

Phytochemical study, antiplasmodial activity and acute toxicity of the aqueous extract of the stem bark of *Alstonia boonei* De Wild

Hermeline Ntalani^{1*}, Mon-Sang César Akouala Bouanga¹, Gelase Fredy Nsonde Ntandou^{3,4}, Dieudonné Mumba Ngoyi², Papy Mandoko Nkoli², Ange Landela², Pascal Robin Ongoka¹, Jean-Maurille Ouamba¹

¹Unité de Chimie du Végétal et de la Vie, Faculté des Sciences et Techniques, Université Marien NGOUABI, BP 69, Brazzaville, République du Congo.

²Laboratoire de Parasitologie, Institut National de Recherches Biomédicales, B.P. 1197, Kinshasa, République Démocratique du Congo.

³Laboratoire de Biochimie et Pharmacologie, Faculté des Sciences de la Santé, Université Marien NGOUABI BP 69, Brazzaville-Congo.

⁴Laboratoire de Physiologie et Physiopathologie Animales, Faculté des Sciences et Techniques, Université Marien NGOUABI, BP 69, Brazzaville- Congo.

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ABSTRACT

Alstonia boonei De Wild is very cited in ethnobotanical surveys and in literature as an antimalarial plant. In the Republic of Congo, traditional therapists use the decoction of *A. boonei* stem bark to treat Malaria. To verify this information obtained from the traditional therapists, we conducted a phytochemical study, evaluated the antiplasmodial activity and the acute toxicity of the decoction of its stem bark. The phytochemical study revealed the presence of alkaloids, tannins, flavonoids and saponins. It also allowed the extraction of total alkaloids with a yield of $6.33 \pm 0.02\%$. The antiplasmodial activity evaluated on the strain isolated from patients infected with *Plasmodium falciparum* was comparable between the decoction ($IC_{50} = 111.2 \mu\text{g}\cdot\text{ml}^{-1}$) and the total alkaloids ($IC_{50} = 116.4 \mu\text{g}\cdot\text{ml}^{-1}$). This activity is therefore due to alkaloids. The lethal dose 50% (LD50) is greater than $5000 \text{ mg}\cdot\text{kg}^{-1}$, the plant is not toxic.

Keywords: *Alstonia boonei*, decoction, phytochemistry, total alkaloids, antiplasmodial, acute toxicity.

*Corresponding author. E-mail: tabunahermeline@gmail.com. Tel: (00242)55559311.

INTRODUCTION

Malaria is one of eradicated diseases in the developed country, with the exception of a few cases of importation, but it remains one of the most widespread and deadly diseases in the world (WHO, 2013). The World Health Organization (WHO) has reported 214 million cases of malaria worldwide in 2015, with 438.000 deaths. About 3.2 billion people, nearly half of the world's population, are at risk of malaria. However, 85% of all malaria cases and 90% of global malaria deaths are concentrated in the African continent (WHO, 2015).

To treat malaria, part of the African population uses traditional medicine (Adebayo and Krettli, 2011; Memvanga et al., 2015; Kodjovi et al., 2017). Africa is rich in biodiversity (UNDP, 2009; Kanouté and Kampmann, 2010, Akaike et al., 2014), and plant biodiversity is an important source of new molecules of therapeutic interest. The most effective antimalarials are quinine and artemisinin (WHO, 2011, 2013), from the *Cinchona calisaya* Weed and *Artemisia annua* L., which are plants used in the traditional Peruvian and Chinese

pharmacopoeia respectively (Bruneton, 2009).

In addition, drawing on quinine and artemisinin, researchers have been able to synthesize other antimalarials such as chloroquine (Surrey and Hammer, 1946) and artesunate (Chekem and Wierucki, 2006). It is therefore very important to have scientific data on traditional remedies. These scientific data may contribute to the discovery of new antimalarial molecules, when resistance to artemisinin has already been reported (WHO, 2011), the current main compound for the treatment of *Plasmodium falciparum* malaria (WHO, 2013), the most dangerous species of *Plasmodium* (Mouchet et al., 2004). The Republic of Congo has a heavy burden of malaria (Moyen et al., 2010, Ntoumi et al., 2013, Koukouikila-Koussounda and Ntoumii, 2016); it is one of the countries with high malaria transmission (WHO, 2016). Combined therapies based on artemisinin or its derivatives (ACT) are used in Congo (Koukouikila-Koussounda et al., 2017; Ndounga et al., 2013), but as in several African countries, in Congo, a portion of the low-income population has a problem of accessibility to drugs (Trapsida et al., 2010). They use traditional medicine to treat malaria (Nsonde-Ntandou et al., 2005; Gando, 2006).

An ethnobotanical survey was carried out among the traditional therapists in Owando, in the Department of the Cuvette, in the Republic of Congo, in order to collect plants used to treat malaria in that locality. During this ethnobotanical survey, *Alstonia boonei* De Wild was selected for being the most cited plant by traditional healers. The purpose of our work is to identify the secondary metabolites, to evaluate the antiplasmodial activity and the acute toxicity of the aqueous extract of *A. boonei* stem bark, in order to verify the information obtained from the traditional therapists on this plant.

MATERIALS AND METHODS

Plant material

The stem bark of *A. boonei* was collected in October 2015, in Brazzaville (Republic of the Congo), on the edge of the TSIEME river, in the District N° 7 named Talangai, and dried for a month at 25°C, out of the sun. A voucher specimen has been deposited at the herbarium of the Institut des Recherches en Sciences Exactes et Naturelles (IRSEN) under reference number 15786.

Animal material

The animal material consisted of Swiss albino mice, (males and females) weighing between 17.06 and 24.67 g, reared at the animal house of the Faculty of Science and Technology of the University Marien NGOUABI in standard conditions (25 ± 5°C, 40 to 70% RH, 12 h light/dark cycle), and fed with standard food with water *ad libitum*.

Ethnobotanical survey

The ethnobotanical survey was conducted in November 2014, in

Owando, in the Department of Cuvette, in the Republic of Congo. Using a questionnaire, the traditional therapists were asked about the plants they use in the treatment of malaria. The vernacular name, the part used and the instructions for use of these plants have been collected.

Phytochemical study

Preparation of the extract

Twenty grams (20 g) of plant material powder were added to 200 ml of distilled water. The resulting mixture was refluxed for 40 min in a 500 ml flask. After heating, the mixture is cooled and filtered with a filter paper and the filtrate is evaporated using a Rotavapor at a temperature of 60°C.

Characterization of secondary metabolites

The tube reaction method was used. Secondary metabolites were characterized in the decoction, but also in the infused, macerated and organic solvents extracts, by the reactions of stains and precipitations with specific reagents according to conventional methods (Bouquet, 1972).

Determination of total alkaloids

20 g of plant material powder were degreased in 300 ml of petroleum ether for 3 h. After filtration, the grounds were dried at room temperature (25 ± 5°C) and macerated for 3 h in 200 ml of distilled water acidified with 2 ml of concentrated sulfuric acid. This operation was repeated until a negative alkaloid identification test was obtained with the MAYER reagent. The various macerates were mixed and obtained mixture was basified with 10% sodium carbonate up to pH = 9. The alkalized solution was then extracted successively with 200 ml of chloroform, until obtaining a negative alkaloid identification test with MAYER reagent. The organic phase was evaporated in Rotavapor to obtain total alkaloids (Bruneton, 2009).

Study of antiplasmodial activity

Collection of infected blood

The study of antiplasmodial activity was carried out at the National Institute of Biomedical Research (I.N.R.B.), which is a National Public Health Laboratory of the Democratic Republic of Congo (DRC). The antiplasmodial activity of the decoction and total alkaloids was evaluated, by optical micro - test method, on parasitized blood, by *Plasmodium falciparum*, taken from a 2 year old child, at the Kingasani Hospital Center in Kimbanséké commune in Kinshasa. This area is known to have a high transmission of resistant *Plasmodium falciparum* malaria. At the time of blood collection, the child had the following characteristics: fever (38°C), no previous taken of antimalarial, positives rapid diagnostic test (RDT) and thick blood smear with 1.3% of *Plasmodium falciparum* parasitaemia.

Realization of the test

Decoction solutions and total alkaloids were prepared with initial concentrations of 10.000 µg.ml⁻¹, then were impregnated into triplicate on the 96-well multiwell plates containing methanol at 50 µl per well. The impregnation on the plates was done with a dilution

of half to half, wells per well, varying the initial concentrations of these solutions from 10.000 to 4.88 $\mu\text{g}\cdot\text{ml}^{-1}$. Quinine (500 mg / 2 ml) and artesunate (120 mg / 12 ml) were used as reference compounds. These compounds were also diluted to concentrations of 6.10 $\mu\text{g}\cdot\text{ml}^{-1}$ for quinine and 0.244 $\mu\text{g}\cdot\text{ml}^{-1}$ for artesunate. The plates impregnated with the solutions were stored at 37°C in a CO₂ incubator for 24 h.

After preparation of the plates, the culture medium was prepared by mixing 20 ml of RPMI 1640 containing 25 mM hepes-L-glutamine buffer with 1.160 ml of 3.6% NaHCO₃; 2 ml of the solution thus obtained were substituted with 2 ml of the serum of the healthy human blood. This culture medium was mixed with 322.5 μl of the parasitized blood and homogenized; this solution containing the parasitized blood was distributed in the plates at a rate of 50 μl /well. The plates were covered, then placed in a jar that was tightly closed. To subtract oxygen and to enrich CO₂ in the jar, a candle was burnt. Whole was then kept in an incubator for 48 h. Then, the thick drops were made from each well. Thick droplet slides were read using an optical microscope (Primo-Star) to determine parasitaemia and calculate the percentage of parasite growth inhibition.

Acute toxicity study

The acute toxicity study of the decoction of bark of *A. boonei* was conducted according to the method described in the OECD Test Guideline No. 423 (OECD, 2011). Three lots of three (3) Swiss albino mice were formed. After three (3) days of acclimation, mice were fasted for 24 h before the experiment. These animals received orally the following doses:

- Lot 1:** control mice receiving distilled water at the dose of 20 ml.kg⁻¹;
- Lot 2:** mice treated with the extract at the dose of 2000 mg.kg⁻¹;
- Lot 3:** mice treated with the extract at the dose of 5000 mg.kg⁻¹.

After administration of the aqueous extract, animals were observed during:

- 3 h to look for a possible change in their general condition (aggression, tail condition, pain sensitivity, mobility and stool condition);
- 48 h to assess mortality;
- 14 days to evaluate the effect of this extract on water intake, food and body weight of mice.

Statistical analysis

Inhibitory concentrations 50% of our extracts were determined using the Origin Pro 8.5 software. Percentage parasite growth inhibition was calculated by the following formula:

$$\text{Inhibition \%} = 100\% - \left(\frac{\text{average of mature parasites}}{\text{average of younger parasites}} \right) \cdot 100\%$$

RESULTS

Ethnobotanical survey

The results of the ethnobotanical survey are presented in Table 1. Eight (8) plants belonging to six families were cited by consulted traditional therapists in this study: Apocynaceae (2), Rubiaceae (2), Costaceae (1),

Aricaceae (1), Euphorbiaceae (1) and Phyllanthaceae (1). *A. boonei* was the most cited (80%). For these traditional therapists, that plant is very effective in the treatment of malaria.

Phytochemical study

Characterization of secondary metabolites

Results of the phytochemical study are presented in Table 2. The chemical families found in the aqueous extract of *A. boonei* bark are alkaloids, tannins, flavonoids and saponins.

Determination of total alkaloids

After a series of three extractions, the quantitative analysis of alkaloids in the bark of *A. boonei* yielded an average mass of 1.2 g of total alkaloids corresponding of 6.33 ± 0.02%, from 20 g of plant material powder.

Antiplasmodial activity

Reading the slides under the microscope and calculating the percentages of inhibition made it possible to obtain the results presented in Table 3. This table shows the different concentrations of the aqueous and alkaloid extracts, and their inhibition percentages. Inhibitory concentrations 50 of our extracts were 111.2 $\mu\text{g}\cdot\text{ml}^{-1}$ for the decoction and 116.4 $\mu\text{g}\cdot\text{ml}^{-1}$ for the alkaloid extract.

Acute toxicity study

Of all the visible signs sought (aggression, tail state, sensitivity to pain, mobility and stool condition), only the reduction in mobility was observed on the day of administration of the extract, in the mice that received the extract at a dose of 5000 mg.kg⁻¹. One death was recorded in lot 3 on the 6th day of the experiment. Figures 1, 2 and 3 show changes in water intake, food consumption, and body weight, respectively, during the 14 days of the experiment.

The observation of Figure 1 shows that the amount of water taken by these animals is greater for lot 3; it increased during the three days following the administration of the aqueous extract of the plant. After three days, this water intake decreased and increased alternately until the end of the experiment. At the level of lots 1 and 2, the water intake has undergone alternately a decrease and an increase from the beginning to the end of the experiment.

Figure 2 shows large food consumption for the mice of lot 3 after the administration of the aqueous extract of the plant. This consumption is greater on the second day.

Table 1. Ethnobotanical survey of plants used to treat malaria in Owando.

Vernacular names	Scientific names	Family	Part of the plant used	Method of preparation
Ekaga (K), Ekaha (M)	<i>Eremospatha cabrae</i> de Wild	Aricaceae	Bark	Decoction
Kongo bololo (L)	<i>Morinda morindoides</i> (Bak). Milne-Red	Rubiaceae	Leaf, stems	Decoction
Motsako (K)	<i>Costus afer</i> Ker-Gawl	Costaceae	Leaves	Decoction
Odjoudjougou (K)	<i>Sarcocephalus latifolius</i> J.E. Smith	Rubiaceae	Root	Decoction
Okocha (K)	<i>Manniphyton fulvum</i> Mull. Arg.	Euphorbiaceae	Bark	Decoction, maceration
Okouga (K)	<i>Alstonia boonei</i> De Wild	Apocynaceae	Bark	Decoction
Onguanguina (M)	<i>Hymenocardia acida</i> Tul.	Phyllanthaceae	Root	Decoction
Oté (K)	<i>Rauvolfia manii</i> Stapf	Apocynaceae	Bark	Decoction

(K): Kouyou, (M): Makoua, (L): Lingala.

Table 2. Chemical screening of the various extracts of *A. boonei*.

Chemical families	Results	
	Decoction	Infused (I), macerated (M) and solvent extracted (S)
Alkaloids	+	+ (M)
Tannins	+	+ (I)
Flavonoids	+	+ (I)
Sterols and tri terpenes	-	- (S - Ether)
Leuco anthocyanins	-	- (I)
Anthracenics free	-	- (S - Chloroform)
Coumarins	-	+ (S -Ethanol)
Saponins	+	+ (M)

+: Positive test; -: Negative test; M: macerated; I: infused; S: solvent extract.

Table 3. Percentage inhibition of parasite growth.

I.C.	10000	5000	2500	1250	625	312.5	156.25	78.125	39.10	19.53	9.77	4.88
I.P.(D)	100	98.99	95.65	88.26	72.67	60.765	52.23	44.32	35.51	22.22	11.67	1.21
I.P.(TA)	100	100	99.53	94.74	82.65	65.24	56.70	43.712	33.82	29.34	15.94	5.20

IC: Inhibitory concentration, I.P.: Inhibition percent, T.A.: Total alkaloids, D: Decoction
0.244 µg.ml⁻¹ of artesunate = 72.81 % IC and 6.10 µg.ml⁻¹ of quinine = 82.10 % IC.

With lot 2, food consumption has alternated a decrease and an increase from beginning to the end of the experience. In lot 1, food consumption decreased from the beginning to the 6th day before to increase and decrease alternately until the end of the experiment.

Figure 3 indicates that the change in body mass of animals is negligible in lots 1 and 2 after the second day. However, this change in body weight is increasing in lot 3. Water intake and food consumption in lot 3 results in weight gain.

DISCUSSION

Ethnobotanical survey

A. boonei was the most cited plant by the traditional therapists during our ethnobotanical survey. In the

literature, *A. boonei* is also cited as an antimalarial by many authors (Iwu, 2014, Adjanohoun et al., 1988). The other listed plants are also mentioned in the literature as an antimalarial: they are: *Hymenocardia acida* (Schmelzer et al., 2008), *Sarcocephalus latifolius* (N'Guessan et al., 2009), *Manniphyton fulvum* (Dibong et al., 2011), *Morinda morindoides* (Zirihi et al., 2010, Tsabang et al., 2017) and *Costus afer* (Iwu, 2014). On the other hand, we did not find bibliographical references evoking the use of *Rauvolfia manii* and *Eremospatha cabrae* in the treatment of malaria.

Phytochemical study

Characterization of secondary metabolites

We were interested in the decoction because the

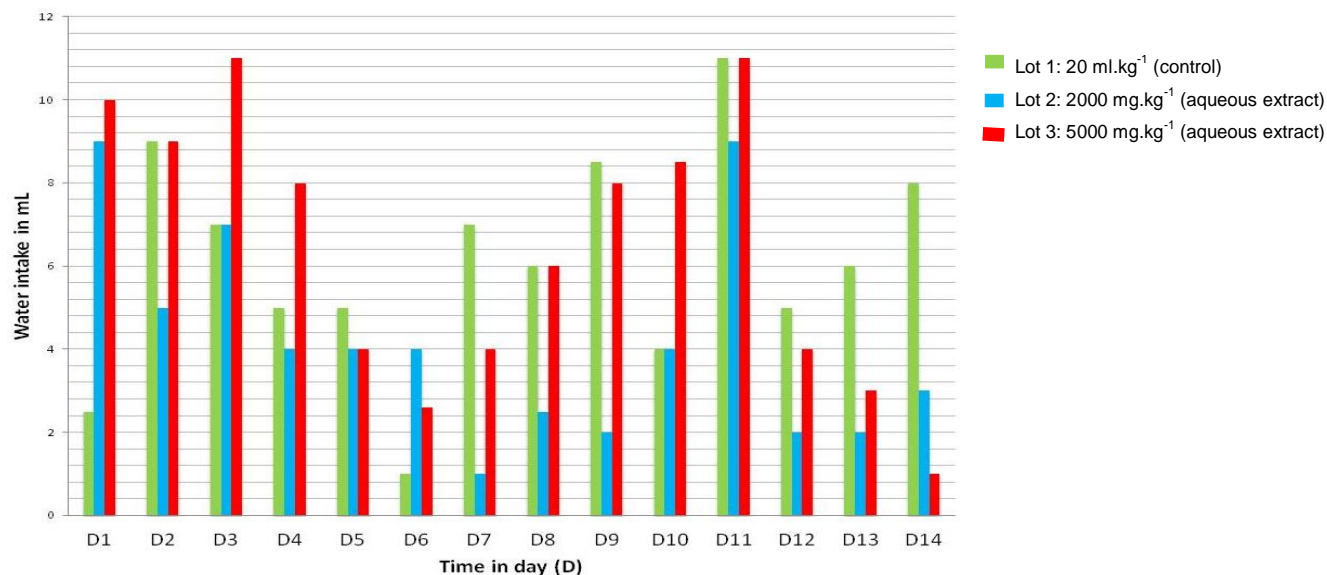


Figure 1. Effects of *Alstonia boonei* De Wild stem bark aqueous extract in water. Results expressed on average $n = 3$.

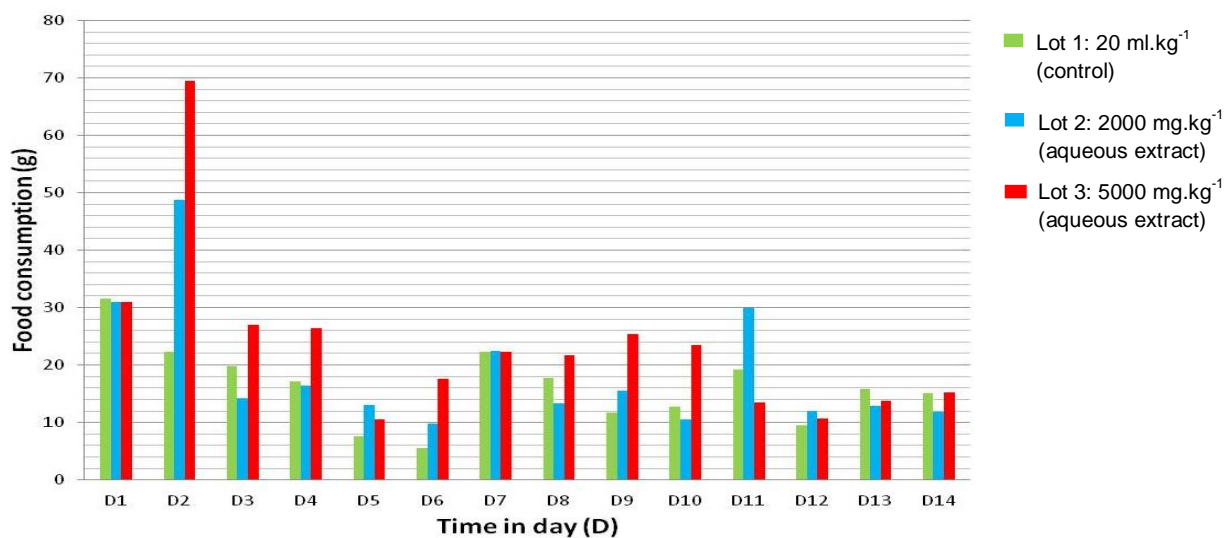


Figure 2. Effects of *Alstonia boonei* De Wild stem bark aqueous extract in food consumption. Results expressed on average, $n = 3$.

traditional therapists use this form of preparation in the treatment of malaria in traditional medicine. The phytochemical study allowed the characterization of alkaloids, tannins, flavonoids and saponins in the decoction of stem bark of *A. boonei*. The flavonoid characterization test gave an orange-yellow color characteristic of flavones. Free sterols and tri-terpenes, leuco anthocyanins and anthracenics were not detected in the various extracts preparation. Coumarins were identified in the ethanolic extract, but not in the decoction. In the literature, Fofana (2004) has also revealed alkaloids, tannins, flavonoids and saponins *A. boonei*

stem bark aqueous extract recolted in the region of Abidjan in Ivory Coast (Fofana, 2004). Alkaloids, saponins and tannins were also observed in the ethanolic extract of *A. boonei* leaves by Dibua et al. (2013) and Olayinka and Maganda (2015). Dibua et al. (2013) also found flavonoids in the ethanol extract of *A. boonei* leaves.

Determination of total alkaloids

The yield of $6.33 \pm 0.02\%$ that we obtained is greater than the following values (0.0436, 0.0430, 0.0318, 0.0196

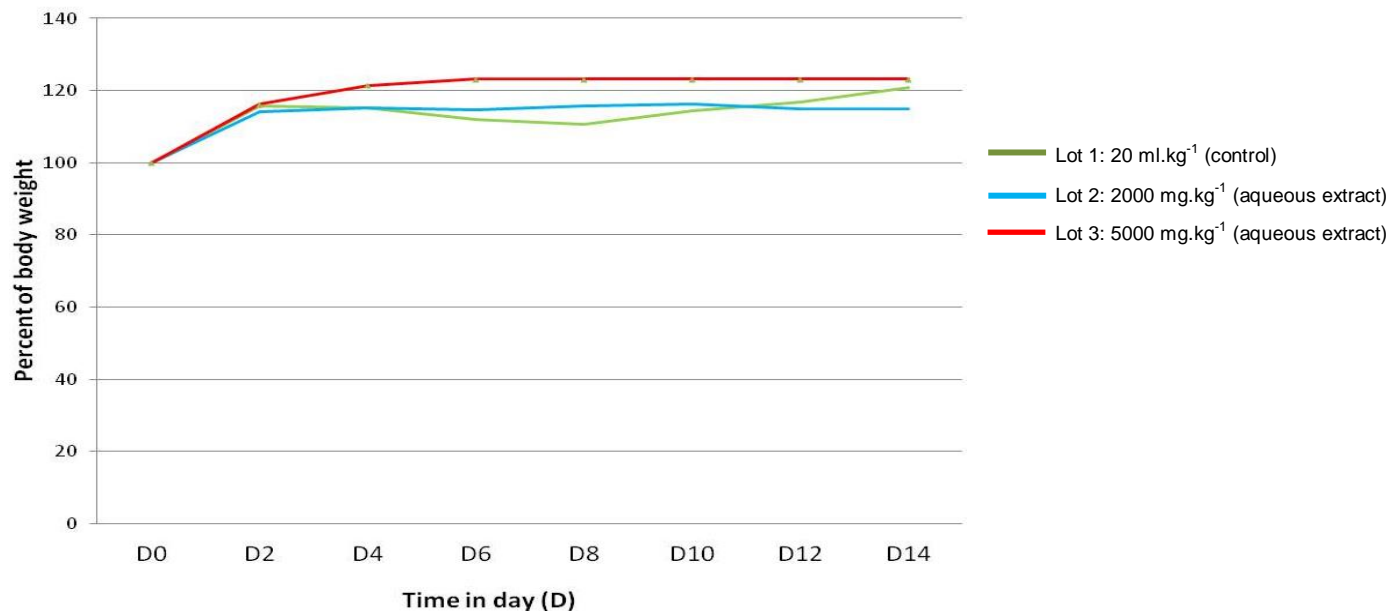


Figure 3. Effects of *Alstonia boonei* De Wild stem bark aqueous extract in weight gain of mice. Results expressed on average $n=3$.

and 0.0174%), which represent the yields obtained by Kémajou et al. (2012), on the ethanolic extract of *A. boonei* bark collected in Zemengoué village in Cameroon and dried at different temperatures. The two solvents used for the preparation of these extracts, not the same polarity and the extraction procedure used, are not the same, which explains the difference observed.

Antiplasmodial activity

The antiplasmodial activity of the aqueous extract ($IC_{50} = 111.2 \mu\text{g.ml}^{-1}$) is comparable to that of the total alkaloids ($IC_{50} = 116.4 \mu\text{g.ml}^{-1}$). These results suggest that the antiplasmodial activity of *A. boonei* is related to the presence of alkaloids in this plant. These two extracts have antiplasmodial activities lower than those of the reference molecules, for each IC_{50} were estimated to be lower than their lowest tested concentrations of $6.10 \mu\text{g.ml}^{-1}$ for Quinine with 82.1% inhibition and $0.244 \mu\text{g.ml}^{-1}$ for artesunate with 72.81% inhibition. The 50% inhibitory concentrations obtained with our two extracts of *A. boonei*, are in the range of those obtained by Sarpong ($IC_{50} > 100 \mu\text{g.ml}^{-1}$) with the ethanolic and ether extracts of the stem bark, of this plant on *Plasmodium falciparum* strains (Sarpong et al., 2016). In the literature, Musuyu et al. (2012) reported an $IC_{50} > 64 \mu\text{g.ml}^{-1}$, for the aqueous extract and Zirihi et al. (2005) an $IC_{50} > 50 \mu\text{g.ml}^{-1}$ for the ethanolic extract of *A. boonei* stem bark on *Plasmodium falciparum* strains.

The low antiplasmodial activity observed in vitro for our two extracts ($IC_{50} = 111.2 \mu\text{g.ml}^{-1}$ and $IC_{50} = 116.4 \mu\text{g.ml}^{-1}$) does not implicate the use of the plant in the

treatment of malaria reported by the traditional therapists. The MALARIAL, for example, marketed in Mali, is a phyto antimalarial drug that has a 50% inhibitory concentration of $600 \mu\text{g.ml}^{-1}$ (Gasquet et al., 1993). Indeed, the molecules constituting an extract may have in vivo interactions that do not occur in vitro.

Acute toxicity study

Compared to the protocol used (OECD 2001), we can say that the aqueous extract of the bark of *A. boonei* has a lethal dose of 50% (LD 50) estimated higher than 5000mg.kg^{-1} . Therefore, this plant is not toxic. This result is similar to that obtained by Nkono, for the study of the acute toxicity of the aqueous extract of the stem bark of *A. boonei* collected at Ombessa in Cameroon (Nkono et al., 2014). Our results are also similar to those obtained by Iyiola et al. (2011) and Dibua et al. (2013) on the *A. boonei* leaves ethanolic extract collected in Shagari and Nsukka (Nigeria).

CONCLUSION

Our study has shown that *A. boonei* plant is mostly used in the treatment of Malaria in traditional medicine, in the department of Cuvette in Congo. Its stem bark decoction extract is not toxic according to the OECD, with a 50% lethal dose that is greater than 5000mg.kg^{-1} . The plant is rich in several secondary metabolites. Its *in vitro* antiplasmodial activity is low and related to alkaloids chemical group.

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