Evaluation of *in vitro* antiplasmodial, antiproliferative activities, and *in vivo* oral acute toxicity of *Spathodea campanulata* flowers

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ABSTRACT

Spathodea campanulata is used in traditional medicine to treat various ailments such as malaria, human immunodeficiency virus (HIV), cancer, fever and urethral inflammation. The aim of this study was to investigate the antiplasmodial, and antiproliferative activities of the extract and resulted fractions from *S. campanulata* flowers, as well as assessing the acute toxicity of its aqueous fraction. The *in vitro* cell-growth inhibition activities were assessed against *Plasmodium falciparum* strain 3D7 for antimalarial activity and three cancer cell lines: Hs683 (human oligo-dendroglioma), MCF7 (human breast carcinoma), and murine B16F10 (mouse melanoma) for antiproliferative activity while the *in vivo* acute oral toxicity was determined according to the modified organisation for Economic Co-operation and Development (OECD) guidelines 423 at a fixed dose on Female Wistar strain laboratory rats. The dichloromethane, ethyl acetate and hexane fractions at a concentration of 25 µg/mL each significantly reduced the viability of 3D7 *Plasmodium* cells with viability percentages of 19.0%, 14.1% and 31.9%, respectively, and IC_{50} of 28.1, 30.2 and 29.7 µg/mL, respectively. The ethyl acetate fraction showed a moderate anti-proliferative activity on mouse melanoma with an I_{50} value of 54.6 µg/mL. Only the dichloro-methane fraction was able to inhibit the 3 cell lines tested with IC_{50} values less than 15 µg/mL. An oral administration of the aqueous fraction did not induce an abnormal variation of the physi-ological 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 for its antimalarial and anticancer potential.

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Introduction

Spathodea campanulata P. Beauv. (Bignoniaceae) is a species native to tropical Africa. Its original range extends in the rainforest regions, approximately from Guinea to Angola along the west coast, to southern Sudan and to eastern Uganda. However, the exact limits of geographic boundaries are uncertain, as the tree being present in various neighboring countries where it could have been introduced by man [1]. As a result of human intervention, this plant is found in most tropical regions of the world, notably in Asia, Southern Africa, America, and Oceania [1].

S. campanulata has many medicinal applications both in the countries where it originates and in those where it was introduced; it could be effective as a prophylactic against malaria and in the fight against Aedes mosquitoes [2–4]. In Cameroon, especially in the central region in the departments of Mefou-and-Afamba, Nyong-and-So'o and Nyong-and-Mfoumou, village healers use a bark decoction for the treatment of hemorrhoids and as an antibiotic [5]. In Kenya, the leaves and bark are used to treat colorectal, skin, and cervical cancers [6]. In Ivory Coast, a decoction of the bark and leaves is used as an antimalarial medication [7]. The ethanol extract of the leaves has shown anticonvulsant and anti-epilepsy activity [8]. Other investigations demonstrated that the methanol extract of its bark has restorative activity for wounds and burns [9].

The flowers of *S. campanulata* have not been studied extensively. In a previous work, we showed that the methanol extract of *S. campanulata* exhibits moderate antiproliferative activity against human cancer cell lines with a half maximal inhibitory concentration (IC_{50}) value of ~78 µg/mL, and moderate antimicrobial activity against *Klebsiella pneumonia* with a minimal inhibitory concentration (MIC) value of 156.2 µg/mL [10]. In another study, the flower and bark ethanol extracts were evaluated for a comparative phytochemical screening [11]. The anthelmintic and anti-hyperglycemic potentials of chloroform and ethyl acetate/n-butanol fractions from *S. campanulata* flowers were also determined, as well as the phytochemical contents [12]. Previous studies of the other part of *S. campanulata* revealed that the stem bark and leaves have aphrodisiac and antimalarial activities [13,14].

The antiplasmodial activities of the flower fractions of *S. campanulata* have not yet been studied; therefore the aim of this work was to evaluate the potential of the methanolic extract and of four sub-fractions from *S. campanulata* flowers. The antiproliferative activity on three cancer cell lines was examined, as well as the safety of the aqueous fraction by acute toxicity test. In addition, phytochemical screening was carried out.

Methodology

Collection and preparation of plant materials

The flowers of *S. campanulata* used in this study were harvested in September 2019, in Bafou, a village located in the West region of Cameroon, and authenticated at the National Herbarium of Cameroon (specimen number 50085/HNC).

Extraction and fractionation

Plant material (*S. campanulata* flowers) was cut into small pieces, air-dried and ground to a powder. Then, 800 g of the powder were macerated at room temperature with 95% ethanol, followed by a liquid-liquid partition using a separating funnel with organic solvents of increasing polarity. After evaporation, the MeOH portion under reduced pressure using a rotary evaporator at 40°C, we obtained 18.1 g of a methanolic extract (denoted MESCf) from which 2.5 g was removed for the subsequent bioassays. Next, 200 mL of water was added to the remaining 15.6 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 ranging from hexane, dichloromethane and ethyl acetate. The total extractable contents (TECs) were 27.9%, 29.7%, 4.0% and 38.5%, for the hexane fraction (HFSCf), dichloromethane fraction (DFSCf), ethyl acetate fraction (EFSCf), and aqueous fraction (AqFSCf) respectively.

Phytochemical screening

Detailed phytochemical screening was performed on the five fractions using standard methods, as reported in the literature [15–18]. Other specific phytochemical tests were also realized, all based on a precipitation reaction via the generation of insoluble complexes called precipitates, and on colorimetry through the formation of colored soluble chemical species. The color reactions were carried out in test tubes in the presence of the reference positive controls. The following tests were used: Drangendorff test (alkaloids), Tannins (gallic tannins), Libermann-Burchard test (steroids and triterpenes), Shinoda (flavonoids), Cardiotonic glycosides (cardiotonic glycosides), Borntrager (anthraquinones), Foam Index test (saponins) and $FeCl_3$ test (polyphenols). All observations were recorded.

General experimental procedure

A cell line of malaria parasites namely the *Plasmodium falciparum* strain 3D7, was provided by the Biomedical Research Center of Rhodes University in South Africa. The culture medium of 3D7 cells was an RPMI 1640 medium containing 2 mM L-glutamate and 25 mM Hepes (Lonza) and supplemented with 5% of Albumax II, 20 mM glucose, 0.65 mM Hypoxantine, 60 µg/ml gentamycin and 2–4% human red blood cells. Three cancer cell lines were used: Hs683 (human oligodendroglioma, primary brain cancer), MCF7 (human breast carcinoma), and murine B16F10 (mouse melanoma). The culture medium of cancer cell lines was an RPMI 1640 medium adjusted with phenol red at 25 mM hepes (Biowittacker, Verviers, Belgium), 10% Fetal Bovine Serum (GIBCO-Lige Technology,

Merelbecke, Belgium), 2% L-glutamine at 200 mM, 2% penicillin-streptomycin at 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin (GIBCO-Life Technology, Verviers, Belgium) and 0.2% gentamicin at 50 mg/mL (GIBCO-Life Technology, Verviers, Belgium). Cancer cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

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 [19,20]. Absorbances from parasite lactate dehydrogenase activity were measured at 620 nm for cultures incubated for 48 h at different concentrations of the *S. campanulata* 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.

Antiproliferative activity

The half maximal inhibitory concentration values (IC_{50}) of all fractions were determined *in vitro* after 3 days of culture using the MTT 3-[4,5]-dimethylthiazol-2yl-diphenyltetrazolium bromide assay (Sigma, Ostende, Belgium), as previously described in a panel of three cancer cell lines [21]. The tested samples are different fractions of a methanolic extract: EFSCf, HFSCf, DFSCf at 0, 10, 50 and 100 µM. The optical densities of the plates were measured at 570 nm by visible spectrophotometry using a 680XR Bio-Rad plate reader. Only three extracts could be tested as we encountered solubility issues with the two others (MESCf and AqFSCf) which could not be dissolved at a solvent level preserving cell integrity (*i.e.* <1% DMSO). Experiments were performed in triplicate. The validation of the tests was done using a positive control *i.e.* emetine at 10 µM (for cell apoptosis). IC_{50} values for cytotoxicity were not determined due to the low inhibition observed in the single concentration screening.

Acute toxicity

The assessment of acute oral toxicity was determined according to the modified OECD guidelines 423 at a fixed dose [22]. Female Wistar strain laboratory ratsaged 8 to 12 weeks were randomly selected and fasted 12 h 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 (bw); 3 rats (group 1) received the aqueous extract at 2 g/kg bw; and 3 rats (group 2) received the aqueous extract at 5 g/kg bw. After the substance was administered, the animals were observed individually at least once during the first 30 min and regularly during the first 24 h after administration, with particular attention during the first 4 h. 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 tests have shown that the crude extract (MESCf) and the different fractions of *S. campanulata* contain 6 classes of compounds *i.e.* alkaloids, galic tannins, cardiotonic glycosides, steroids, triterpenoids, and saponins, while polyphenols, anthraquinones and flavonoids were not detected. The Table 1 summarizes the results of our phytochemical screening using various standard tests.

The viability percentage of *Plasmodium falciparum* 3D7 cells and the standard deviation obtained for each sample is reported in

Table 1
Phytochemical screening results of *Spathodea campanulata* flower extracts

Type of test	Chemical compound family	Observation	Fractions				
			HFSCf	DFSCf	EFSCf	AqFSCf	MESCf
Dragendorf	alkaloids	orange coloring	+	–	–	–	+
Tannins	gallic tannins	blue green	–	–	+	–	+
Libermann Burchard	steroids	blue-purple coloring	–	–	–	–	+
Libermann Burchard	triterpenes	brick red coloring turning purple	–	–	–	–	+
Schinoda	flavonoids	orange or purplish pink	–	–	–	–	–
Cardiotonic glycosides	cardiotonic glycosides	dark reddish layer of brown color	+	–	–	+	+
Borntrager	anthraquinones	pink or purple	–	–	–	–	–
Foam index	saponins	presence of foam	–	–	+/-	+/-	+/-
Iron chlorid (FeCl3)	polyphenols	more or less dark blackish blue or green coloration	–	–	–	–	–

+ = presence; - = absence; +/- = presence in small quantity. HFSCf = hexane fraction of *S. campanulata* flowers; DFSCf = dichloromethane fraction of *S. campanulata* flowers; EFSCf = ethyl acetate fraction of *S. campanulata* flowers; AqFSCf = aqueous fraction of *S. campanulata* flowers; MESCf = methanol extract of *S. campanulata* flowers.
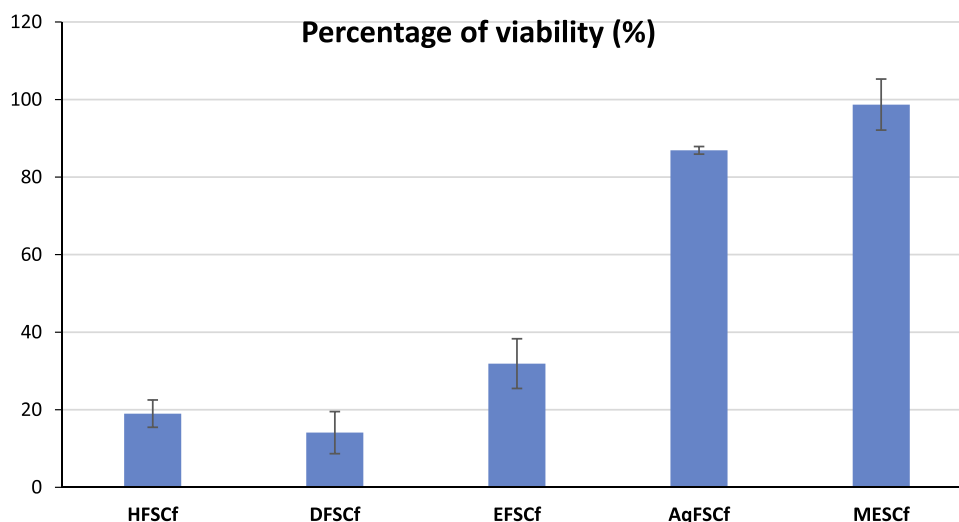
Fig. 1. Viability percentage of *Spathodea campanulata* flower extracts on *Plasmodium falciparum* 3D7 cells. The values are the means of 3 independent experiments with standard error bars indicated. Microsoft Excel 2016 was used for data analysis.

Fig. 1. The hexane (HFSCf), dichloromethane (DFSCf) and ethyl acetate (EFSCf) fractions significantly reduced the viability of *plasmodium falciparum* 3D7 at a concentration of 25 µg/mL with values of 19.0%, 14.1% and 31.9%, respectively. Subsequently, the IC_{50} of HFSCf, DFSCf and EFSCf fractions 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 29.7, 30.2 and 28.1 µg/mL, for fractions HFSCf, DFSCf and EFSCf, respectively (Fig. 2).

The dose-response curves of *in vitro* growth inhibitory activity of *S. campanulata* fractions on a panel of three cancer cell lines was reported (Fig. 3) and the IC_{50} values of the different fractions tested were determined (Table 2). The study of global growth activity by MTT tests enable to determine the IC_{50} of fractions HFSCf, DFSCf and EFSCf. All samples exhibited an antiproliferative activity on the B16F10 mouse cell line, with IC_{50} values in the range of 14.9–54.6 µg/mL. Moreover, only fraction DFSCf demonstrated a significant antiproliferative activity on the human cancerous (MCF7 and Hs683) cell lines, with IC_{50} values lower than 10 µg/mL.

The curves are the logarithm of the tested concentration expressed in µM as a function of percentage of cell viability. The fractions tested were EFSCf (ethyl acetate fraction of *S. campanulata* flowers), HFSCf (hexane fraction of *S. campanulata* flowers) and DFSCf (dichloromethane fraction of *S. campanulata* flowers). The three cell lines were B16F10 (mouse melanoma), MCF7 (human carcinoma) and Hs683 (human oligodendroglioma).

In agreement with the result of phytochemical screening, acute toxicity was performed on the aqueous fraction of *S. campanulata* flowers because this fraction contains the main part of cardiotonic glycosides. The LD_{50} is the concentration of substance in mg/kg
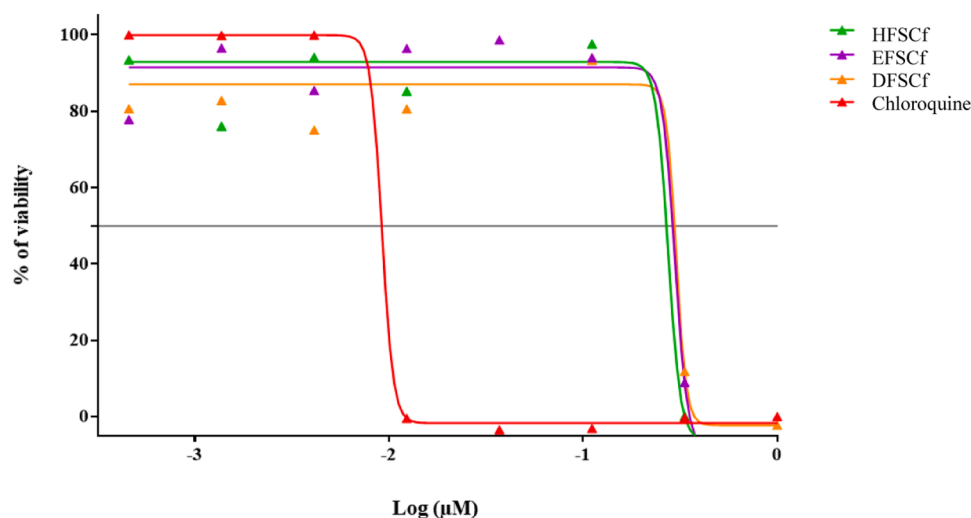

Fig. 2. Dose-response curves of the antiplasmodial assay of *Spathodea campanulata* flower extracts. Positive control was pure chloroquine compound with an IC_{50} value of 2.9 µg/mL. GraphPad Prism program version 5.02 was used for data analysis.
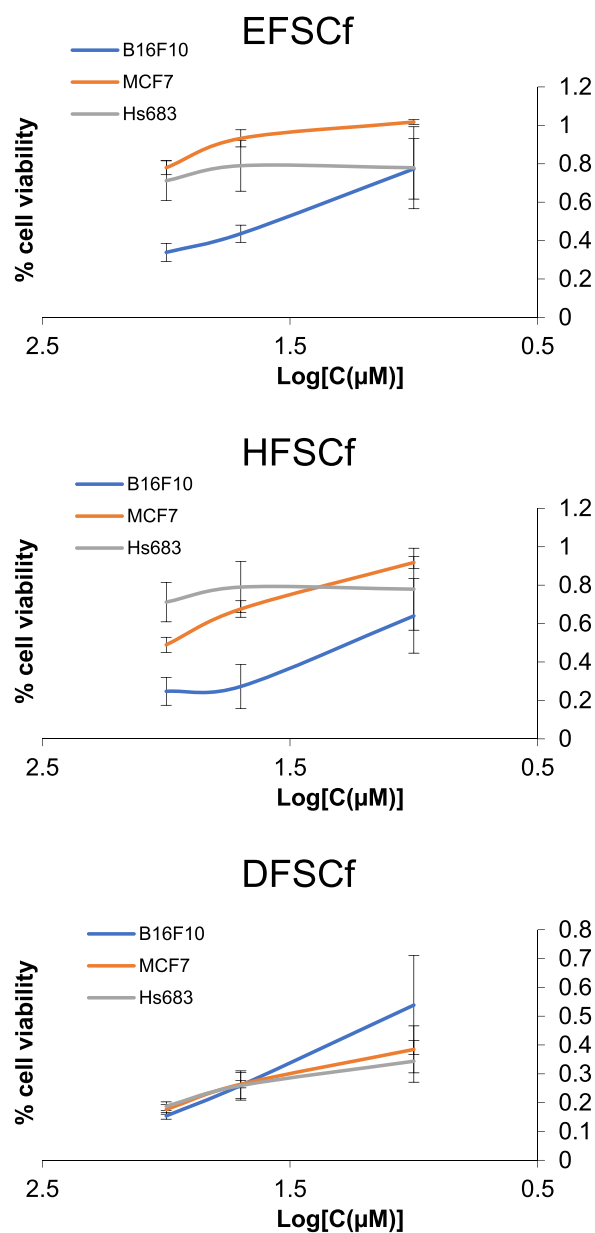
Fig. 3. Dose-response curves of *in vitro* growth inhibitory activity of *Spathodea campanulata* fractions on a panel of three cancer cell lines (B16F10, MCF7 and Hs683).

Table 2

In vitro growth inhibitory activity of *Spathodea campanulata* fractions on a panel of three cancer cell lines

Cancer cell lines	IC$_{50}$ (µg/mL)		
	EFSCf[a]	HFSCf[b]	DFSCf[c]
Mouse melanoma B16F10	54.6 ± 16.5	18.4 ± 6.3	14.9 ± 6.1
Human carcinoma MCF7	> 100	100.0 ± 7.4	< 10
Human oligodendroglioma Hs683	> 100	73.2 ± 4.0	< 10

The *in vitro* IC$_{50}$ growth inhibitory concentrations were determined using the MTT colorimetric assay. Values given are the mean of six replicates ± SD.

[a] EFSCf = ethyl acetate fraction of *S. campanulata* flowers.

[b] HFSCf = hexane fraction of *S. campanulata* flowers.

[c] DFSCf = dichloromethane fraction of *S. campanulata* flowers.

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. After administration, the animals were individually weighed at least once every 7 days: an evolution of the weight mass of the rats during the study was therefore recorded. The result of Table 3 shows, in the same way as for the determination of the LD_{50}, that the aqueous fraction has no impact on the weight mass of the rats.

Discussion

Phytochemical tests have shown that the crude extract (MESCf) and the different fractions of *S. campanulata* contain 6 classes of secondary metabolites *i.e.* alkaloids, galic tannins, cardiotonic glycosides, steroids, triterpenoids, and saponins, while polyphenols, anthraquinones and flavonoids were not detected. Our results are in accordance with a previous phytochemical screen of *S. campanulate* flowers [11]. However, flavonoids and polyphenols were not observed, in contrast with the reported presence of anthocyanins, a class of flavonoids [23]. Considering that the test for flavonoids and phenolic compounds was negative for all samples, this could explain why other parts of the plant, like leaves and bark are used in traditional medicine to treat colorectal, skin, and cervical cancers [6].

According to the traditional pharmacopeia, the barks and leaves of *S. campanulata* would present a prophylaxis in the fight against malaria. For this reason, we evaluated the antiplasmodial activity of the flowers of this plant, demonstrating thus that hexane, dichloromethane, and ethyl acetate fractions effectively reduced the viability of *P. falciparum* by 81%, 85% and 68%, with IC_{50} values of 28.1, 29.7 and 30.2 μg/mL, respectively. These results compare well with those of Makinde et al. in 1998 where the alcoholic extract of the leaves showed an antiplasmodial activity against *Plasmodium berghei* [13]. In another study, it has been shown that the hexane and chloroform fractions of the bark possessed a schizonticidal blood action, and the chloroform fraction has also a prophylactic potential [24]. However, the total methanolic extract MESCf was found to only reduce the viability of the 3D7 cells of *P. falciparum* by 1.25% while the tested fractions have a stronger effect. Therefore, the weak antiplasmodial activity of the total extract could be assigned to an antagonistic effect of the compounds present in the phytocomplex with respect to antiplasmodial activity. According to the phytochemical screening, it could be suggested that the activity of the hexane fraction could be due to the presence of alkaloids.

Concerning antiproliferative activity, all samples exhibited an activity on the B16F10 mouse cell line, with IC_{50} values in the range of 14.9–54.6 μg/mL and only fraction DFSCf demonstrated a significant antiproliferative activity on the human cancerous (MCF7 and Hs683) cell lines, with IC_{50} values lower than 10 μg/mL. The cytotoxicity activity criteria for crude extracts and fractions as established by the NCI is an $IC_{50} \leq 30$ μg/mL in the preliminary assay [25]. These results corroborate our previous studies, which reported that the methanol extract of the flowers exhibited an inhibitory activity on three cancer cell lines with IC_{50} around 80 μg/mL [10]. Shehab et al. in 2014 reported that the hexane, chloroform, ethyl acetate and butanol fractions of the ethanolic extract of the flowers could be endowed with anticancer properties on 2 cell lines, namely cancer colon (HCT116) and breast carcinoma (MCF7) with ICs_{50} in the range of 17 to 30 μg/mL [12]. According to the phytochemical screening, the activity of the hexane fraction could be associated with the presence of alkaloids, knowing that an alkaloid (bisindole) is used in anti-tumor chemotherapy on rat hepatoma in cell culture [26]. Dichloromethane fraction did not exhibit the presence of alkaloid and was the least active fraction. On the other hand, the ethyl acetate fraction which was the most active fraction on the three cell lines tested did not reveal the presence of any class of secondary metabolite tested according to the phytochemical screening. Its activity could be due to an untested class of compound or to a synergy between several tested classes if we exclude false negatives. From these results and compared to the studies conducted by Mbosso et al. in 2016, it emerges that the different fractions HFSCf, DFSCf and EFSCf have a better antiproliferative activity than the total extract on the tested cell lines [10]. In that study, the total extract of leaves exhibited a weak activity against all tested human cell lines (against lung, glioma, and melanoma) with a mean IC_{50} value above 78 μg/mL. As *S. campanulata* is used in traditional pharmacopeia to treat cancer, the significant anti-proliferative activity of the hexane, dichloromethane, ethyl acetate fractions of *S. campanulata* flowers against melanoma, carcinoma and glioma confirms the potential of this plant to produce phytomedicine [6,10].

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 [21]. Therefore, these results are in line with those of Talla in 2017 in which the aqueous extract from the trunk barks of the same plant was found to be non-toxic after oral administration of doses from 50 to 5000 mg/kg [14]. Another study by Klaasen et al. in 1995 highlighted that the ethanolic extract of the leaves was non-toxic after oral administration of doses from 1000 to 5000 mg/kg [27].

Conclusion

In summary, the phytochemical screening of a methanolic extract of *S. campanulata* flowers revealed the presence of alkaloids, galactic tannins, steroids, terpenoids, cardiotonic glycosides and saponins. The same extract was fractionated with hexane, dichloromethane, ethyl acetate and water leading to fractions HFSCf, DFSCf, EFSCf and AqFSCf, respectively. EFSCf showed the presence of tannins, AqFSCf, revealed the presence of the cardiotonic glycosides, while the presence of tested classes of secondary metabolite was found in DFSCf. As far as the antiplasmodial property is concerned, only the hexane, dichloromethane and ethyl acetate fractions demonstrated a significant antiplasmodial activity with IC_{50} values of 28.1, 29.7 and 30.2 μg/mL, respectively. The total extract and the aqueous fraction did not significantly reduce the viability of *Plasmodium falciparum* 3D7 cells at a concentration of 25 μg/mL. These results confirm the use of *S. campanulata* for its antimalarial properties, suggesting the use of flower extracts to treat

Table 3
General observations in acute toxicity.

	Witness batch (distilled water 10 mL/kg bw)	batch 1 (extract at 2000 mg/kg bw)	batch 2 (extract at 5000 mg/kg bw)
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;.

malaria as already the case for the barks and leaves in traditional pharmacopoeia. DFSCf was endowed with the best antiproliferative activity against all cancer cell lines (IC$_{50}$ < 10 μg/mL) compared to the other fractions and our previous study. This deserves further investigations for its use in traditional medicine in the treatment of colorectal, skin and cervical cancers. The toxicity study showed that *S. campanulata* P. Beauv. did not exhibit acute oral toxicity at dose limits of 2000 and 5000 mg/kg. These results highlight the biological potential of this species and could also form a preliminary basis for the selection of candidate plants for further phytochemical research for new bioactive molecules of plant origin. However, investigation on isolation and identification of the bioactive phytochemical compounds from the flowers of *S. campanulata* and qualitative standardization as well as other toxicity studies are still required.

Ethics approval and consent to participate

All animal handling and procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85–23, revised 1996). The experimental procedure was approved by The Institutional Ethics Committee for Research (n° 1295 CEI-UDo/02/2018/T, University of Douala, Republic of Cameroon, February 9, 2018).
Consent to participate was not applicable.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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