to 85 min), moderately polar compounds were progressively eluted from the stationary phase to the M*t*BE-rich mobile phase. The corresponding fractions from F_8 to F_{24} represented 16.5 % of the crude extract mass (469.9 mg). Water-soluble tannins and sugars were then recovered in fraction **F_{25}** by extrusion [14] of the CPC column. This fraction represented 72.8 % of the crude mass extract (2071.9 mg). All the 25 fractions were analyzed by ^1H and ^{13}C NMR spectroscopy in order to identify the main class of compounds in the extract and unambiguously identify at least one major member of each class.

Six fractions (**F_5**, **F_7**, **F_{11}**, **F_{13}**, **F_{18}** and **F_{25}**) containing the main characteristic compounds of *A. leiocarpa* bark extract were selected to be tested against *Toxoplasma gondii* RH strain. Fraction **F_{18}** (35.2 mg, 1.2% w/w of the extract) contained purified sericoside **(1)**; fraction **F_{13}** (22.8 mg, 0.8% w/w of the extract) contained purified ellagic acid **(2)**; fraction **F_5** (15.7 mg, 0.6% w/w of the extract) was approximately a 66/33 (based on ^1H NMR signal integration) mixture of 3,3'-di-*O*-methylellagic acid **(3)** and 3,4,3'-tri-*O*-methylflavellagic acid **(4)**; fraction **F_{11}** (28.0 mg, 1.0% w/w of the extract) corresponded to purified trachelosperogenin E **(5)**; fraction **F_7** (11.4 mg, 0.4% w/w of the extract) was approximately 1/3 (based on ^1H NMR signal integration) mixture of trachelosperogenin E **(5)** and catechin **(6)**; fraction **F_{25}** corresponded to water-soluble

tannins and sugars (2071.9 mg, 72.8% w/w of the extract). The sample **F_0** corresponded to the crude extract without water soluble sugars and tannins (CBEWT) obtained after partitioning in the M*t*BE/ CH_3CN/H_2O (4/1/5, v/v) biphasic solvent system. Metabolites **1** to **6** were identified thanks to ^1H and ^{13}C NMR spectral data (see 1S Table). Their molecular structures were validated by further 2D NMR analyses (HSQC, HMBC and COSY) and by comparison with reference NMR data from the literature [15,16]. Their chemical structures are given in Fig 1.

DMSO was able to solubilize all samples, hence it was selected to test all of them (crude bark extract, **F_0**, **F_5**, **F_7**, **F_{11}**, **F_{13}**, **F_{18}** and **F_{25}**) [17].

The cytotoxicity of the crude bark extract, **F_0**, **F_5**, **F_7**, **F_{11}**, **F_{13}**, **F_{18}** and **F_{25}**, was assessed on Vero cells which have significant advantages such as yield and viability for *T. gondii* culture (Table II). The 20% cytotoxic concentration (CC_{20}) values ranged from 7.27 to 85.62 µg/mL and corresponded to the acceptable limit of cell viability. Moreover, the values of 50% cytotoxic concentration (CC_{50}) ranged from 38.57 to more than 200 µg/mL. These results were confirmed microscopically after coloration with the RAL 555 kit. A concentration of 200 µg/mL of the crude bark extract was the most cytotoxic concentration for Vero cells, without inducing a complete cell death (see 2S Fig). Moreover, the fraction **F_5** containing 3,3'-di-*O*-methylellagic acid (**6**) and 3,4,3'-tri-*O*-methylflavellagic acid (**5**) was still very cytotoxic at 20 µg/mL and 40 µg/mL. Lower concentrations showed no cytotoxicity. These two compounds were previously reported as cytotoxic agents against human cells [18]. Nevertheless these data could be useful for other studies focusing on the antiparasitic activity of *A. leiocarpa* metabolites on another intracellular parasite that can also invade Vero cells.

The crude bark extract of *A. leiocarpa* was tested for its potential antitoxoplasmic activity. Selectivity index (SI) was calculated for each tested sample (Table II). The ethanol crude bark extract of *A. leiocarpa* was effective with an IC_{50} of 59.30 ± 9.16 µg/mL(Fig 2A) with a low selectivity index (SI 2.32), confirming that the African birch contains efficient molecules against protozoan parasites as previously described [11]. Remarkably this IC_{50} was in the same order of magnitude of the IC_{50} of sulfadiazine, which is 77 µg/mL (307.66 µM) [6], even though sulfadiazine is generally combined to other active molecules when used as an antitoxoplasmic treatment. This is interesting since there are very few treatments currently available against *T. gondii*. Seven chemically simplified fractions or purified compounds obtained after CPC fractionation of the crude extract were thus tested in order to understand which compound induced

this antitoxoplasmic activity. Tannins (**F_{25}**) and sericoside (**F_{18}**) showed no antitoxoplasmic effect (Table II), that is consistent with previous results obtained on *P. falciparum* [19,20]. The crude bark extract without tannins (**F_0**) showed a lower average IC_{50} of 12.83 ± 1.85 µg/mL (Fig 2B) with an increased selectivity index (SI 9.61). Ellagic acid (**F_{13}**) showed a low antitoxoplasmic activity, with an IC_{50} value of 74.06 ± 19.60 µg/mL (245.08 ± 64.68 µM). This result may seem surprising insofar as it is very active against *P. falciparum* [21]. The mixture of 3,3'-di-*O*-methylellagic acid and 3,4,3'-tri-*O*-methylflavellagic acid (**F_5**) showed an antitoxoplasmic activity, despite its cytotoxicity, with an IC_{50} value of 13.78 ± 3.52 µg/mL but with a low selectivity index SI of 2.80. On the contrary, interestingly, trachelosperogenin E (**F_{11}**) showed an average IC_{50} of 26.63 ± 0.94 µg/mL (51.14 ± 1.81 µM), with a selectivity index of 9.75 (higher than 5) and parasites were not detected at 100 µg/mL (Fig 2C). Trachelosperogenin E (**5**) could explain, at least in part, the antitoxoplasmic activity of *A. leiocarpa*. Finally, a mixture containing mainly catechin with a small amount of trachelosperogenin E (**F_7**) showed no activity against *T. gondii*. Consistently, catechin was previously reported to have no activity on *P. falciparum* [22] and as trachelosperogenin E being the minor compound of this mixture (about 30%), its concentration may not be enough to induce any antiparasitic effect. The IC_{50} of the reference drug, pyrimethamine, was measured at 0.29 ± 0.19 µg/mL (1.20 ± 0.79 µM) (Table II) for the *T. gondii* RH strain. These results were visually confirmed by fixation of the cultures with cold methanol and coloration with the kit RAL 555 for microscopic analyses (see 3S Fig).

Since the crude bark extract without tannins (**F_0**) and trachelosperogenin E (**F_{11}**) were the most active with the most satisfying SIs (9.61 and 9.75, respectively), their mechanism of action was investigated during the cell invasion by RH strain tachyzoites before invading Vero cells. The used concentrations for the cell invasion assay were 20 µg/mL for **F_0** and 30 µg/mL (57.61 µM) for **F_{11}**. Results showed a significant inhibition of the invasion process, with 24% inhibition for CBEWT (**F_0**) and 56% inhibition for trachelosperogenin E (**F_{11}**) (Fig 3). As expected, pyrimethamine showed no effect on *T. gondii* invasion (Fig 3). These results were also confirmed microscopically. Considering their respective efficiency against invasion, it appeared that most of the **F_0** and trachelosperogenin E (**F_{11}**) activity took place during this process. The highest activity against cell invasion by *T. gondii* was indeed due to trachelosperogenin E, whereas CBEWT had a weak effect against it. Regarding its

global activity on *T. gondii* growth, several active molecules should be contained in this extract and could also alter other processes such as parasite multiplication.

Considering these promising results, the *in vivo* antitoxoplasmic activity of the crude bark extract without tannin (**F_0**) and trachelosperogenin E (**F_{11}**) was also assessed. Mice were infected with ME49 strain tachyzoites. DMSO-treated group showed no significant difference in tachyzoites growth compared to untreated infected mice (control group). Only the crude bark extract without tannin provided a protection against brain invasion by *T. gondii* in our chronic toxoplasmosis model. Moreover, CBEWT had a significant activity against the parasite compared to trachelosperogenin E at J_0 by increasing mice survival by 50% ($p < 0.05$) and at J_3 compared to control group ($p < 0.05$). Even if it was not significant, it also increased mice survival rate by 30% at J_{+1}. Nevertheless, a prophylaxis treatment (J_{-1}) had no effect on the parasite load (Fig 4). It was also observed that CBEWT was able to significantly inhibit *T. gondii* growth in a chronic toxoplasmosis model ($p < 0.05$) (Fig 5).

Finally, trachelosperogenin E showed no significant antitoxoplasmic activity in our chronic toxoplasmosis mouse model (Fig 4). Even though CBEWT tended to be more efficient against toxoplasmosis compared to untreated mice at J_0 and J_1, more animals would be required to highlight a significant difference between CBEWT and untreated control group.

In this study, we investigated both the *in vitro* and *in vivo* activity of several extracts, fractions and pure compounds from *A. leiocarpa* bark on *T. gondii*. For the first time, we highlighted that the crude bark extract without tannins and trachelosperogenin E (**5**) showed a promising *in vitro* antitoxoplasmic activity. More specifically trachelosperogenin E was able to inhibit host cells invasion by *T. gondii* tachyzoites. However, molecular analyses should be considered to accurately understand the mechanism of action of *A. leiocarpa* metabolites on *T. gondii*. We also proved that the crude bark extract without tannins had an interesting antiparasitic activity in our chronic toxoplasmosis murine model. In order to enrich our results, it would be interesting to test the active compounds (CBEWT and trachelosperogenin E) on different toxoplasmosis murine models and/or in combination with a classical treatment such as sulfadiazine or pyrimethamine.

Materials and Methods

Chemicals and plant materials

Methyl-*tert*-butyl ether (M*t*BE), *n*-heptane, and acetonitrile (CH_3CN), dimethylsulfoxide (DMSO) were purchased from Carlo Erba Reactifs SDS.

The ethanol (99%) bark extract of *A. leiocarpa* was kindly provided by the company Givaudan (Active Beauty Department, Givaudan France).

Apparatus and operation conditions

Separation were accomplished by Centrifugal Partition Chromatography (CPC) on a column with a capacity of 303.5 mL (FCPE300®, Rousselet-Robatel-Kromaton) containing seven partition disks engraved with 231 twin partition cells (≈ 1 mL per twin cell). The CPC column was filled with the lower phase at a minimal rotation speed of 200 rpm. The rotation speed was then increased up to 1200 rpm. The sample solution was loaded into the column by progressively pumping the less polar upper phase (first mobile phase) from 0 to 20 mL/min in 3 minutes in the ascending mode (KNAUER Preparative Pump 1800® V7115). The upper phase was pumped for 50 minutes to ensure the elution of all hydrophobic compounds. The moderately polar middle phase (second mobile phase) was then pumped for 33 minutes to elute compounds with a medium polarity. Finally, the column was extruded to recover the most hydrophilic compounds retained inside the column (tannins). Fractions of 20 mL were collected over the whole experiment using a Pharmacia Superfrac collector. The separation was monitored by UV at 210, 254, 280 and 366 nm (UVD 170S detector, Dionex).

All CPC fractions were checked by Thin Layer Chromatography (TLC) on Merck 60 F254 pre-coated silica gel plates and developed with ethyl acetate / toluene / acetic acid / formic acid (70:30:11:11 v/v). UV light was used to detect ellagic acid and its derivatives at 254 and 365 nm. TLC plates were then sprayed with a vanillin (5% w/v in EtOH) and H_2SO_4 solution (50% v/v in MeOH), followed by heating at 100-120°C for 2-3 min. About 20 mg of each sub-fraction were dissolved in 600 µL DMSO-*d6* and analyzed by nuclear magnetic resonance (NMR) spectroscopy at 298 K on a Bruker Avance AVIII-600 spectrometer. ^{1}H and ^{13}C NMR spectra were acquired at 600.15 MHz and 150.91 MHz, respectively. Additional heteronuclear single quantum coherence (HSQC), heteronuclear multiple bound correlation (HMBC), and homonuclear correlation spectroscopy (COSY) 2D-NMR experiments were performed

on fractions containing putatively identified compounds using standard Bruker microprograms.

Crude extract fractionation

First of all, 504 mg of the crude bark extract of *Anogeissus leiocarpa* were partitioned in 10 mL of a biphasic solvent system composed of M*t*BE, CH_3CN and H_2O in the proportions 4/1/5 (v/v) in order to separate the highly abundant sugars and water-soluble tannins from the other potentially active secondary metabolites of lower polarity (triterpenes, methylated ellagic acid derivatives, flavonoids and saponins). The upper phase was recovered and evaporated to dryness, resulting in a residue of 141 mg (28% of the crude extract mass). This residue, corresponding to the crude bark extract without tannins, was annotated CBEWT and corresponded to sample $\mathbf{F_0}$.

In parallel, the crude bark extract of *A. leiocarpa* was also fractioned by CPC using method previously fully described [12]. Briefly, a three-phase solvent system was prepared by mixing *n*-heptane (700 mL), M*t*BE (700 mL), CH_3CN (700 mL) and water (700 mL) in a separatory funnel. After separation of the *n*-heptane rich upper phase, 700 mL of M*t*BE were added to the mixture of middle and lower phases in order to slightly reduce the polarity of the remaining two-phase solvent system. For the injection step, 3 g of the crude bark extract of *A. leiocarpa* were dissolved in a mixture of lower phase / middle phase / upper phase (45:10:5 v/v) and the pH was adjusted to 4-5 with sodium hydroxide. All CPC fractions were combined on the basis of TLC composition similarities, resulting in a series of 25 sub-fractions or purified compounds (Table I).

NMR analyses of the CPC fractions

On the basis of NMR spectral profiles, six fractions exhibiting a chemical composition representative of the main molecular classes present in the crude bark extract of *A. leiocarpa* were selected to perform the *in vitro* antitoxoplasmic assays. These selected fractions were $\mathbf{F_5}$ (3,3'-di-*O*-methylellagic acid + 3,4,3'-tri-*O*-methylflavellagic acid), $\mathbf{F_7}$ (trachelosperogenin E + catechin), $\mathbf{F_{11}}$ (trachelosperogenin E), $\mathbf{F_{13}}$ (ellagic acid), $\mathbf{F_{18}}$ (sericoside) and $\mathbf{F_{25}}$ (water-soluble tannins) (Table I). Residual solvent percentages were not evaluated prior to experiments since solvents were evaporated after the extraction of the natural compounds.

T. gondii strains

RH (type I) and ME49 (type II) strains of *T. gondii* were provided by the French Biological *Toxoplasma* Resource Centre (Biological resource center *Toxoplasma*, France).

Parasites growth

RH and ME49 strains tachyzoites were cultured on Vero cell monolayers (American Type Culture Collection (ATCC), CCL-81) at 37°C, 5% CO_2 in a humidified incubator. Both cells and parasites were grown in complete medium Iscove's Modified Dulbecco's Medium/Glutamax (IMDM) (Invitrogen) supplemented with 2% (v/v) fetal calf serum (Biowest) and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin) (GIBCO). Host cells were infected at a 1:1 parasite to cell ratio. Cells and tachyzoites were counted using a Kova Slide counting chamber with Trypan blue (v/v).

In vitro chemosensitivity of *T. gondii* and cytotoxicity evaluation

The *in vitro* chemosensitivity of *T. gondii* (IC_{50}) was assessed as previously described [6]. The crude bark extract, the crude bark extract without tannins (**F_0**), **F_5**, **F_7**, **F_{11}**, **F_{13}**, **F_{18}** and **F_{25}** were tested at ten concentrations obtained by twofold dilution series from a stock solution (crude bark extract and samples **F_0**, **F_7**, **F_{11}**, **F_{13}**, **F_{18}** and **F_{25}**: 0.39 to 200 µg/mL; **F_5**: 0.078 to 40 µg/mL) for 72 hours. Pyrimethamine (Pyr) was used as a positive control. *T. gondii* growth was determined by an enzyme-linked immunosorbent assay (ELISA) on the fixed infected cultures with an anti-*T. gondii* surface antigen-1-horseradish peroxidase (SAG-1-HRP) conjugated monoclonal antibody (Argene Biosoft). Spectrophotometric readings (FLUOstar Omega microplate reader, BMG Labtech) were made at 450 nm, corrected at 630 nm. For a visual control, the last well of each condition was fixed with cold methanol, stained with kit RAL 555 (RAL Diagnostics) and examined microscopically (AxioVert 200 M, Zeiss) at magnification 20x.

The *in vitro* cytotoxicity (CC_{20} and CC_{50}) of samples was evaluated on Vero cell cultures using the UptiBlue viable cell counting assay (Interchim): wells were emptied and washed with cold Phosphate Buffered Saline (PBS) (Sigma-Aldrich). Then, 100 µL of IMDM supplemented with 2% (v/v) fetal calf serum and 10% (v/v) UptiBlue were added in each well. Afterwards plates were incubated at 37°C for 3 hours. The protocol

was slightly the same as described above, except that no parasite was inoculated in wells. Spectrophotometric readings (FLUOstar Omega microplate reader, BMG Labtech) were made at 570 nm, corrected at 600 nm. Visual control was made as described above.

IC_{50} data analysis

The effect of the crude extract and fractions at various concentrations was determined by data plotting as previously described [23] with minor modifications to the y axis. Results were averaged, optical densities (OD) values for cultures without drug treatment were used at 100% value of parasites growth and plotted as a function of the logarithm of each compound concentration. The IC_{50} value was calculated as the sample concentration for which 50% of parasite growth was inhibited.

Selectivity indexes

A selectivity index (SI) was calculated for each sample, as the ratio between cytotoxic and antiparasitic activities:

$SI_{\textit{Toxoplasma}} = CC_{50\ \text{Vero}} / IC_{50\ \textit{Toxoplasma}\ \text{RH}}$

The antitoxoplasmic effect was considered selective if SI > 5. This cut-off point was based on previous studies focused on anti-*Toxoplasma* activities in which SIs had to be > 4 [24] or 6 [25] to be considered selective.

Invasion assay

In order to determine which part of *T. gondii* life cycle was inhibited by the most selective compounds, an invasion assay was performed for each compound. A previously described protocol was adapted to our model [26] . Briefly, in a 96-well plate, 40,000 Vero cells were incubated for four hours at 37°C and 5% CO_2. The wells containing Vero cells were then emptied and refilled with 100,000 tachyzoites (RH strain) preincubated for 20 minutes with active compound or pyrimethamine at room temperature in 100 µL of parasite/active compound suspension per well, except for eight wells as negative control. Eight other wells were refilled with 100,000 tachyzoites without active compound and were used as invasion positive control. The plate was incubated for one hour as described above and wells were washed with phosphate buffered saline (PBS). The plate was then fixed with cold methanol as described

previously and rehydrated with 100 µL of PBS per well for 10 minutes. Wells were emptied again before adding the anti-*T. gondii* SAG-1-HRP conjugated monoclonal antibody (Argene Biosoft) and incubated at 37°C for one hour. They were then emptied and washed before adding an anti-mouse antibody coupled with AlexaFluor 488 and incubated for one hour at room temperature. Spectrofluorimetric readings (FLUOstar Omega microplate reader, BMG Labtech) were made at 490 nm and blank readings were made on the mean value of the eight negative control wells. Microscopic analysis were performed at magnification 40x (AxioVert 200M, Zeiss).

In vivo chemosensitivity of *T. gondii*

The *in vivo* experimentations were approved by the Comité d'Ethique en Experimentation Animale de Reims Champagne-Ardenne (C2EA 56) on July 17th, 2018, acceptation number APAFIS#6851-2016092210116940v3.

The *in vivo* antitoxoplasmic activity of the *in vitro* active and selective compounds was assessed in 8-months old female Swiss mice, weighing 20 grams and provided by Charles River Laboratories. Immunocompetent mice were infected with *T. gondii* ME49 strain (type II) tachyzoites to mimic chronic toxoplasmosis. This strain was chosen due to the high predominance of genotype II strains in Europe [27]. The parasites inoculation and treatments injections were made intraperitoneally. A 1% (v/v) DMSO injection was used as a negative control. In total 95 mice were used for the experiment. The group was divided into four subgroups, each of which was submitted to the following conditions:

I: control group (n = 5): ME49 parasites, no treatment.

II: DMSO group (n = 10): ME49 parasites, injection of 1% (v/v) DMSO 10 minutes after parasites infestation (J0).

III: CBEWT group (n = 10 for each condition): ME49 parasites, injection of CBEWT (200 µg/mL) solubilized in 1% (v/v) DMSO at J0, J+1 (24 hours after infestation), J+3 (72 hours after infestation) or J-1 (24 hours before infestation).

IV: TrachE group (n = 10 for each condition): ME49 parasites, injections of trachelosperogenin E (200 µg/mL) solubilized in 1% (v/v) DMSO at J0, J+1, J+3 or J-1.

Surviving mice were sacrificed 30 days after tachyzoites infestation (J+30) and brains were collected. The samples were then analyzed by Real Time-qPCR to measure the parasite load as previously described [28,29]. If mice died before J+30, the day of death was recorded (J+X). Data were analyzed as survival curves.
Mice were under constant survey during the experiment in order to avoid any suffering or distress induced by the parasite load or the treatments.

Statistical analysis

For CC_{50} and IC_{50} assays, values were expressed as mean ± standard deviation. For the invasion assay results comparison, a one-way ANOVA test ($p < 0.05$) followed by a Holm-Hidak's multiple comparisons test were performed. The software used was GraphPad Prism 7.0.
For the *in vivo* active compounds, statistical analyses were performed using a nonparametric Kruskal-Wallis test followed by a *post hoc* nonparametric Wilcoxon-Mann-Whitney test. P-values < 0.05 were considered to be statistically significant.

Supporting Information

^{13}C NMR assignments of *A. leiocarpa* as well as cytotoxic activity of the crude bark extract and optical microscopic observation of the RH strain of *T. gondii* growth in presence of each compound/fraction from *A. leiocarpa* are available as Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

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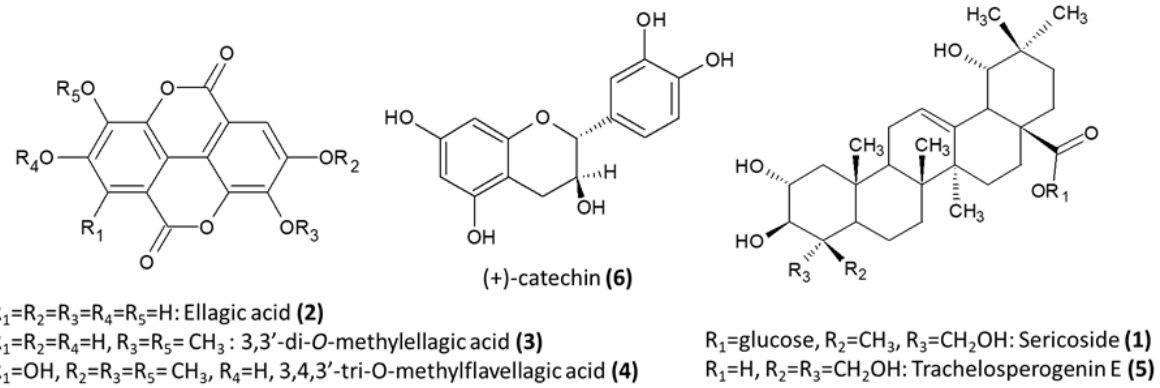
Table I: Mass repartition of the CPC fractions obtained from the ethanolic extract of *Anogeissus leiocarpa*.

CPC fractions*	Mass (mg)	% of the total extract mass	Composition
F_1-F_4	264.1	9.3%	Complex metabolite mixture
F_5	15,7	0,6%	3,3'-di-*O*-methylellagic acid **(3)** + 3,4,3'-tri-*O*-methylflavellagic acid **(4)**
F_6	14,3	0,5%	Complex metabolite mixture
F_7	11,4	0,4%	Trachelosperogenin E **(5)** + catechin **(6)**
F_8-F_{10}	60.5	2.1%	Complex metabolite mixture
F_{11}	28	1,0%	Trachelosperogenin E **(5)**
F_{12}	19,5	0,7%	Complex metabolite mixture
F_{13}	22,8	0,8%	ellagic acid **(2)**
F_{14}-F_{17}	114.2	4.1%	Complex metabolite mixture
F_{18}	35,2	1,2%	pure sericoside **(1)**
F_{19}-F_{24}	189.7	6.8%	Complex metabolite mixture
F_{25}	2071,9	72,8%	Water-soluble tannins and sugars

**in bold, selected fractions for in vitro assays*

Table II. Cytotoxic and antitoxoplasmic activities of the eight samples obtained by CPC and pyrimethamine (Pyr). Values are expressed as 95% confidence intervals. Underlined value is theoretical based on the trend line since CC_{50} was not reached.

Compound	**CC_{50} on Vero cells (µg/mL)**	**CC_{20} on Vero cells (µg/mL)**	**IC_{50} on *T. gondii* (µg/mL)**	**Selectivity index**
Crude bark extract	119.2 – 156.0	19.3 – 44.5	46.6 – 72.0	2.3
Crude bark extract without tannin - CBEWT (**F_0**)	106.1 – 140.5	5.3 – 9.2	10.3 – 15.4	9.6
Sericoside (**F_{18}**)	> 200	74.7 – 89.3	118.2 – 151.2	< 5
Ellagic acid (**F_{13}**)	> 200	9.9 – 32.7	46.9 – 101.2	> 2.7
Methylated ellagic acid derivatives (**F_5**)	26.8 – 50.3	3.6 – 13.1	8.9 – 18.7	2.8
Trachelosperogenin E (**F_{11}**)	<u>252.4 – 266.6</u>	11.2 – 13.6	25.3 – 27.9	9.7
Trachelosperogenin E + catechin (**F_7**)	> 200	58. 2 – 113.0	135.6 – 169.5	< 5
Water-soluble tannins + sugars (**F_{25}**)	> 200	36.4 – 55.8	105.9 – 112.4	< 5
Pyrimethamine (Pyr)	2.1 – 3.0	1.1 – 2.2	0.0 – 0.5	9.0



R_1=R_2=R_3=R_4=R_5=H: Ellagic acid **(2)**
R_1=R_2=R_4=H, R_3=R_5= CH_3 : 3,3'-di-*O*-methylellagic acid **(3)**
R_1=OH, R_2=R_3=R_5= CH_3, R_4=H, 3,4,3'-tri-O-methylflavellagic acid **(4)**

R_1=glucose, R_2=CH_3, R_3=CH_2OH: Sericoside **(1)**
R_1=H, R_2=R_3=CH_2OH: Trachelosperogenin E **(5)**

Figure 1: Chemical structures of the main metabolites recovered from the crude bark extract of *Anogeissus leiocarpa* and selected for biological evaluation against *Toxoplasma gondii*.

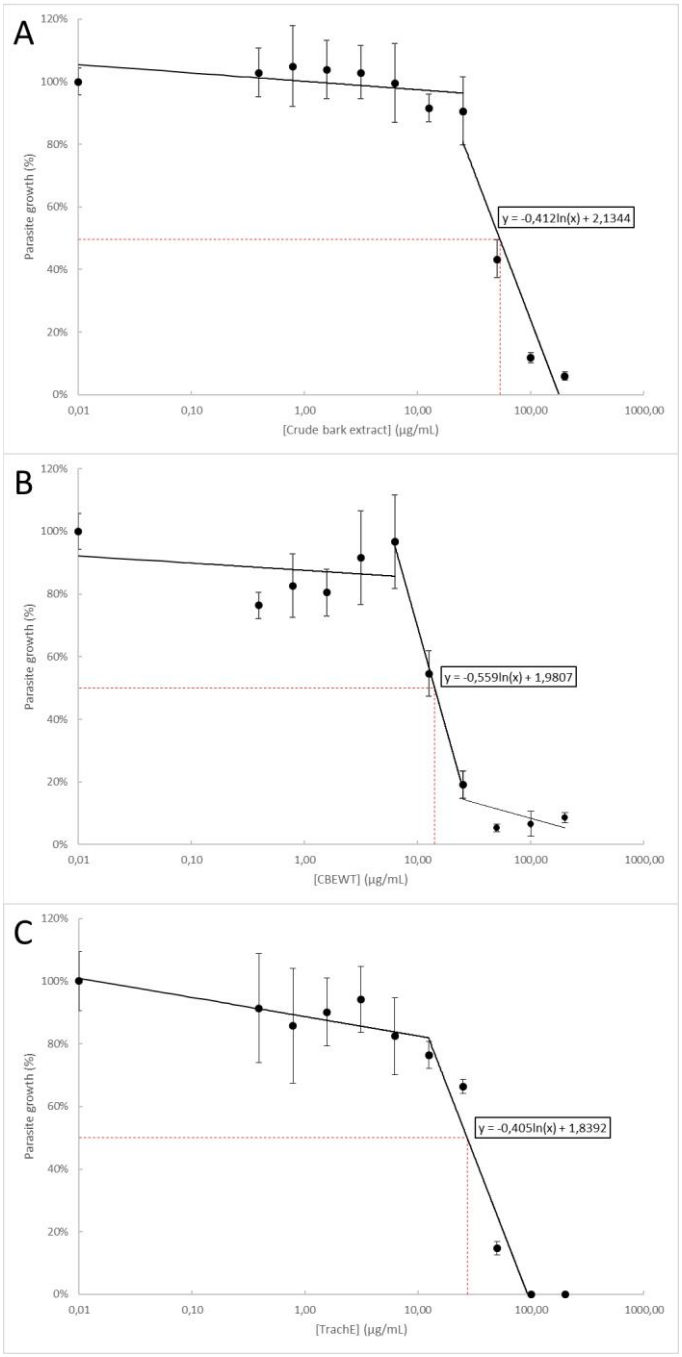


Figure 2: Representative dose-response curves of the *in vitro* effect of crude bark extract (A), $\mathbf{F_0}$ (B) and $\mathbf{F_{11}}$ (C) from *A. leiocarpa* on *T. gondii* RH strain growth after 72 h of incubation (n = three replicates). Each graph shows the tachyzoites growth compared to a positive control and determined by ELISA with infected monolayers (*y* axis) versus compound concentration (*x* axis). Values are expressed as mean ± SD.

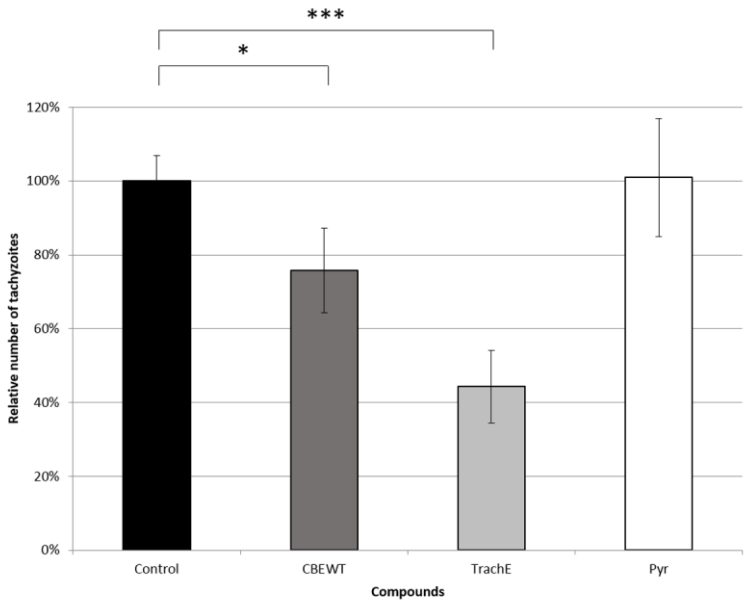


Figure 3: Relative number of parasites after incubation of tachyzoites with crude bark extract without tannin (CBEWT), trachelosperogenin E (TrachE) or pyrimethamine (Pyr). This experiment reflects the inhibition of the cell invasion by *T. gondii* tachyzoites induced by the natural compounds that showed an *in vitro* activity. *p < 0.05, ***p < 0.007 compared to the control group (n = three replicates).

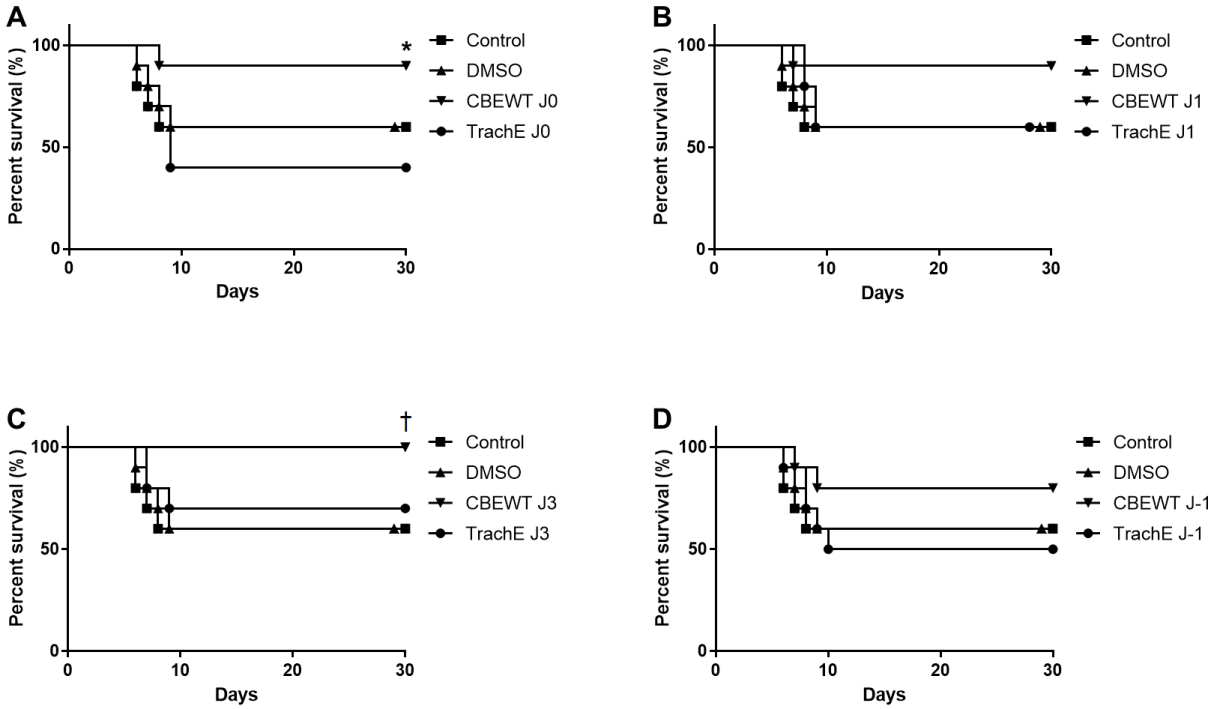


Figure 4: Antitoxoplasmic activity of the crude bark extract without tannins (CBEWT) and trachelosperogenin E (TrachE) in our chronic toxoplasmosis mouse model induced by ME49 strain tachyzoites. Treatments were intraperitoneally injected at J_0 (A), J_{+1} (B), J_{+3} (C) and J_{-1} (D). Control consisted in infected mice without treatment. DMSO consisted in 1% (v/v) DMSO injected in infected mice as a negative control (*$p < 0.05$ compared to TrachE-treated group; †$p < 0.05$ compared to control group).

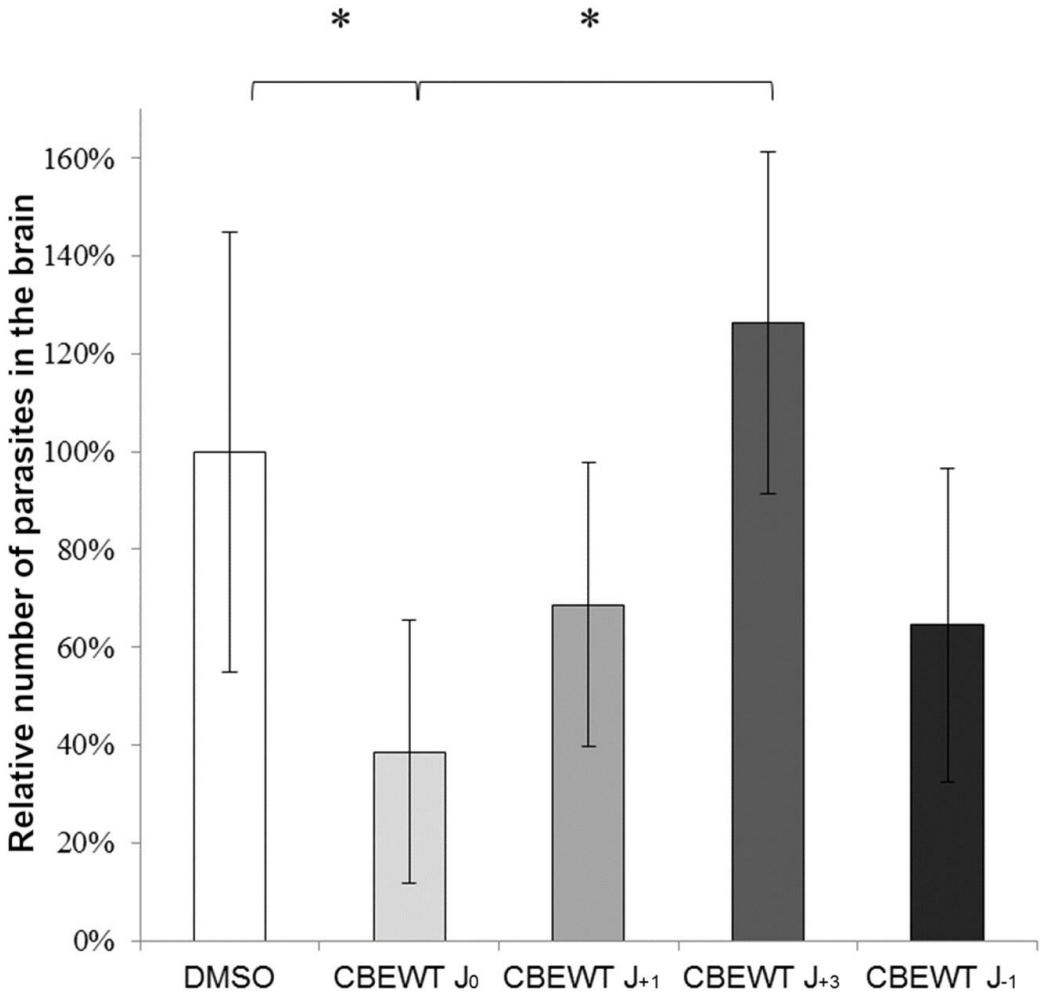


Figure 5: Relative number of parasites detected in brain in a chronic toxoplasmosis mouse model induced by ME49 strain tachyzoites. Ten mice per group were treated with the crude bark extract without tannins (CBEWT) 10 minutes after inoculation (J_0), 24 hours after inoculation (J_{+1}), 72 hours after inoculation (J_{+3}) or 24 hours before inoculation (J_{-1}) (*$p < 0.05$ compared to DMSO control group).