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Jean Emmanuel MbossoTeinkela
Professor, Department of Biological Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O.Box. 2701 Douala, Cameroon

Lisette Pulchérie Yoyo Ngando
Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Hans Denis Bamal
Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Eugène Ekounè Kamè,
Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Albert Emmanuel Mpondo Mpondo
Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Jules Clément Assob Nguedia
Department of Biological Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Simone Véronique Fannang
Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Corresponding Author:
Jean Emmanuel MbossoTeinkela
Professor, Department of Biological Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O. Box. 2701 Douala, Cameroon

Evaluation of *in vitro* antiplasmodial, anti-inflammatory activities and *in vivo* oral acute toxicity of *Trichoscypha acuminata* Engl (Anacardiaceae) stem bark

Jean Emmanuel MbossoTeinkela, Lisette Pulchérie Yoyo Ngando, Hans Denis Bamal, Eugène Ekounè Kamè, Albert Emmanuel Mpondo Mpondo, Jules Clément Assob Nguedia and Simone Véronique Fannang

Abstract
The promotion of traditional medicine by regulating the search for a new adequate therapeutic response and a low price is one of the objectives of the WHO strategy for the years 2014-2023. The genus *Trichoscypha* of the family Anacardiaceae is used in traditional medicine to treat bronchial diseases, rheumatic diseases, sterility, anemia and cancer. Therefore, our work focused on the evaluation of the antiplasmodial, of the total extract and resulted fractions from *Trichoscypha acuminata* Engl (Anacardiaceae) stem bark, as well as the anti-inflammatory activities and the acute oral toxicity of the total extract.
The *in vitro* cell-growth inhibition activities were assessed against *Plasmodium falciparum* strain 3D7 for the antiplasmodial activity. The anti-inflammatory activity was tested only on the total extract (TETAsb) by quantification of the percentage of denaturation of hen egg albumin with (HEA) a concentration range of 25 to 1600 µg/mL of tested extract. while the *in vivo* acute oral toxicity was determined according to the modified Organisation for Economic Co-operation and Development (OECD) guidelines 423 at 2000 mg/kg and 5000 mg/kg body weight on female Wistar strain laboratory rats.
Phytochemical screening revealed the presence of tannins, phenols, triterpenes, flavonoids, alkaloids and glycosides. The antiplasmodial activity showed that only the HFTAsb and DFTAsb fractions at a concentration of 20 µg/mL reduced the viability of *P. falciparum* 3D7 cellswith viability percentages of 39.43% and 40.61%, respectively, and IC_{50} of 29.90 and 28.16 µg/mL, respectively. The total MeOH extract (TETAsb) showed greater anti-inflammatory activity than the reference molecule Diclofenac with a maximum of 428.33% following the HEA denaturation test. An oral administration of the aqueous fraction did not induce an abnormal variation of the physiological parameters in female Wistar laboratory rats, at non-toxic doses up to 5000 mg/kg body weight for 14 days. These results confirm the use of this plant in traditional medicine to treat rheumatic diseases. These studies are the first biological studies conducted on the genus *Trichoscypha* and the species *Trichoscypha acuminata*. All these results constitute a biological basis of this species and this genus.

Keywords: *Trichoscypha acuminata*, phytochemical analysis, antiplasmodial, anti-inflammatory, acute toxicity

Introduction
Traditional medicine is an important and sometimes underestimated component of health services. In some countries, traditional or non-conventional medicine may be called complementary medicine. Traditional medicine has a long history of being practiced to maintain health or to prevent and treat disease, especially chronic diseases. The World Health Organization (WHO) Strategy for Traditional Medicine 2014-2023 was developed in response to a World Health Assembly resolution on traditional medicine [1]. The objective of this strategy is to assist states in harnessing the potential contribution of traditional medicine to health, wellness, and person-centered health care, and to promote the safe and effective use of traditional medicine through regulation, research, and the integration of traditional medicine products, practices, and practitioners into health systems [1].
The use of plants for therapeutic purposes (herbal medicine) has a long history and is currently experiencing renewed public interest. It is possible to use whole plants or the products of their extraction. In this perspective of valorization of traditional medicine, we therefore focused our work on the study of *Trichoscypha acuminata* Engl (Anarcadiaceae).

The choice of this plant was motivated on the one hand by its traditional use: the bark is used as a remedy against constipation in infants [2], infertility and dysmenorrhea for woman, rheumatism, as face wash against pustules and by women for bleeding during pregnancy [2]. A decoction is also used in steam baths and lip frictions for bronchial ailments, headaches, feverish stiffness, side or stomach pain, as a vermifuge and aphrodisiac [2]. This plant is also used for the treatment of cancer in the southern regions of Cameroon. On the other hand, we should mention here that no biological studies have been conducted on *T. acuminata* to the best of our knowledge, but plants of the Anacardiaceae family are known to possess anti-inflammatory and antiplasmodial properties [3]. We can nevertheless note the phytochemical study of the aqueous extract of the fruits of *T. acuminata* which made it possible to isolate a pentahydroxy dihydrobiflavonol also called acuminatanol [4]. The present study therefore focuses on the evaluation of antiplasmodial and anti-inflammatory activities and acute toxicity of extracts from the stem bark of *T. acuminata.*

Materials and Methods

Sample collection and Extraction

The plant materials were obtained from the Sam Timber Group Cameroon Ltd forestry exploitation in the Littoral Region, Cameroon and identified at the Cameroon National Herbarium, by comparison to the sample of J and A RAYNAL No. 13449, registered under N°12964/HNC. The stem bark, of *T. acuminata* was harvested, air-dried away from light for 8 weeks and pulverized. Exactly 2.3 kg of the pulverized stem bark was cold extracted in 96° methanol for 48 hours (h) with occasional shaking. The extract was then filtered through Whatman's N°1 filter paper and the methanol filtrates were separately concentrated to dryness *in vacuo* using a rotary evaporator at 60 rpm [5]. The methanolic extract (TETAsb: 89.3 g) was obtained and 15 g were removed for the subsequent bioassays. The extraction yield was then calculated according to the following formula:

$$Yield = \frac{\text{weight of the dried crude extract}}{\text{weight of the powder}} \times 100$$

Next, 200 mL of water was added to the remaining 74.3 g of methanolic dry extract in order to carry out successive liquid-liquid extractions using a separating funnel and with different solvents of increasing polarity, namely hexane, dichloromethane and ethyl acetate, in that order. The total extractable contents (TECs) were 8.14% (6.05 g), 4.8% (3.57 g), 22.94% (17.05) and 64.1% (47.63), for the hexane fraction (HFTAsb), dichloromethane fraction (DFTAsb), ethyl acetate fraction (EFTAsb), and aqueous fraction (AqFTAsb) respectively.

Phytochemical analysis of plant extracts

The total extract and the four fractions were tested for the presence of secondary metabolites (steroids, triterpenes, phenols, flavonoids, tannins, alkaloids, saponins, coumarins), anthocyanins, heterosides, glycosides) following common procedures described by Ronchetti et Russo (1971), Hegnauer (1973), Wagner (1983), Békro *et al*. (2007), with slight modifications [6-9].

General experimental procedure

We used malaria parasites (*Plasmodium falciparum* 3D7 strain). They were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 25 mM Hepes (Lonza). This medium was supplemented with 5% Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 ug/ml gentamycin and 2-4% human red cell hematocrit. Chloroquine was used as the reference drug. 20% DMSO was used as solubilizing solvent. L-lactic acid, APAD (acetylpyridine adenine dinucleotide), NBT (nitro blue tetrazolium), PES (phenazine ethosulphate), triton X-100 and trizma base were used as reagents. For the study of anti-inflammatory activity we used hen's egg albumin (HOA), a phosphate-buffered saline solution (PBS, Ph 6.4). Diclofenac was used as the reference drug. Female Wistar rats were used for acute toxicity.

Antiplasmodial activity

The activity against *Plasmodium falciparum* chloroquine-sensitive 3D7 strain was assessed following the procedure described by Makler & Hinrichs in 1993 and Mbosso *et al*. in 2018 [10, 11]. Absorbances from parasite lactate dehydrogenase activity were measured at 620 nm for cultures incubated for 48 h at different concentrations of the *T. acuminata* methanolic extract and fractions in 96-well plates to assess parasite viability. Chloroquine was used as the reference medicine, 10% DMSO as solvent, L-lactic acid, acetylpyridine adenine dinucleotide (APAD), nitroblue tetrazolium (NBT), phenawine ethosulphate (PES), triton X-100 and trizma were used as reagents. Experiments were performed in triplicate.

Anti-inflammatory activity

The *in vitro* anti-inflammatory activity of the total extract of *T. acuminata* stem bark sample (TETAsb) was carried out using the protein denaturation inhibition method, involving the preparation of three solutions:

- The test solution (5 mL) consisting of 0.2 mL Chicken Egg Albumin (CEA), 2.8 mL saline and 2 mL test extract with a dilution range from 1600 µg/mL to 25 µg/mL of order 2 (in 7 test tubes).
- Test control solution (5 mL) composed of 0.2 mL CEA, 2.8 mL saline solution and 2 mL distilled water.
- Test standard solution (5 mL) consisting of 0.2 mL CEA, 2.8 mL saline and 2 mL Diclofenac sodium standard solution with a concentration range of 1600 µg/mL to 25 µg/mL.

The various mixtures were incubated at room temperature for 20 min, and then heated to 70°C for 5 min. After cooling, absorbance was measured at 660 nm. The control represents 100% of denatured proteins; and results were compared with Diclofenac sodium. The experiments were performed in triplicate. The percentage of inhibition of protein denaturation (% Inh) is calculated using the following formula:

$$\%\, Inh = \left(\frac{Vt}{Vc - 1}\right) X\ 100$$

Vt: absorbance of sample
Vc: absorbance of control

Acute toxicity

The assessment of acute oral toxicity was determined according to the modified OECD guidelines 423 at a fixed

dose [12]. Female *Wistar* strain laboratory ratsaged 8 to 12 weeks were randomly selected and fasted 12 hours before the test by receiving *ad libitum* water. After this fast, the rats were weighed (D0) and the test substance was administered to them orally using an orogastric tube according to the following distribution: the control group (3 rats) received distilled water at 10 mL/kg body weight; 3 rats (group 1) received the total extract of *T. acuminata* stem bark at 2000 mg/kg bw; and 3 rats (group 2) received the total extract of *T. acuminata* stem bark at 5000 mg/kg bw. After the substance was administered, the animals were observed individually at least once during the first 30 minutes and regularly during the first 24 hours after administration, with particular attention during the first 4 hours. They were observed for 14 days following the administration of the substance.

The observations focused on changes in the skin, body hair, somato-motor activity, and behavior. Particular attention was paid to various manifestations such as tremors, convulsions, diarrhea, lethargy, sleep and coma. The rats underwent weighing during a study period respectively each day: on D0 (the day of administration), to D13 (the 14th day after administration) to assess the weight change.

Results

Phytochemical screening

Phytochemical tests have shown the presence of tannins, triterpenes, phenols and heterosides in the 5 different fractions. Anthraquinones were found in 3 fractions (TETAsb, EFTAsb, AqFTAsb), steroids in 3 fractions (TETAsb, DFTAsb, AqFTAsb). The TETAsb and HFTAsb fractions revealed the presence of alkaloids. The Table 1 summarizes the results of our phytochemical screening using various standard tests.

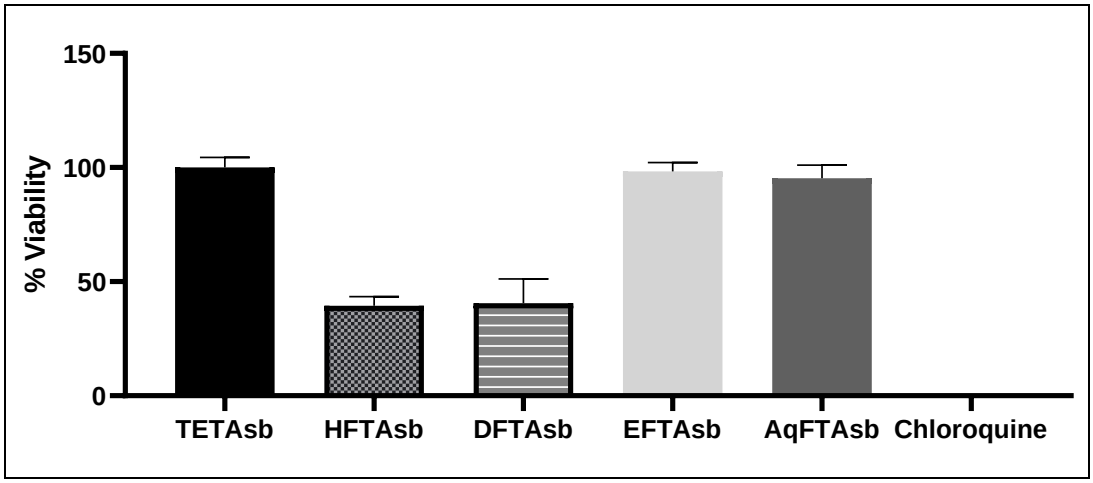
Table 1: Phytochemical screening of the total methanol extract of *Trichoscypha acuminata* stem bark and its various fractions

Phytochemicals	TETAsb	HFTAsb	DFTAsb	EFTAsb	AqFTAsb
Heterosides	+	+	+	+	+
Triterpenes	+	+	+	+	+
Polyphenols	+	+	+	+	+
Tannins	+	+	+	+	+
Flavonoids	+	+	+	-	-
Alkaloids	+	+	-	-	-
Steroids	+	-	+	-	+
Anthraquinones	+	-	-	+	+
Saponins	-	-	-	-	-
Glycosides	-	-	-	-	-
Anthocyanins	-	-	-	-	-
Coumarins	-	-	-	-	-

Legend: + = presence; - = absence; TETAsb = total extract of *Trichoscypha acuminata* stem bark; HFTAsb = hexane fraction of *Trichoscypha acuminata* stem bark; DFTAsb = dichloromethane fraction of *Trichoscypha acuminata* stem bark; EFTAsb = ethyl acetate fraction of *Trichoscypha acuminata* stem bark; AqFTAsb = aqueous fraction of *Trichoscypha acuminata* stem bark.

Assessment of antiplasmodial activity

The viability percentage of *Plasmodium falciparum* 3D7 cells and the standard deviation obtained for each sample is reported in Figure 1 Only the hexane (HFTAsb) and the dichloromethane fraction (DFTAsb) significantly reduced the viability of *Plasmodium falciparum* 3D7 by 39.43 and 40.61% respectively at a concentration of 25 µg/mL. The other fractions did not reduce viability and showed percentages of viable cells of 100.08% for the total extract (TETAsb); 98.26% for the ethyl acetate fraction (EFTAsb) and 95.34% for the aqueous fraction (AqFTAsb).

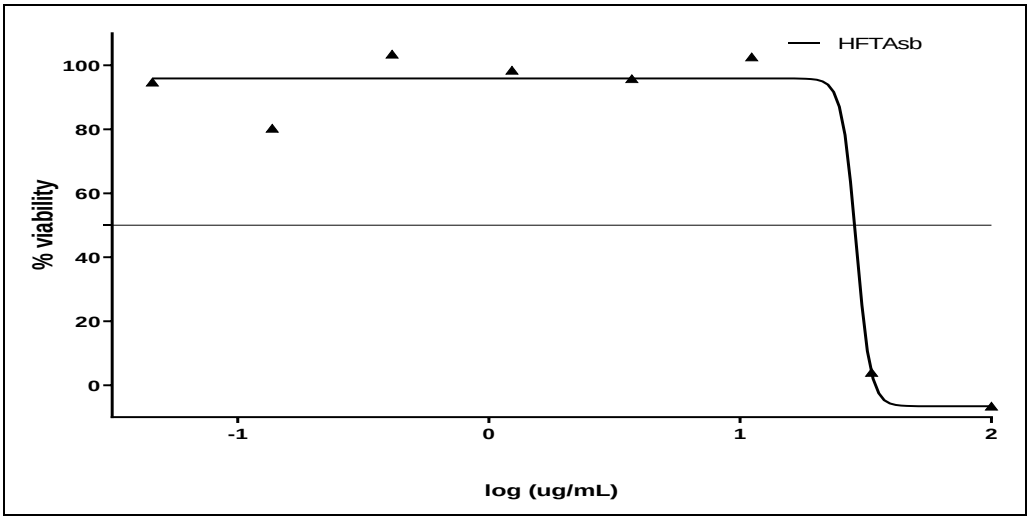


Legend: TETAsb = total extract of *Trichoscypha acuminata* stem bark; HFTAsb = hexane fraction of *Trichoscypha acuminata* stem bark; DFTAsb = dichloromethane fraction of *Trichoscypha acuminata* stem bark; EFTAsb = ethyl acetate fraction of *Trichoscypha acuminata* stem bark; AqFTAsb = aqueous fraction of *Trichoscypha acuminata* stem bark.; Choroquine = reference drug used

Fig 1: Percentage of viability of *Plasmodium falciparum* 3d7 cells on *Trichoscypha acuminata* stem bark total extract and fractions. The values are the means of 3 independent experiments with standard error bars indicated

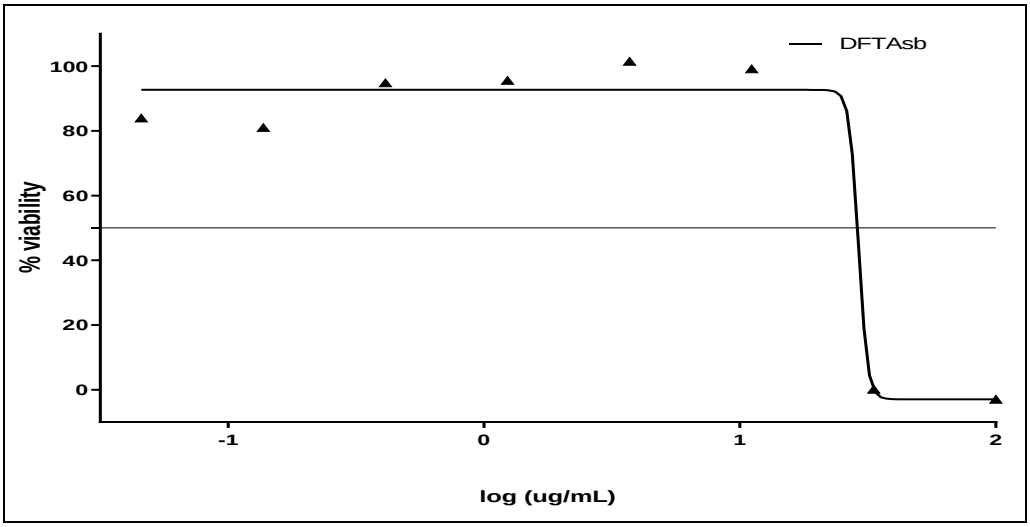
Samples TETAsb, EFTAsb and AqFTAsb did not significantly reduce parasite viability at a concentration of 20 µg/mL. However, samples HFTAsb and DFTAsb showed a 60.57% and 59.39% reduction in parasite viability respectively.

Subsequently, the IC_{50} of hexane fraction (HFTAsb) and the dichloromethanefraction (DFTAsb) were determined by graphical regression method on dose-response curves at a fixed-concentration of parasite (25 µg/mL). Promising antiplasmodial activities were obtained with IC_{50} of 29.90 µg/mL for hexane fraction (Figure 2) and 28.16 µg/mLfor the dichloromethane fraction (Figure 3).



Legend: HFTAsb = hexane fraction of *Trichoscypha acuminata* stem bark

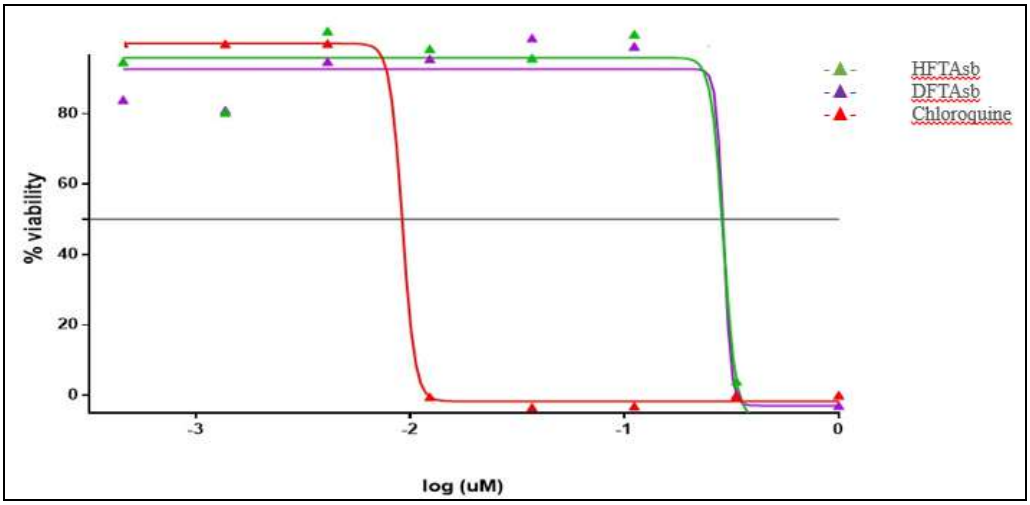
Fig 2: Dose-response curves of the antiplasmodial assay of hexane fraction of *Trichoscypha acuminata* stem bark. GraphPad Prism program version 5.02 was used for data analysis.



Legend: DFTAsb = dichloromethane fraction of *Trichoscypha acuminata* stem bark

Fig 3: Dose-response curves of the antplasmodial assay of dichloromethane fraction of *Trichoscypha acuminata* stem bark. GraphPad Prism program version 5.02 was used for data analysis.

For comparison purposes, chloroquine (an antimalarial drug) was used as a standard with IC_{50} of 0,015 µM (Figure 4).



Legend: HFTAsb = hexane fraction of *Trichoscypha acuminata* stem bark; DFTAsb = dichloromethane fraction of *Trichoscypha acuminata* stem bark; Chloroquine = reference drug used

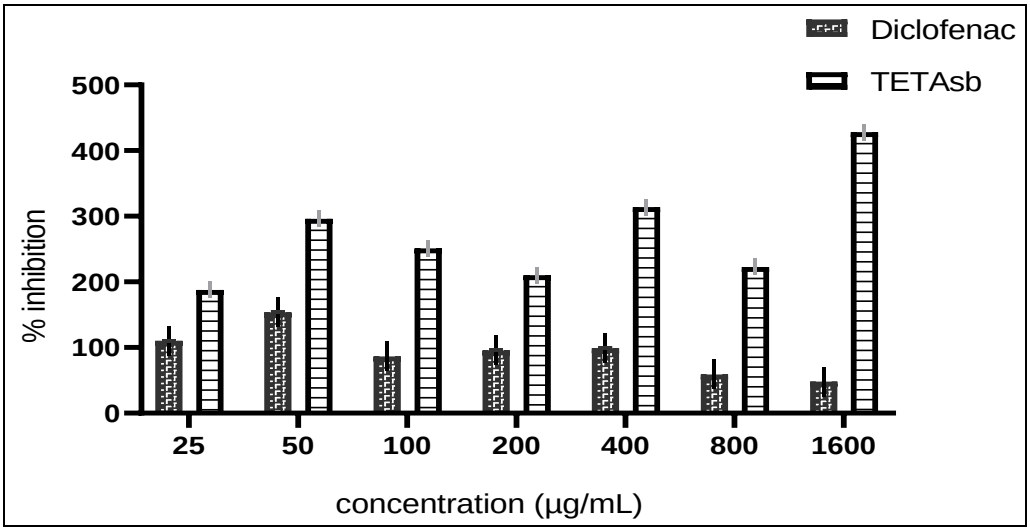
Fig 4: Comparison of dose-response curves for chloroquine, the hexane fraction and the dichloromethane fraction

This figure shows that Chloroquine has a more significant antiplasmodial activity than the other two fractions.

Assessment of anti-inflammatory activity

The percentage inhibition of Chicken Egg Albumin (CEA) denaturation of the total extract of *Trichoscypha acuminata* stem bark (TETAsb) was evaluated. According to the results observed (figure 5), the TETAsb sample significantly inhibits CEA denaturation over the concentration range 1600 µg/mL to 25 µg/mL. TETAsb's percentage inhibition ranged from 187.5% to 428.33%.

The results obtained for this extract were compared with those obtained for Diclofenac sodium, an anti-inflammatory drug used as a standard, which exerted a percentage inhibition ranging from 48.33% to 153.75% at the same concentrations.



Legend: TETAsb = total extract of *Trichoscypha acuminata* stem bark; Diclofenac = reference drug used

Fig 5: Comparison of percent inhibition of CEA denaturation of Diclofenac and TETAsb

This figure shows that the TETAsb sample has a higher percentage of AOP denaturation inhibition than Diclofenac and consequently possesses more significant anti-inflammatory activity.

Acute toxicity

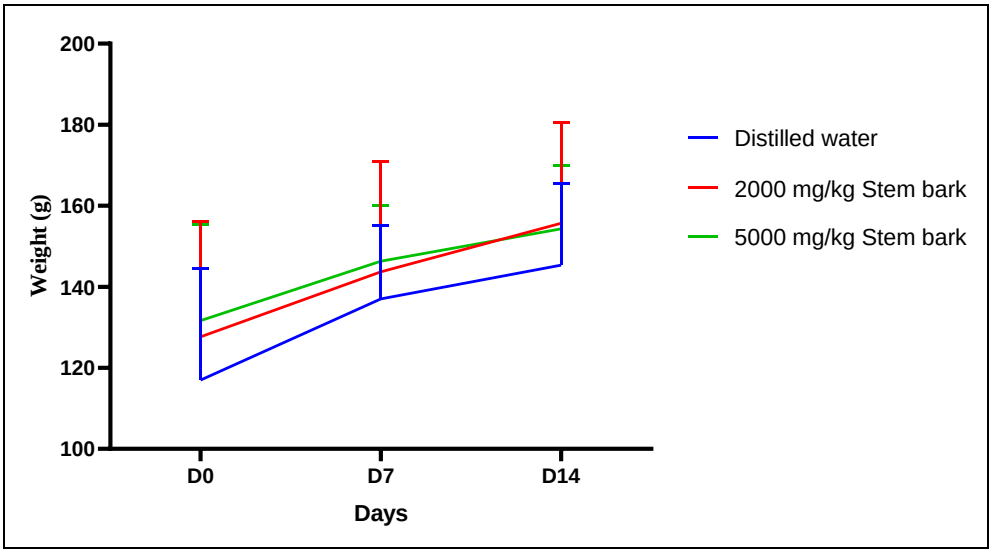
Total extract of *T. acuminata* stem bark was administered to rats on a body weight basis. The LD_{50} is the concentration of substance in mg/kg causing the death of 50% of a given animal population under precise experimental conditions. After oral administration of a single dose of the aqueous fraction, abnormal variation of the physiological parameters was not observed during 14 days of the assay for groups 1 and 2 of rats compared to the rat control group, and therefore was considered as non-toxic at doses of 2000 and 5000 mg/kg. Consequently, the LD_{50} can be considered as greater than 5000 mg/kg. The result of Table 2 shows, in the same way as for the determination of the LD_{50}, that the aqueous fraction has no impact on the physiological parameter of the rats.

Table 2: General observations in acute toxicity.

	Witness batch (distilled water 10 mL/kg)	batch 1 (extract at 2000 mg/kg)	batch 2 (extract at 5000 mg/kg)
Number of rats	3	3	3
Coat modification	A	N	N
Eyes modification	A	A	A
Regurgitation	A	A	A
Trembling	A	A	A
Convulsions	A	A	A
Impaired gait	A	A	A
Nervousness	N	N	N
Mating attitude	A	N	N
Salivation	A	N	N
Lethargy	A	N	N
Slumber	N	N	N
Itching	N	N	N
Agitation	N	N	N
Vomiting	A	A	A
Intense thirst	N	N	N
Nutrition	N	N	N
Bizarre behaviors	A	A	A

A= absence; N= normal;

After administration, the animals were individually weighed at least once every 14 days (Figure 6), after which the rats were sacrificed and an autopsy was performed on a macroscopic level: an evolution of the weight mass of the rats during the study was therefore recorded



Legend: TETAsb = total extract of *Trichoscypha acuminata* stem bark

Fig 6: Evolution of rats' weight gain after administration of TETAsb

Despite the difference in size between the rats that received the extract at a dose of 2000 and 5000 mg/kg.bw, the average weight of the rats generally increased over the 14-day span, regardless of the batch.

Discussion

Methanol extraction of *Trichoscypha acuminata* stem bark was carried out by double maceration. This polar solvent was chosen for our study on one hand for its low boiling temperature of around 65°C (a temperature that minimizes the risk of damage to secondary metabolites during evaporation on rotary evaporator) and on the other hand for its capacity to dissolve a large proportion of polar and non-polar compounds [13].

Fractionation was carried out using solvents of increasing polarity, giving us the hexane, dichloromethane, ethyl acetate and aqueous fractions. The yields resulting of this fractionation showed that the aqueous fraction (AqFTAsb) had the highest yield with a value of 64.1%, followed by the ethyl acetate fraction (EFTAsb) with 22.94%, then the hexane fraction (HFTAsb) with 8.14%, then the dichloromethane fraction (DFTAsb) with 4.8%, relatively to the total extract (TETAsb). These values would mean that *T. acuminata* stem barkcontains more apolar than polar compounds. Phytochemical test shave shown that the crude extract (TETAsb) and the different fractions of *T. acuminata* stem bark contain 8 classes of compounds i.e. alkaloids, tannins, steroids, triterpenes, flavonoids, anthraquinones, polyphenols, heterosides, while glycosides, anthocyanins, saponins and coumarines were not detected, in accordance with the work carried out by Nyangono in 2017 [14], which revealed that the genus *Trichoscypha* (*T. oddini*) would contain, in addition to the metabolites revealed in our study, other metabolites such as saponins and coumarins which were not revealed in our study. This could be due to the insolubility of the total extract and fractions in water.

Evaluation of the *in vitro* antiplasmodial activity of the crude extract and fractions of *T. acuminata* stem bark of at a concentration of 20 µg/mL, on a 3D7 line of *P. falciparum* showed that only the HFTAsb and DFTAsb fractions reduced the viability of the parasite by around 60.57% and 59.39%. Determination of the IC_{50} of the HFTAsb and DFTAsb fractions that reduced malaria viability enabled us to obtain IC_{50} values of 29.90 µg/mL for the HFTAsb fraction and 28.16 µg/mL for the DFTAsb fraction. The activity of these fractions was determined according to the classification established by Jonville *et al*. in 2008, which stipulates that: an extract is very active if its $IC_{50} < 5$µg/ml; an extract is active if $5 \leq IC_{50} < 15$ µg/mL; it has moderate activity if $15 \leq IC_{50} \leq 50$ µg/ml; and the extract is considered inactive if its $IC_{50} > 50$ µg/mL [15]. As a result, we can say that the HFTAsb and DFTAsb fractions with an IC_{50} of 29.90 and 28.16 µg/mL respectively have moderate activity on *Plasmodium falciparum*. These HFTAsb and DFTAsb fractions having reacted positively to the flavonoid and alkaloid tests (HFTAsb) could justify their antimalarial activity. The TETAsb extract did not show any activity, which could suggest antagonism between the different molecules present in this extract and this could be the reason why it is not known in the traditional pharmacopoeia for treating malaria. This antiplasmodial activity observed on the *Trichoscypha* genus (*T. acuminata*) corroborates the studies carried out by Horgen *et al*. in 1997 and Roumy in 2007, which showed respectively that the *Swintonia genus* (*S. foxworthyi*) and the *Tapirira* genus (*T. guianensis*), also from the Anacardiaceae family, have moderate (CI_{50} =19.2 µg/mL for *T. guianensis*) and very active (CI_{50} = 3.5 µg/mL for *S. foxworthyi*) antiplasmodial properties [16, 17], all tested on *Plasmodium falciparum* strains.

Comparing the inhibitory effect of different concentrations of total extract (TETAsb) of *T. acuminata* on protein denaturation as shown in Figure 5 and that of the standard (Diclofenac) at the same concentration range from 1600 to 25 µg/mL, we observe a significant inhibition of CEA denaturation in a dose-dependent manner. We find that TETAsb extract is more active than Diclofenac with a difference of at least 70% for the concentration at 25 µg/mL and at most 380% for the concentration at 1600 µg/mL.

Protein denaturation is one of the causes of inflammation [18, 19]. The possible mechanism of denaturation involves alteration of the electrostatic, hydrogen, hydrophobic and disulphide bonds that maintain the three-dimensional structure of proteins [18, 20]. Several anti-inflammatory proteins have shown a dose-dependent ability to inhibit thermally induced protein denaturation. Protein denaturation is indeed the cause of inflammation in conditions such as rheumatoid arthritis. These anti-inflammatory proteins against protein denaturation, which was the main mechanism of action of NSAIDs postulated by Mizushima and kobayashi in 1968 before the discovery of the inhibitory effect on cyclooxygenase by Vane in 1971, may play an important role in the anti-rheumatic activity of NSAIDs [19, 21]. The inhibitory activity of the denaturation of CEA can be attributed to the richness of this extract (TETAsb) according to the results of the phytochemical screening in tannins, flavonoids, Anthraquinones, all having an anti-inflammatory activity. The results are in agreement with previous reports where phenolic compounds, including flavonoids, showed anti-inflammatory activity through various mechanisms [22, 23], and also by the fact polyphenols and alkaloids are known for their antioxidant properties [24, 25]. These results also corroborate the studies carried out by Aouissa *et al*. in 2002, which showed that the *Mangifera indica* species of the Anacardiaceae family also has anti-inflammatory activities [26]. These results would therefore justify the use of this plant in traditional medicine to treat rheumatic conditions.

After oral administration of a single dose of the aqueous fraction, abnormal variation of the physiological parameters was not observed during 14 days of the assay, and therefore was considered as non-toxic at doses of 2000 and 5000 mg/kg. According to Clarke's work, a LD_{50} greater than 5000 mg/kg of body weight is considered as non-toxic for animal testing [27]. The parameters that appeared during the observation phase could be due to effects associated with the administration of methanol. This study corroborates the work carried out by Aouissa *et al*. in 2002, which showed that the *M. indica* species of the Anacardiaceae family was not toxic at the same doses administered during our study [26].

Conclusion

The phytochemical screening revealed the presence of secondary metabolites such as tannins, triterpenes, phenols, sterols, flavonoids and alkaloids. This would justify the use of this plant in traditional medicine for the treatment of infections. The total MeOH extract (TETAsb) showed anti-inflammatory activity, justifying its use in traditional medicine to treat rheumatic conditions. The acute toxicity test revealed that the total extract of *T. acuminata* has a lethal dose of over 5,000 mg/kg and is therefore not toxic at this dose. This is the first biological study carried out on the genus *Trichoscypha* and the species *Trichoscypha acuminata*. All the results of this study form a biological basis for this species and genus.

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Conflicts of interest

The authors have declared that there is no conflict of interest.

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