Studies on *Cassytha filiformis* from Benin: isolation, biological activities and quantification of aporphines

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Abstract

Cassytha filiformis (Lauraceae), a widely distributed parasitic plant, contains several aporphine alkaloids and is often used in African folk medicine to treat cancer, African trypanosomiasis and other diseases. Samples of *C. filiformis* from Benin were studied. We isolated and identified 6 aporphines and analysed the *in vitro* cytotoxic properties of four of them on different cancer and non-cancer cell lines. The major alkaloids (actinodaphnine, cassythine, and dicentrine) were also shown to possess antitrypanosomal properties *in vitro* on *Trypanosoma brucei brucei*. In order to elucidate their mechanism of action, the binding mode of these molecules to DNA was studied. The results of the optical measurements showed that these three alkaloids bind to DNA and behave as typical intercalating agents. Biochemical experiments also indicated that they interfere with the catalytic activity of topoisomerases. These interactions with DNA may explain, at least in part, the effects observed on cancer cells and on trypanosomes.

In order to quantify these alkaloids in samples from different origins, a sensitive and accurate procedure based on an alkaloid extraction coupled to a HPLC-UV-MS determination has been developed for the separation and quantification of the major aporphines in *C. filiformis*. The extraction parameters and the liquid chromatography conditions were optimized in order to improve the selectivity of the method, which was completely validated using cassythine, one of the major aporphines in our samples, as reference standard. This procedure was successfully applied to the determination of these pharmacologically interesting aporphines in seven different batches of *C. filiformis*. The results showed variations in the total alkaloid content in samples, which ranged from 1.1 to 4.3%.

Introduction

C. filiformis is a sprawling parasitic herb which is widely distributed along the seashores throughout the tropics. Several aporphinoid alkaloids have been isolated from samples originating from Taiwan, Brazil, Australia and New Guinea but compositions are quite variable among origins[1-3]. A methanolic extract of a Chinese sample was found

to exhibit a significant vasorelaxing activity as well as an inhibitory effect on platelet aggregation induced by several aggregating agents. A bio-guided fractionation of this extract allowed to isolate cathafiline, cathaformine, predicentrine, ocoteine, actinodaphnine and *N*-methylactinodaphnine which all have antiplatelet actions. The last two compounds also showed vasorelaxing properties and ocoteine was shown to be a selective α_1-adrenoceptor antagonist in isolated rat thoracic aorta[4]. It is known in Mauritius as "liane sans fin" and is, according to PLARM[5], one of the 215 most used plants in the Indian Ocean islands. In African traditional medicine the practitioners use the whole plant mostly as an aqueous extract to fight several infections, parasites or to treat cancers[6]. In order to confirm these last traditional uses, we investigated the potential cytotoxic and antitrypanosomal activity of a Beninese *C. filiformis* extract and its major alkaloids.

As topoisomerases have been proposed as potential targets for aporphines[7,8] and because Woo *et al*[8] have suggested that dicentrine behaves as a DNA targeted "adaptative" intercalating agent, we investigated the interaction of the aporphines isolated from *C. filiformis* with DNA and studied their potential inhibition of the DNA-associated enzymes topoisomerases I and II, which both serve to resolve constraints in the genome.

Data on the alkaloids from *C. filiformis* showed that compositions were quite variable depending on the origin of the samples. The composition could also vary according to the host plant of this parasitic plant and the season of the year. Therefore we decided to quantify the aporphines in several samples. The quantification methods for aporphines in plant samples described in the literature, when applied to the tested samples, did not allow a good separation of the major aporphines nor an ESI detection[9-13]. Therefore, a new HPLC method coupled to UV detection had to be developed and validated for the separation and quantification of these pharmacologically interesting aporphines in *C. filiformis*. In order to confirm the structure of these compounds and demonstrate the

specificity of the present HPLC-UV method, the mobile phase was selected to be compatible with ESI-MS detection which has not been used in routine analysis. The method was validated by considering different parameters such as selectivity, response function, trueness, precision, accuracy and linearity. Finally, the method was successfully used to quantify aporphines in different batches of *C. filiformis*.

Materials and Methods

Plant material

The first sample of *Cassytha filiformis* L. (Lauraceae) was collected at Sèmè, Ouémé, Benin and identified by Prof V Adjakidjé**.** A voucher has been deposited at the Herbarium of the Belgian National Botanical Garden, at Meise (BR-S.P. 848 105). Plant materials used for quantification were taken on various host plants and at different seasons of the years 2002-2003 (dry or rainy seasons) (Table 1). The collected plants were air-dried and kept at room temperature until analysis. Just before extraction, the dried plant materials were ground and passed through a sieve (Retsch® 355 µm) to obtain homogeneous powders.

Table 1: Various batches of *C. filiformis* collected on different hosts and seasons (dry or rainy) of the year 2002-2003. Ekpe and Sèmè are geographically close and have the same type of soil.

Batch of *C. filiformis*	Collecting place	Season of the collecting	Host plant of *C. filiformis*
1	Ekpe	Short rainy season	*Acacia auriculiformis* (Mimosaceae)
2	Ekpe	Long dry season	*Acacia auriculiformis (*Mimosaceae*)*
3	Sèmè	Long rainy season	*Anacardium occidentale* (Anacardiaceae)
4	Sèmè	Long rainy season	*Mangifera indica* (Anacardiaceae)
5	Sèmè	Long dry season	*Mangifera indica* (Anacardiaceae)
6	Sèmè	Long rainy season	*Azadirachta indica* (Meliaceae)
7	Sèmè	Long dry season	*Hyptis suaveolens* (Lamiaceae)

Preparation of the crude alkaloidal extract for in vitro tests

Air-dried aerial parts (1708 g) of *C. filiformis* were powdered, macerated during 24 h with MeOH-HOAc (99:1) and exhaustively percolated with the same solvent mixture. After concentration of the percolate under reduced pressure and filtration, the acidic aqueous solution was washed with ether, alkalinised by $NaHCO_3$ to pH 8 and extracted with CH_2Cl_2. NH_4OH 25% was then added to pH 11 and the solution was repeatedly extracted with CH_2Cl_2. The CH_2Cl_2 layers were dried over anhydrous Na_2SO_4 and concentrated to give respectively 3.61 and 0.62 g of residues.

Preparation of the alkaloid extract for quantifications

50 g of dried and powdered samples of *C. filiformis* were macerated four times with 250 mL MeOH acidified with 1% of acetic acid at 50 °C in a refluxed water bath for 1 h. Between each maceration the residue was filtered and washed with 50 mL of the same solvent.

The extracts were combined and concentrated under reduced pressure. The residue was dissolved in 400 mL of an aqueous solution acidified by acetic acid (1%) then filtered. The filtrate was washed three times with 150 mL ether. The aqueous acid layer was basified (pH 9.5) with NH_4OH 25% and extracted three times with 200 mL CH_2Cl_2. The CH_2Cl_2 layers were combined and dried over anhydrous Na_2SO_4 and evaporated to dryness. 50 mg of the residue obtained was dissolved in 50 mL MeOH. The solution of alkaloid extract (1 mg/mL) was passed through 0.45 μm membrane and 20 μL of this filtered extract were directly injected into the HPLC system. This standardized protocol was applied to each sample of *C. filiformis*. For HPLC-MS experiments, the solution of alkaloid extract (1 mg/mL) was diluted with MeOH to obtain solutions of about 500 μg/mL before membrane filtration.

Purification of alkaloids

Alkaloids were purified as previously described[14] using High Speed Counter Current Chromatography, MPLC on Toyopearl® HW-40S, preparative TLC on silica gel and RP-18 Lobar® columns. Structural identification of (**2-7**) (Figure 1) was carried out by analysis of their spectroscopic data (^1H, ^{13}C NMR and MS) and comparison with previously reported values[3,15,16].

Boldine (**1**) (Figure 1) was purchased from Federa (Brussels, Belgium).
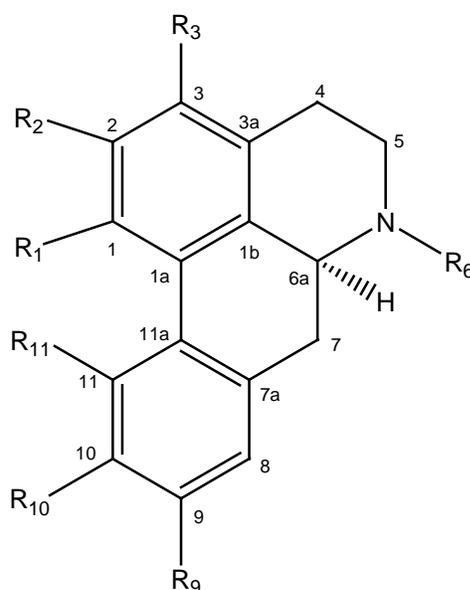
Compound	R_1	R_2	R_3	R_6	R_9	R_{10}	R_{11}
1 Boldine	O-CH$_3$	OH	H	CH$_3$	OH	O-CH$_3$	H
2 Actinodaphnine	-O-CH$_2$-O-		H	H	OH	O-CH$_3$	H
3 Cassythine	-O-CH$_2$-O-		O-CH$_3$	H	OH	O-CH$_3$	H
4 Dicentrine	-O-CH$_2$-O-		H	CH$_3$	O-CH$_3$	O-CH$_3$	H
5 Norneolitsine	-O-CH$_2$-O-		H	H	-O-CH$_2$-O-		H
6 Neolitsine	-O-CH$_2$-O-		H	CH$_3$	-O-CH$_2$-O-		H
7 Cassythidine	-O-CH$_2$-O-		O-CH$_3$	H	-O-CH$_2$-O-		H

Figure. 1: Structure of boldine (1) and of the identified aporphines (2-7) of *C. filiformis*

Cytotoxic assays

Cytotoxicity of crude extract and isolated compounds was determined after 72 hours incubation on HL-60, mouse 3T3 fibroblasts, human HeLa and melanoma Mel-5 cell lines using tetrazolium salts MTT (Sigma) and WST-1 (Boehringer) colorimetric assays[17]. The relative absorbance was expressed as a percentage of the corresponding control considered as 100%. Means and standard errors were calculated. Student t-test was performed (statistical significance was set at $p < 0.05$* and 0.001**). The results are expressed by IC_{50} values (concentration of compounds causing 50% inhibition of cell growth) calculated from graphs using at least five different concentrations of each alkaloid.

Antitrypanosomal activity

Trypanosoma brucei brucei (*T. b. brucei*) (strain 427) bloodstream forms were used for determination of the *in vitro* antitrypanosomal activity of the crude extract and the major *C. filiformis* alkaloids as explained in the literature[18], after 72 hours of incubation, by the Alamar Blue™ (Immunosource, Halle-Zoersel, Belgium) test[19]. The emanating fluorescence was expressed as percentage of the control, considered as 100%, and IC_{50} values (concentration of compound that reduced fluorescence intensity by 50%) were calculated by linear interpolation selecting values above and below the 50% mark according to Hills *et al*[20]. The antitrypanosomal drugs, diminazene aceturate (Berenil®, Hoechst AG) and suramin (Sigma), were used as positive controls[19]. All experiments were performed at least in triplicate.

Absorption spectra and melting temperature studies

Melting curves and absorption spectra were measured using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat as previously described[18]. For the melting curves, the measurements were performed in BPE buffer pH 7.1 at an

alkaloid concentration of 20 µM together with CT-DNA or poly(dA-dT)$_2$ at 20 µM (DNA-phosphate/drug ratio (P/D) = 1). The "melting" temperature Tm was taken as the mid-point of the hyperchromic transition.

Absorption spectra measurements were performed in 1 mM sodium cacodylate buffer (pH 6.5) at a compound concentration of 20 µM together with the CT-DNA at 200 µM (DNA-phosphate/drug ratio (P/D) = 10). A DNA blank at the same nucleotide concentration was performed concomitantly and used as a reference in the recording of absorption spectra.

Electric linear dichroism (ELD)

ELD measurements were performed with a computerized optical measurement system using the procedures previously outlined[21]. All experiments were conducted with a 10 mm pathlength Kerr cell having 1.5 mm electrode separation. The samples were oriented under an electric field strength varying from 1 to 14 kV/cm. Each alkaloid was studied at a DNA-phosphate/drug ratio (P/D) = 20.

Circular dichroism (CD)

CD measurements were recorded on a Jobin-Yvon CD6 dichograph. The same solutions as in the ELD experiments were used. Solutions of alkaloids and/or nucleic acids in 1 mM sodium cacodylate buffer pH 6.5 were scanned using a 1 cm pathlength cell. Three scans were accumulated and automatically averaged.

Topoisomerase-mediated DNA relaxation experiments

Topoisomerase I and II-mediated DNA relaxation assays were performed as described earlier[18,22]. For the topoisomerase I assays, supercoiled pLAZ3 DNA (130 ng) was incubated with topoisomerase I in a relaxation buffer. Camptothecin (Sigma) was used as a positive control.

For the topoisomerase II assays, supercoiled pLAZ3 DNA (130 ng) was incubated with human topoisomerase II in a relaxation buffer. Etoposide (Sigma) was used as a positive control.

For both assays, reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (5 μL) and electrophoresed at room temperature in 1% agarose gels.

HPLC chromatographic conditions

A LiChrospher 60, RP-select B column, (250 × 4 mm, 5 μm particle size) was used throughout all chromatographic experiments and was equipped with a guard column packed with the same sorbent (4 × 4 mm) manufactured by Merck. The HPLC separations were carried out at room temperature as described previously[23]. The mobile phase consisted of (A) water containing 10 mM ammonium acetate adjusted to pH 3 with acetic acid-acetonitrile (90:10, v/v) and (B) acetonitrile. A gradient elution program was applied as follows: 0-2 min linear increase from 0 to 5% B; 2-5 min hold on 5% B; 5-31 min linear increase to 10% B; 31-40 min hold on 10% B; 40-45 min linear increase to 15% B; 45-74 min linear increase to 20% B; 74-79 min linear increase to 40% B; 79-81 min linear decrease to 0% B; kept to 90 min. The flow rate was kept constant at 0.7 mL/min, and the injection volume was 20 μL. Peaks were detected at 307 nm. Each solution was injected three times.

Standard stock solutions

Stock solutions of boldine (**1**) (Figure 1) (Federa, Brussels, Belgium) (S1) and cassythine (S2) were prepared independently by dissolving the appropriate amount of each compound in methanol in order to obtain a final concentration of 52 and 1000 μg/mL, respectively. Furthermore, three different concentrations of cassythine (20, 165 and 510 μg/mL) were added to a solution of 1 mg/mL of the alkaloid extract.

Peak identification

Alkaloids were identified by comparison of their retention times, UV and mass spectra with the corresponding pure compounds previously isolated from *C. filiformis*[14,24]. Identification of compound A (Figure 2) is not complete as we did not manage to obtain enough pure compound for refined NMR structure determination. Nevertheless, MS data showed that it may be nor-boldine or one of its isomers[24].

Data analysis

The validation data were recorded and processed by e.noval version 1.0 software (Arlenda, Belgium) and the statistical data of the dosage with SPSS 10.1.4 for Windows software (SPSS Inc, Chicago, IL, USA).

Results and Discussion

Cytotoxicity and antitrypanosomal activity

The alkaloid fraction showed a cytotoxic activity *in vitro* against HeLa cells (IC_{50} = 35.2 µg/mL). Purification of this fraction led to the isolation of four known aporphine alkaloids: neolitsine (**6**), dicentrine (**4**), cassythine (**3**) and actinodaphnine (**2**) (Figure 1)[14]. Furthermore, two other alkaloids were identified later: norneolitsine (**5**) and cassythidine (**7**)[23,24].

Compounds (**2,3,4,6**) were tested for their ability to inhibit *in vitro* the growth of HeLa, Mel-5, HL-60 cancer cells and 3T3 non-cancer cells (Table 2).

Table 2: **Cytotoxic activities of alkaloids isolated from *C. filiformis***

Compound	IC_{50} (µM)[a]			
	HeLa*	3T3*	Mel-5*	HL-60**
Neolitsine (**6**)	21.6	21.4	42.1	54.8
Dicentrine (**4**)	33.3	35.1	36.3	28.3
Cassythine (**3**)	44.3	57.6	24.3	19.9
Actinodaphnine (**2**)	30.9	66.4	25.7	15.4
Camptothecin	0.04	2.6	0.4	0.005

[a]IC_{50} (concentration of drug causing 50% inhibition of growth), *MTT test, **WST-1 test
Camptothecin was used as a positive control.

Our results also showed differences in the sensitivities between the tested cell lines to neolitsine, cassythine and actinodaphnine. In general these compounds do not display a selective activity on cancer cell lines, an exception seems to be actinodaphnine which is at least twice less toxic on non cancer 3T3 cells. This is also in agreement with the findings of Boustie *et al*[25] who observed an IC_{50} of 107 µM on non cancer Vero cells. Dicentrine and actinodaphnine have also been shown to have *in vivo* anti-tumor properties[26,27]. The presence of these four alkaloids may thus explain the cytotoxic activity of the extract and support the traditional use of *C. filiformis* in Benin to treat cancers.

As *C. filiformis* is traditionally used to treat African trypanosomiasis, a disease for which appropriate and affordable treatment is scarce[28], we decided to analyze the *in vitro* effect of a crude alkaloid extract and its major isolated compounds on *Trypanosoma brucei brucei*, one of the causative agents of this disease. The crude extract revealed a significant antitrypanosomal activity (IC_{50} = 2.2 µg/mL). The activity of its major alkaloids is given in Table 3.

Table 3: Antitrypanosomal activity of alkaloids (2-4)

Compound	IC_{50} (μM)[a] *T. b. brucei*[b]
Actinodaphnine (**2**)	3.2
Cassythine (**3**)	6.0
Dicentrine (**4**)	14.6
Suramin	0.06
Diminazene aceturate	0.02

[a]Values indicate 50% inhibitory concentrations (IC_{50}) in μM
[b]*Trypanosoma brucei brucei* bloodstream forms

DNA interaction and binding mode

As Woo *et al*[8] have suggested that dicentrine behaves as a DNA targeted "adaptative" intercalating agent, we also investigated the interaction of these aporphine alkaloids with DNA and their potential inhibitiory effect on the DNA-associated enzymes topoisomerases I and II.

Absorption measurements were performed at 20 μM in the absence and presence of 200 μM calf thymus DNA to estimate the interaction of the compounds with DNA. The absorption spectrum of each alkaloid at 20 μM was recorded in the absence and presence of 200 μM calf thymus DNA. Addition of all three alkaloids (**2**,**3**,**4**) to DNA induced significant hypochromic effects and bathochromic shifts in the absorption band of the aporphine chromophore centered at 305 nm, reflecting the interaction between the electronic states of the chromophore and the DNA bases[18]. Melting temperature (Tm) studies were performed with CT-DNA and the alternating polynucleotide poly(dA-dT)$_2$. The difference in Tm values between the drug-DNA complexes and free DNA or polynucleotide in solution provides a useful means to evaluate qualitatively the interaction of the molecules with double stranded DNA. The results of these Tm measurements show that all three molecules stabilized duplex DNA against heat denaturation[18]. As expected, the stabilization due to these three compounds is significantly higher for the alternating poly(dA-dT)$_2$ polymer (ΔTm > 6 °C) than for

CT-DNA (ΔTm < 6 °C) due to the lower intrinsic Tm of the synthetic polymer compared to the naturally occuring DNA from CT which contains 42% GC base pairs and therefore melts at a higher temperature than poly(dA-dT)$_2$ (Tm of 42° and 61°C for poly(dA-dT)$_2$ and CT-DNA, respectively)[18].

Additionally, two spectroscopic methods with polarized light were applied to define the DNA binding process for the studied aporphines. The ELD experiments provided direct information on the DNA intercalating properties of the tested compounds. For all 3 aporphines, the reduced dichroism $\Delta A/A$ was negative in the alkaloid absorption band (300-312 nm) reflecting the orientation of the chromophore perpendicular to the DNA helix axis (or electric field direction)[18]. All the CD spectra of the complexes between the aporphines and CT-DNA revealed weak amplitude variations compared to the spectra obtained with the molecule alone in the absorption band of this latter one.

From the experiments using circularly and linearly polarized light, we concluded that the 3 aporphines are oriented parallel to the DNA base pairs, as expected for an intercalative binding mode. This behavior is in accordance with an intercalation into DNA[18].

Topoisomerase I and II inhibition

To analyze in greater detail the mode of action of these molecules, we evaluated their effects on the catalytic activity of topoisomerases I and II using a relaxation assay with supercoiled DNA.

Closed circular DNA was incubated with topoisomerase I in the absence and in the presence of the 3 aporphines (**2**,**3**,**4**). The results[18] indicate that the relaxation of DNA was already altered by the three alkaloids isolated from *C. filiformis* at a concentration of 5 μM, as observed with the reference topoisomerase I poison, camptothecin. Ethidium-prestained gels indicated that none of the aporphines promoted DNA cleavage

by topoisomerase I. In other words, the alkaloids did not stabilize topoisomerase I-DNA complexes, as typically observed with topoisomerase I poisons such as camptothecin[29]. So we can conclude that actinodaphnine, dicentrine and cassythine exhibit a non-specific topoisomerase I inhibition, most likely due to intercalation into DNA.

Similar experiments performed with human topoisomerase II have revealed that none of the aporphines are poisons of this enzyme[18].

Quantification of aporphines by HPLC-UV

Method development

Optimisation of the alkaloid extraction from C. filiformis

Different procedures were tested to obtain an optimal and well-suited time extraction of the alkaloids from the plant material. The best results were obtained with the quadruple maceration at 50 °C as proposed by Kartal *et al*[30] for the extraction of other types of alkaloids. In addition to these preliminary extraction experiments[14], the liquid-liquid extraction step was standardized, a pH value of 9.5 and a 200 mL-volume of dichloromethane in triplicate were selected.

Chromatographic separation

The chromatographic separation parameters were optimised to allow a good separation of all aporphines (Figure 2). In these conditions, the resolution values between each aporphine peak-pair were higher than 1.5 and no significant interferences were observed at the time of retention of the different alkaloids of interest.

Method validation

The overall validation strategy involves four steps: evaluation of the extraction efficiency, determination of the cassythine content in one batch of alkaloid extract, a pre-validation phase and the validation phase or formal validation step. As no alkaloid

identified in the tested samples of *C. filiformis*[23] was commercially available, cassythine (**3**), one of the major aporphines from our batch of *C. filiformis*[14] was selected as reference standard to express the results. However, it was checked that the UV response factors of the other aporphine alkaloids were very close.
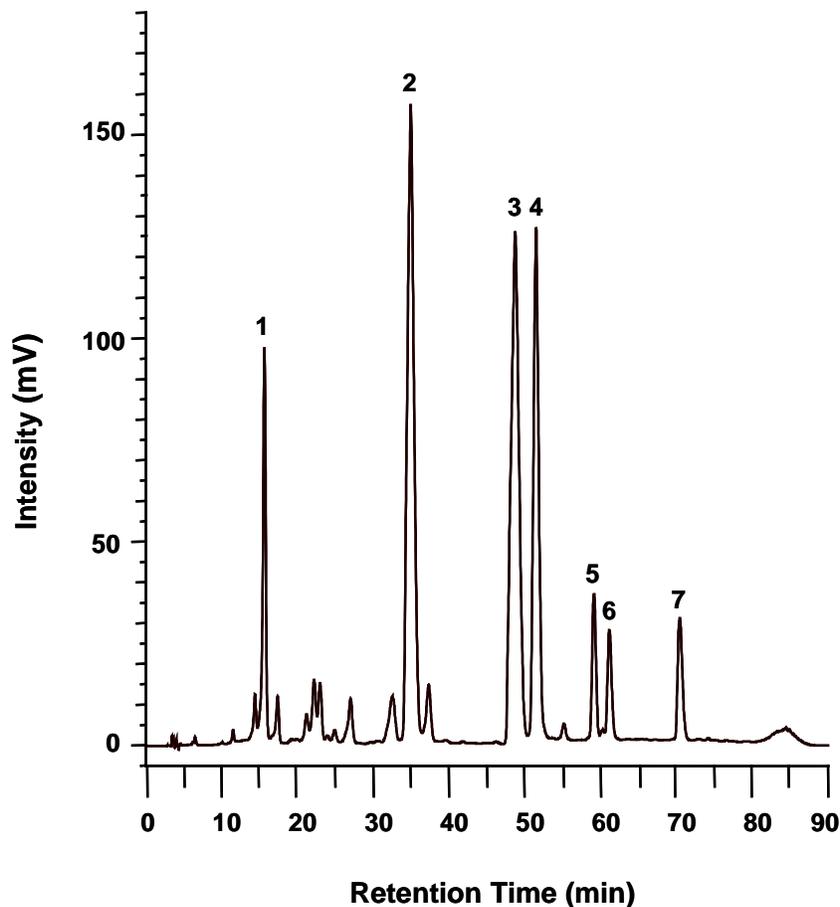
Figure 2: Typical chromatogram of the total alkaloid extract (1 mg/mL) obtained by using the extraction step on the batch 5 of *C. filiformis* coupled to LC method. Key to peak identity: 1, compound A; 2, actinodaphnine; 3, cassythine; 4, dicentrine; 5, norneolitsine; 6, neolitsine; 7, cassythidine.

Determination of the extraction efficiency

In the present study, a recovery test was performed using boldine (**1**) to evaluate the efficiency of the standardized extraction method[31,32] first without the matrix showing a mean recovery of about 101%. When the same amount (13 mg) of boldine was added to

three 50 g samples of *C. filiformis* and extracted by the standardized procedure, the mean recovery was about 75% indicating the influence of the matrix (Table 4). However, as the relative standard deviation values for the mean recoveries (within and without matrix) were very close, this process was shown to be sufficiently reproducible.

Table 4: Extraction efficiency

Boldine (1)	Samples	Recovery (%) (n^a =3) mean ± SD^b
without matrix	1	100.2 ± 4.7
	2	104.2 ± 5.8
	3	99.1 ± 0.4
	Mean ± SD^b (N^c=9)	101.2 ± 4.4
within matrix	1	82.1 ± 2.2
	2	71.9 ± 1.6
	3	71.0 ± 3.6
	Mean ± SD^b (N^c=9)	75.0 ± 5.8

[a]Number of replicates (n)
[b]Standard deviation (SD)
[c]Total number of experiments (N)

Detemination of the cassythine content in the alkaloid extract

Two different quantitative methods were used to determine the amount of cassythine in the alkaloid extract (batch 1). In the first technique, a standard curve using known amounts of pure cassythine dissolved in methanol at three different concentration levels ranging from 20 to 1000 µg/mL was constructed (n=3; k=3). In the second technique, the standard addition method[33], three different concentration levels of cassythine of about 20, 160 and 510 µg/mL were added to a methanolic solution of 1 mg/mL of the alkaloid extract of *C. filiformis* batch 1[23]. Each concentration level was injected three times each day for three days. The concentrations calculated by these two different quantitative methods were not significantly different. These preliminary experiments seem to demonstrate that no matrix effect can be allotted to the alkaloid extract and that higher concentration levels (1000 µg/mL of cassythine in methanol) could be used for the routine analysis of the different batches of the plant materials.

Prevalidation step

Due to its influence on the other criteria, the stability of the analyte investigated was checked before starting the prevalidation step. In the present study, the stability of a stock solution of cassythine as well as the solution of the alkaloid extract stored for 30 days at 4 °C was demonstrated. No significant degradation of the compounds of interest was observed.

On the basis of the prevalidation protocol proposed by the "Société Française des Sciences et Techniques Pharmaceutiques (SFSTP)" Commission[31,34], the experiments achieved during the prevalidation step deal with the analysis of the response function and the selection of the appropriate model for the calibration curve in the validation step. For that purpose, the SFSTP approach based on two-sided 95% β-expectation confidence intervals for total measurement error –including both bias and precision – of the calibration standards has been used[34]. An acceptance criteria of 15% was assessed according to the Food and Drug Administration (FDA) document[35]. In order to determine the response function, the alkaloid extract of batch 1 of *C. filiformis* was used as the matrix. Three calibration curves in the matrix (k=3) were constructed with known amounts of cassythine in the range comprised between 20 and 510 µg/mL by selecting three concentration levels (m=3). Each level of concentration was injected three times (n=3). Before calculation of the response function, the response corresponding to the initial concentration present in the matrix under investigation plus its standard error was then systematically removed from each signal. As illustrated in Figure 3, once this computation has been performed, the response function can be determined by applying different regression models. According to this visual analysis, better results were obtained using a quadratic regression model (Figure 3f) but as other profiles (c.f. Figure 3a, 3c, 3d and 3e) were within the acceptance limits, a linear regression through 0 fitted with only the highest concentration level, could be used as regression model[34]. Consequently, a calibration curve built from the highest concentration level (1000

µg/mL) of cassythine in methanol was selected for the validation step in order to avoid time-consuming analysis of a standard calibration curve with different concentration levels[23].
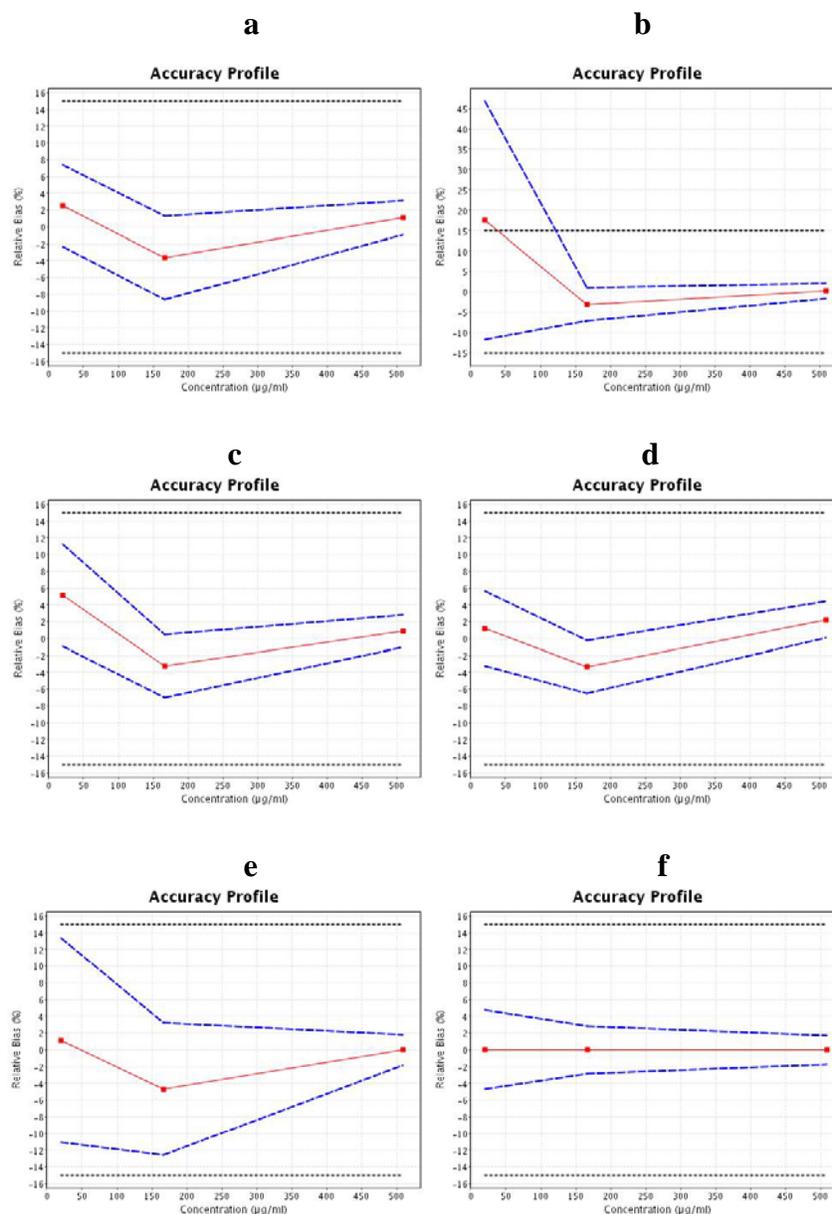
Figure 3: Accuracy profiles of cassythine (concentration in µg/mL) using a (3a) weighted linear regression model with a weight equal to 1/X, (3b) linear regression model, (3c) linear regression model after square root transformation, (3d) linear regression model after logarithm transformation, (3e) linear regression through 0 fitted with only the highest concentration level, (3f) quadratic regression

Validation step

Selectivity

The selectivity of the analytical method was investigated in order to assume that the method could be used to quantify cassythine and the other aporphine alkaloids in presence of other constituents in the alkaloid extract. Peaks were identified by comparison of their retention time (R_T) with the corresponding pure compounds previously isolated from *C. filiformis* and HPLC-MS analysis at different levels of the peaks. The HPLC-MS data confirmed that no interferences were observed at the time of retention of the different alkaloids under investigation.

Response function

The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample. The validation results for the response function[31,32,35] are presented in Table 5.

Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one. Compared to the regulatory requirements fixed[23], the proposed method was quite acceptable since the bias (Table 5) did not exceed the values of 15% irrespective of the concentration level[23].

Precision

For each concentration level of the validation standards, the variances of repeatability and of time dependent intermediate precision as well as the corresponding relative standard deviations (RSD) were computed from the estimated concentrations. The RSD values presented in Table 5 were relatively low, less than 4% for the lowest

concentration level of the range, and illustrated the good precision of the proposed method.

Table 5: Validation results

Validation Criterion		Cassythine
Response function	Range	0 – 1016.0 µg/mL
(n[a] = 3)	Slope	62430
Trueness (k[b] = 3; n[a] = 3)		
Absolute bias: µg/mL (relative bias: %)		
20.0 µg/mL		1.25 (6.2)
166.7 µg/mL		0.19 (0.1)
509.3 µg/mL		25.62 (5.0)
Precision (k[b] = 3; n[a] = 3)		
Repeatability (RSD[c] %)		
20.0 µg/mL		2.2
166.7 µg/mL		1.3
509.3 µg/mL		0.8
Intermediate precision (RSD[c] %)		
20.0 µg/mL		3.9
166.7 µg/mL		2.6
509.3 µg/mL		0.8
Accuracy (k[b] = 3; n[a] = 3)		
β-expectation confidence limit (µg/mL)		
20.0 µg/mL		19.2 – 23.3
166.7 µg/mL		155.4 – 178.3
509.3 µg/mL		526.8 – 543.1
Linearity	Range (µg/mL)	20 – 509.3
(k[b] = 3; n[a] = 3)	Slope	1.054
	Intercept	- 3.545
	r^2	0.9995
LOD[d]	µg/mL	13.1
LOQ[e]	µg/mL	20.0
Effect of dilution	Factor (µg/mL)	recovery ± SD (%)
(n[a] = 3)	2 (1000 µg/mL)	101.6 ± 2.1
	5 (2500 µg/mL)	102.0 ± 3.8

[a]Number of replicates (n) [b]Number of series (day) (k)
[c]Standard deviation (RSD) [d]Limit of detection (LOD)
[e]Limit of quantitation (LOQ)

Accuracy

Accuracy is an expression of the concordance of one assay value and the conventionally accepted value. The accuracy takes into account the total error, *i.e.* systematic and random errors, related to the test results[34]. The upper and lower *β*-expectation confidence limits expressed in µg/mL are presented in Table 5 as a function of the introduced concentrations. As can be seen from the results, the proposed method was accurate, since the different confidence limits of the bias did not exceed the acceptance limits at each concentration level.

Linearity

The linearity of an analytical method is its ability to give results directly proportional to the quantity of analyte within a definite range. In order to verify the method linearity, a regression line was fitted on the estimated or back-calculated concentrations as a function of the introduced concentrations by applying the linear regression model based on the least squares method[31,32,34]. The linearity of the present method was demonstrated since the absolute *β*-expectation confidence limits were within the absolute acceptance limits[23].

Detection and quantification limits

The limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression[33]. By applying this computation method, the LOD was equal to 13 µg/mL. Moreover, as the accuracy profile did not go outside the acceptance limits, the limit of quantification (LOQ) was fixed to 20 µg/mL, i.e. the smallest concentration level quantitatively determined with a well-defined accuracy[34](Table 5).

Effect of the dilution

Due to the unknown amount of alkaloid expected in the plant materials, the influence of the dilution procedure, which is intended to be used in routine for samples with a concentration higher than the upper limit of the range, has to be checked[31,32]. In the present study, two dilution factors (2 and 5) were studied and no significant effect was observed (Table 5).

Application to samples of C. filiformis

According to the validation step, a linear calibration curve passing through zero built from one concentration of pure cassythine (1000 µg/mL) was used for calibration. 1 mg/mL solutions of alkaloid extracts of each sample of *C. filiformis* obtained by the standardized protocol were injected three times and analysed by HPLC-UV. In each sample, the seven major separated aporphines were quantified and expressed in cassythine[23]. The results of the quantification (mean of three injections) for the seven samples are given in Figure 4.
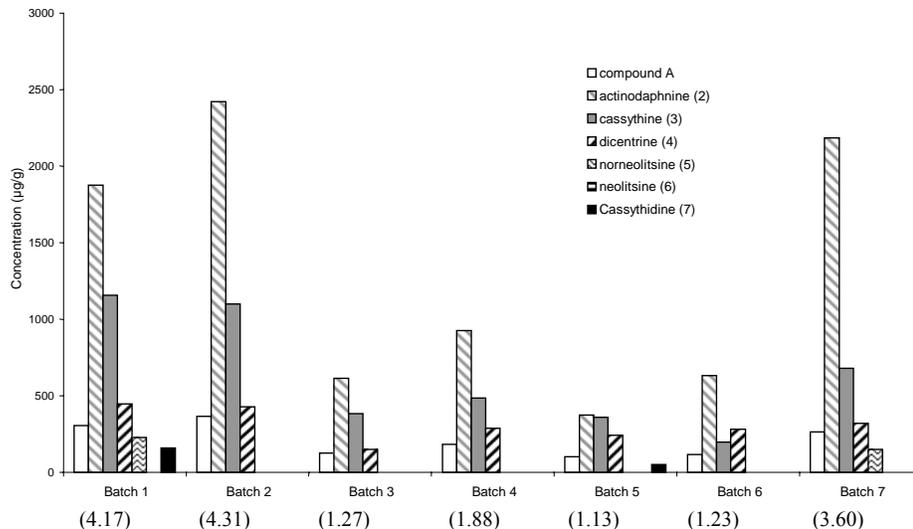
Figure 4: Different concentrations (µg aporphines expressed in cassythine by g of dried plant of *C. filiformis*) of quantifiable aporphines in the seven batches analyzed of *C. filiformis*. Values in parenthesis are the total alkaloid content in each batch expressed in percentage (%)

Comparison of the contents of the seven samples of C. filiformis

The comparison was first done individually for each aporphine. In other words the contents of a single compound are compared for all the samples injected. To allow this comparison a statistical ANOVA test was applied to the data. P values < 0.05 indicated a significant difference between samples. All batches were found to be significantly different from each other except samples 3 and 6 for actinodaphnine and samples 4 and 6 for dicentrine. The total amount of quantifiable aporphines was also found to be significantly different from each other. Even if the same standardized extraction was applied to all the batches of plant, the yields of total quantifiable aporphines could vary from 1.1 to 4.3 % of dried plant. The host plant and the period of the collection had both an effect on the quantity of aporphines and their relative proportions but a clear relationship according to the general effect of the season or the host plant cannot be deduced. Samples collected on the same host plant but at diverse seasons of the year were all different. The same facts were observed for samples collected at the same period but on different host plants (variations are more important). Nevertheless the four major aporphines are the same in all samples (Figure 4), but in variable proportions. According to the literature this does not seem to be the case with samples from other continents for which the major aporphines are different[1-3]. This may be due to an additive effect of the soil. We plan now to apply the validated procedure to other samples of *C. filiformis* from different countries to obtain further information on the role of these factors on the aporphine contents.

Conclusions

In conclusion we have shown that the crude alkaloid extract of a Beninese sample of *C. filiformis* possessed *in vitro* cytotoxic and antitrypanosomal activities. These properties may be partially explained by the presence of aporphine alkaloids. Six of them have been isolated and identified: actinodaphnine, cassythine, dicentrine, neolitsine, norneolitsine and cassythidine. The first four aporphines were shown to possess

cytotoxic properties on cancer (HeLa, Mel-5 and HL 60) and non-cancer (3T3) cell lines, while actinodaphnine, dicentrine and cassythine were also active on *Trypanosoma brucei brucei*, one of the causative agents of African trypanosomiasis. Spectroscopic and biochemical experiments have indicated that these three aporphines intercalate into DNA and non specifically inhibit topoisomerases.

As aporphines isolated up to now from samples of *C. filiformis* varied considerably according to the origin of the sample, we decided to develop a selective and accurate procedure for the quantitative determination of the major aporphines in *C. filiformis*. The extraction step and the chromatographic conditions were optimised in order to improve the selectivity of the procedure, which was fully validated using cassythine as reference standard. This procedure was successfully applied to the determination and quantification of these aporphines in seven different batches of *C. filiformis* indicating that both the season of collection and the host plant influence the levels and proportions of aporphines in *C. filiformis*.

Acknowledgements

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