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Original Research

Antiparasitic activity of *Alstonia boonei* De Wild. (Apocynaceae) against *Toxoplasma gondii* along with its cellular and acute toxicity

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ABSTRACT:

Toxoplasmosis (*Toxoplasma*) and malaria (*Plasmodium*) are two disease caused by parasites of the phylum Apicomplex. The treatment of toxoplasmosis is similar to that of malaria. Traditionally, *Alstonia boonei* De Willd. (Apocynaceae) is a plant widely used in the treatment of malaria. This study was conducted to assess the antiparasite activity of the 70% hydroethanolic extract of *A. boonei* on *Toxoplasma gondii* and to study its cellular and acute toxicity. The *in vitro* parasitic and cytotoxic assays were performed on HFF cells while the acute *in vivo* toxicity was performed on (Swiss) mice. The ethanolic extract stopped the proliferation of *T. gondii* with an IC_{50} of 0.13 mg/mL. This extract is neither toxic to HFF cells nor to mice at doses lower than 15000 mg/kg/Vo bw. No signs of behavioural toxicity were observed at this same dose. This confirms the traditional use and its safety in the treatment of *phylum* apicomplex parasites.

Keywords:
Alstonia boonei, Toxoplasmosis*, Toxoplasma gondii,* HFF cells, toxicity.

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INTRODUCTION

Toxoplasmosis is caused by a parasite *Toxoplasma gondii*. The parasite mostly infects warm-blooded animals; for example humans, though the target host is cat (Torda, 2001). Toxoplasmosis is a benign or an asymptomatic infection in the majority of cases affecting immunocompetent subjects. The severity of this infection is related to the risk of transmission of the parasite to the fetus in the case of primary infection of the mother during pregnancy. The severity of the manifestations (chorioretinitis, brain damage) can go as far as spontaneous abortion, depending on the stage of pregnancy during which the infection occurs. This congenital form of toxoplasmosis remains a major public health problem in both developed and developing countries (Botté, 2007). The disease is present all over the world and it is estimated that one third of the world's population is infected with *T. gondii*. Its prevalence in humans is variable. Although present worldwide, it is much more common in areas around the equator, particularly in Latin America, Africa and Madagascar (Camara, 2011). In Côte d'Ivoire, the prevalence is between 40 and 60%, according to the World Health Organization (WHO). These figures are based on the old data. The current prevalence may be higher than these figures as there is no systematic screening for the disease in pregnant women. Toxoplasmosis is transmitted from the mother to her fetus. The risk that the fetus get infected, and the severity of the infection, depends on the stage of pregnancy. The risk is less than 2% before two months of pregnancy in this case the fetal damage is serious. The risk could be 70% at the end of pregnancy and the fetus will then suffer essentially with ocular lesions (Berger, 2003). Synthetic drugs remain the most effective method of treatment against this parasite in mainly immunosuppressed patients with toxoplasmosis. The use of antifolates as antibiotics is more marginal, their use as antiparasitic medication remains essential. In general, these molecules are effective on both malaria (*Plasmodium* species) and toxoplasmosis (*Toxoplasma* species), two infections caused by the parasites of phylum Apicomplexa. However, a problem very often encountered in the case of treatment of infectious diseases has been the emergence of resistances that develop against almost all known inhibitors. Antifolates are not excluded from this resistance occurrence and different types of resistance have been identified that involve in chromosome mutations at most cases (Camara, 2011). These resistance phenomena have prompted us to continue seeking for new bioactive molecules which replace those that have become ineffective for the well-being. Moreover, the populations, mostly in the developing countries, due to difficult economic condition, could not afford imported pharmaceutical products. As a result, they are increasingly using medicinal plants for their treatment (Gnahoué *et al*., 2015). Regarding this situation, it becomes necessary to initiate a scientific research using plants available from our pharmacopoeia in order to achieve the production of new herbal medicines (phytomedicaments). Therefore, following an ethnobotanical survey, *Alstonia boonei* (Apocynaceae) was selected for its many traditional uses but especially as an antimalarial plant for pharmacological tests.

MATERIALS AND METHODS

Preparation of the extract

The trunk bark of *Alstonia boonei* De Willd. (Apocynaceae) harvested and cut, were rinsed with water and dried away from sunlight. After drying, they were crushed to a fine powder using an electric grinder. One hundred grams (100 g) of drug powder was homogenized in 1 liter of ethanol-water solution (70/30) in a blender (Mixer) of the brand Life's Superb (LS-317) at room temperature. The homogenate obtained was filtered on a square of white fabric, on hydrophilic cotton and then on Watman paper successively. After

three extraction cycles (extraction by exhaustion), the volume of the final filtrate was put in an oven set at 50 ° C to remove the extraction solvent (Béné, 2017). The dried evaporate was recovered in the powder form and constitutes the 70% hydroethanolic extract (Eeth70%).

HFF cells

The biological material utilized was a cell line HFF (Human Foreskin Fibroblast) provided by the research facility of Adaptation and Pathogenesis of Microorganisms (LAPM) of Grenoble, France. They are human cells from the prepuce of new born. A confluent cell layer was formed after 96 h and they quit dividing by contact inhibition. After 24 h in culture, they experience mitosis (or dividing cells). These cells were utilized to assess the antiparasitic activity to assess cytotoxicity (Camara *et al*., 2016).

***Toxoplasma gondii* strain and proliferation test**

The *T. gondii* RH-YFP2 strains (RH strain expressing two genes encoding the yellow fluorescent protein) were maintained in culture by passing on the confluent HFF cell layer in D10 medium. The parasites were dropped on glass slides covered with a layer of confluent HFF cells. After 30 sec of centrifugation at about 100 g to accelerate the sedimentation of parasites on HFF cells, the culture plates were incubated at 37°C for 15 min. The cell layer was then washed for three times with PBS medium (phosphate buffer saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) to remove extracellular parasites. The nucleus of HFF cells was stained with Hoechst 33258; and a blue colour was obtained. For intracellular treatment, synchronized invasion with approximately 100 parasites per glass cover, covered with a layer of confluent HFF cells was performed. The culture medium was replaced by D10 medium supplemented with plant extracts (0-1 mg/mL). After 24 h culture, parasitized cells were fixed. The number of parasites inside the parasitophorous vacuoles were counted. The parasites expressing YFP were visualized directly under the epifluorescence microscope.

Cytotoxicity tests

The toxicity study was performed following the method of Mossman (1983). Human Foreskin Fibroblasts (HFF) cells were used in this work. Both HFF (confluent and divisional) cell types were cultured at 37°C, 5% CO_2 in D10 (Dulbecco Minimum Essential Medium, Gibco) supplemented with 10% fetal bovine serum; glutamine 1%; penicillin 50 $U.mL^{-1}$ and streptomycin 50 $\mu g.\mu l^{-1}$.

To measure the cytotoxicity of the ethanolic extract, the HFF cells were seeded in 96-well plates (Cell Star) at a rate of 3000 to 5000 cells per well in 100 µl of D10 medium. Later, the cells were permitted to interact with the plant extract solubilized in PBS buffer for 24 h at various concentrations (0 - 1000 µg/mL). This was done in triplicate (Camara *et al*., 2016).

The cell viability test was performed utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The tetrazolium ring it contains was reduced to formazan by the mitochondrial succinate dehydrogenase of metabolically active cells, producing a purple colour. The amount of precipitate formed is proportional to the number of living cells. In each well, the MTT is added at a concentration of 500 µg/mL and incubated for three hours at 37°C. The formazan crystals are solubilized in 10 mM dimethylsulfoxide (DMSO). Optical density was at 544 nm utilizing a Safir spectrophotometer (Tecan); this estimation of the absorbance made it conceivable to detect the relative amount of living and metabolically active cells. The results were reported as percentage cell viability versus control without plant extract utilizing the following formula (Camara *et al*., 2016):

$$\text{Viability rate} = \frac{\text{Abs544 nm extract}}{\text{Abs544 nm control}} \times 100$$

Acute toxicity study

The determination of acute toxicity was

performed according to the method of Lichtfield and Wilcoxon (1949). The animals used in this study consisted of 60 white mice of Swiss strain (males and females) weighing 18-20 g and provided by the pet farm of the faculty of pharmacy and biological sciences of Félix Houphouët-Boigny. The mice fasted for 12 h before testing. They were acclimatized for one week before the start of the experiment. They were housed in aluminium cages of medium size and were divided into six lots of ten mice each including a control group. The cages were placed in a well-ventilated room. Five doses of ethanolic extract of *Alstonia boonei* were tested (9000, 11250, 15000, 22500 and 45000 mg/kg of body weight). This corresponded to the concentrations of 300, 375, 500, 750 and 1500 mg/mL respectively. The mice in each test-lot received a concentration at the rate of 10 mL/kg of body weight given by gastric gavage while the mice in the control group received only distilled water at the rate of 10 mL/kg of body weight. Two hours later, the mice were given a normal diet. The mortality rate and all clinical signs of toxicity in each lot were noted. The mice were observed at one hour, two hours, four hours, twelve hours, twenty four hour, 2 days, 3 days, 6 days and 12 days. Some parameters were evaluated:

The maximum tolerated dose (MTD)

The lethal dose 100% (LD_{100});

The lethal dose 50% (LD_{50}) (Behrens and Karber formula, 1935) which takes into account: the LD_{100}, the average of the sum of the deaths between two successive doses, the difference between two successive doses and the average of the number of animals used per lot. The formula of Behrens and Karber (1935) is used:

$LD_{50} = LD_{100} - \Sigma (a \times b) / n$

where LD_{100} = minimum dose always deadly; Σ = average of the sum of the deaths between two successive doses; a = difference between two successive doses; b = the average number of animals used per batch; n = average of the total number of animals used.

The behaviour of the mice was observed at these different times and symptomatic disorders such as movement difficulties, grooming, drowsiness, dyspnea and death were observed.

RESULTS

Antiparasitic activity

Figure 1 shows the effect of ethanolic extract of *Alstonia boonei* on the proliferation of *T. gondii*, there is a gradual decrease in the percentage of proliferating as the concentration increases. At the concentration of 0.8 mg/mL, there is a complete inhibition of the multiplication of parasites. An IC_{50} value of 0.13 mg/mL (concentration which inhibits 50% of the proliferation) is then obtained. It can be said that the ethanolic extract of *A. boonei* is effective in blocking the proliferation of *T. gondii*.

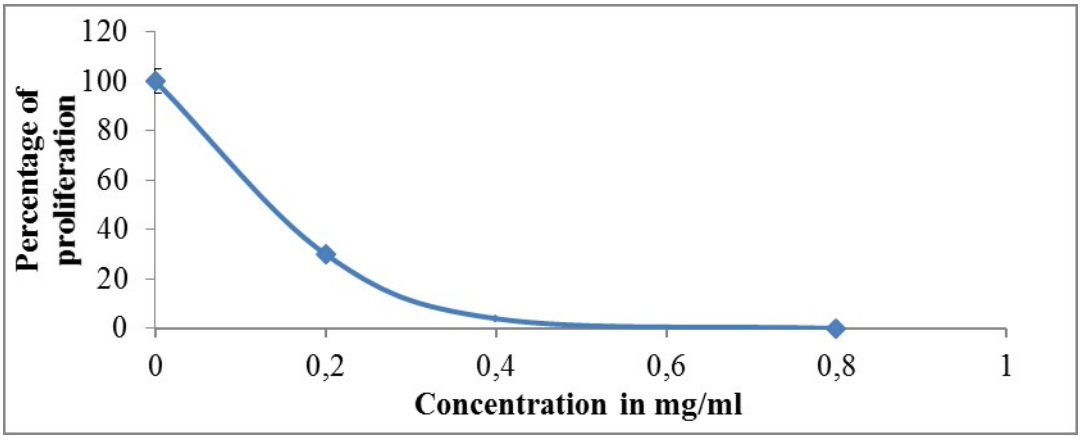

Figure 1. Effect of ethanolic extract of *Alstonia boonei* on the proliferation of *T. gondii* parasite inside human fibroblasts (HFF)

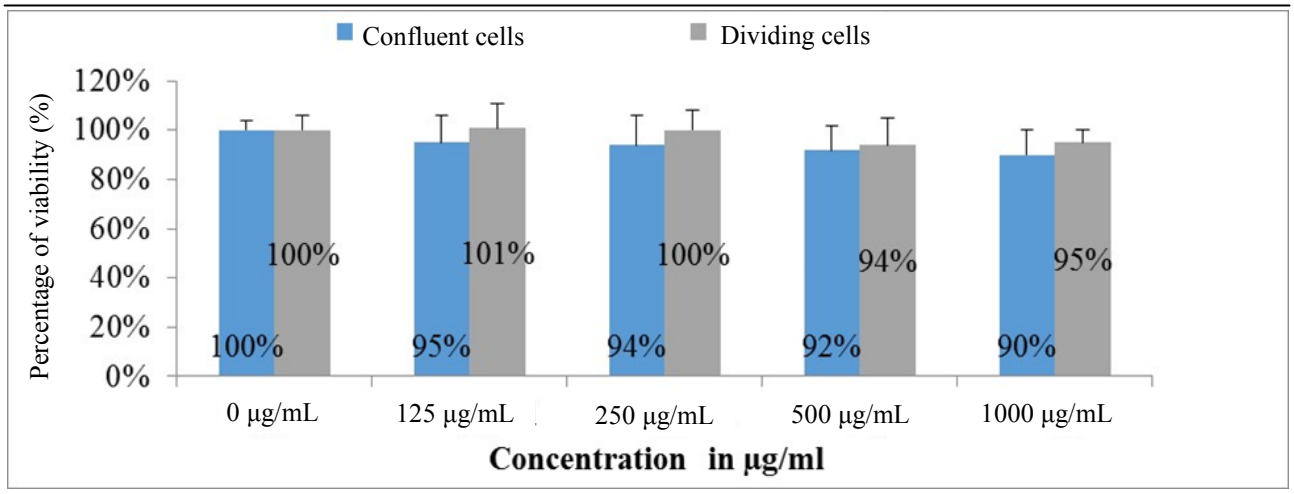

Figure 2. Effect of ethanolic extract of *Alstonia boonei* at different concentrations on the viability of human HFF cells

Cytotoxic activities

Figure 2 shows the effect of 70% ethanolic extract of *Alstonia boonei* on the viability of human HFF cells. For the confluent cells, the percentage of surviving cells decreased from 100% to 90% at a concentration of 1000 µg / ml whereas in the dividing cells, a slight decrease from 100% to 95% at 1000 µg/ mL was observed. Whether the cells are dividing or stopping mitosis, *Alstonia boonei* has very little effect on cell viability. We could deduce that the *Alstonia boonei* extract is not toxic on human HFF cells at the concentrations tested.

Acute toxicity

Determination of LD_{50}

Table 1 shows the values of the percentage of mortality after the administration of the extract. The acute toxicity study carried out after oral administration of the ethanolic extract of *Alstonia boonei* to mice at the different lots and it, did not show any deaths. The highest concentration of ethanolic extract administered was 1500 mg/mL at 10 mL/kg body weight, a maximum dose of 45000 mg/kg body weight. The absence of deaths observed in the treated mice did not make it possible to determine the LD_{50}

Assessment of behavioural parameters

• From 0 to 15000 mg / kg / Vo

Oral doses up to 15000 mg/kg/Vo caused no change in the behaviour of mice (Table 2). No signs of toxicity such as decreased sensitivity to pain, noise or reduced movement were observed.

• From 15000 mg / kg / hour to 45000 mg / kg / hour

At various doses in this range, oral administration of 70% ethanolic extract of *Alstonia boonei* caused symptomatic disorders, increased movement difficulties, dyspnea and drowsines in the treated mice (Table 3). After 24 h these symptomatic disorders disappeared.

Table 1. Toxicological characteristic observation after oral gavage of the mice with the ethanolic extract of *Alstonia boonei*

S. No	Substance administered	Distilled water	Plant extract				
		Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
1	Concentration (mg/mL)	0	300	375	500	750	1500
2	Corresponding dose (mg/Kg/Vo)	0	9000	11250	15000	22500	45000
3	Number of mice per lot	10	10	10	10	10	10
4	Number of dead mice	Nil	Nil	Nil	Nil	Nil	Nil
5	Mortality (%)	Nil	Nil	Nil	Nil	Nil	Nil

Table 2. Effects of ethanolic extract of *Alstonia boonei* on some physiological and behavioural parameters in mice over time. Doses<15000 mg/kg/Vo

Mice with extract 0-15000 mg/kg/Vo										
S. No	**Period**	**1 h**	**2 h**	**4 h**	**12 h**	**24 h**	**2 j**	**4 j**	**6 j**	**12 j**
1	Movement	N	N	N	N	N	N	N	N	N
2	Toilettage	N	N	N	N	N	N	N	N	N
3	Drowsiness	N	N	N	N	N	N	N	N	N
4	Dyspnea	N	N	N	N	N	N	N	N	N
5	Death	0	0	0	0	0	0	0	0	0

N: Normal; A: Aggressive; D: Decreased; P: Pasty; R: Reduced; Au: Increased

Table 3. Effects of *Alstonia boonei* extract on some physiological parameters in mice over time. Doses>15000 mg/kg/Vo

Mice with extract between 15000 mg/kg/Vo and 45000 mg/Kg/Vo										
S. No	**Period**	**1h**	**2 h**	**4 h**	**12 h**	**24 h**	**2 j**	**4 j**	**6 j**	**12 j**
1	Movement	R	R	R	N	N	N	N	N	N
2	Toilettage	D	D	D	N	N	N	N	N	N
3	Drowsiness	Au	Au	Au	N	N	N	N	N	N
4	Dyspnea	Au	Au	Au	N	N	N	N	N	N
5	Death	0	0	0	0	0	0	0	0	0

N: Normal; A: Aggressive; D: Decreased; P: Pasty; R: Reduced; Au: Increased

DISCUSSION

Alstonia boonei De Willd. (*Apocynaceae)* is a plant highly recommended traditionally in the treatment of malaria (malaria). In Côte d'Ivoire, according to Bouquet and Debray (1974) and Fofana (2004), the decoction of the bark is used against malaria, to clean suppurative wounds and for the treatment of open fractures; macerated bark is used against jaundice (jaundice); its sap is used against cough and throat pain and externally against skin infections; the leaf paste also reduces edema. According to the same authors, the bark, the root and the leaves are also used to relieve rheumatic pains and several other pains. This present study has revealed a total inhibition of the multiplication of *Toxoplasma gondii* by the stem bark of 70% ethanolic extract of *A. boonei* at a concentration of 0.8 mg/mL. Toxoplasmosis (*Toxoplasma*) and malaria (*Plasmodium*) are two infections caused by parasites of the phylum apicomplex. The molecules are effective on both malaria and toxoplasmosis (Camara, 2011). Phytochemical studies carried out by Akinmoladun *et al.* (2007) and Chime *et al.* (2013) on the stem bark of *Alstonia boonei* revealed the presence of several chemical compounds including alkaloids that are endowed with antiparasitic activities. The presence of these compounds could justify the action of the ethanolic extract of *A. boonei* on *Toxoplasma gondii*. Indeed, the *in vitro* antiplasmodial activity of alkaloids of *A. Boonei* against the sensitive and resistant strains of *Plasmodium falciparum* and the *in vivo* activity against *Plasmodium berghei* in mice have been reported several years back by authors such as Vasanth *et al.* (1990), Awe and Opeke (1990), Wright *et al.* (1993), Osadebe (2002) and Majekodunmi *et al.* (2008). In addition, Phillipson and Wright (1991) as well as Kipré *et al.* (2018) revealed that alkaloids belonging to the beta-carboline group have various activities, including antiparasitic activities.

The MTT assay allows to visualize the activity of a mitochondrial enzyme: Succinate Dehydrogenase (SDH). SDH is an enzyme involved in mitochondrial respiration, when in a cell, SDH is very active, this implies that the cell is metabolically very active (Béné, 2017). *Alstonia boonei* has very little effect on cell viability, since the percentage of viability varied slightly. It shows the safety of the 70% ethanolic extract of the plant on HFF cells. Indeed, according to the work of Coularie (2012), when the cell viability is greater

than 30%, the substance tested is not toxic. In this case, it is assumed that SDH is not activated, since with both cell types, a high variation in cell viability is not observed.

According to the results of the acute toxicity study, it can be said that the plant is safe because no deaths were observed in mice, even at the highest dose. At doses below 15000 mg/kg/Vo, no signs of toxicity were observed. The LD_{50} was not determined because the highest dose resulted in no deaths.

CONCLUSION

This study investigated the evaluation of the anti -parasitic activity of *Toxoplasma gondii* and the toxicity of the ethanolic extract of *Alstonia boonei* De Willd. (*Apocynaceae*). This extract blocked the proliferation of *T. gondii* with an IC_{50} of 0.13 mg/mL. The ethanolic extract is neither toxic to HFF cells nor to mice at doses, especially below 15000 mg/kg/Vo. No evidence of behavioural toxicity was observed for both the acute toxicity at this same dose. On the other hand, it is true that the doses of the extract higher than 15000 mg/kg/ Vo caused no mortality of the mice, but these doses caused symptomatic disorders, movement difficulties, dyspnea and drowsiness in the treated mice. This study could be used for the development of an effective and improved traditional medicine that can treat toxoplasmosis.

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