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TRYPANOCIDAL ACTIVITY OF EXTRACTS AND CASSANE DITERPENOIDS ISOLATED FROM THE LEAVES OF *ERYTHROPHLEUM SUAVEOLENS* (GUILL. ET PERR.) BRENAN (FABACEAE)

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ABSTRACT
Erythrophleum suaveolens is a plant species of ivorian flora used to treat several pathologies including Buruli ulcer, tuberculosis and certain parasitic diseases. To our knowledge, no phytochemical study has been conducted on leaves of this species. This work carried out on the leaves is in line with development of medicinal plants in Côte d'Ivoire through the evaluation of trypanocidal activity of extracts and molecules isolated from different parts of the species. The phytochemical study was carried out using classical chromatographic methods (silica column chromatography, Sephadex column chromatography) and structural determination (NMR, IR, MS). The evaluation of trypanocidal activity against *Trypanosoma brucei brucei* was performed using the method used by N'Guessan et al.[1] The chemical study led to the isolation of two amide- and amine-functional cassane diterpenoids. These are Nor-cassamine (**1**) and Norcassamide (**2**), isolated for the first time from the leaves of this species. Biological tests showed that cold methanolic extract and Norcassamine (**1**) have trypanocidal activity, even if this remains moderate compared to the reference molecule, Melarsoprol.

KEYWORDS: *Erythrophleum suaveolens*, Norcassamine, NMR, trypanocidal activity.

1. INTRODUCTION
The use of plants in medicine is very old. According to a report by the World Health Organisation,[2] plants have an important role for humans in the field of food, clothing, habitat and health. The world's population, especially in Africa, has always relied on traditional medicine to treat several diseases. Howerver, the difficulty lies in the certification of African traditional medicine by the WHO. Despite the difficulties encountered, the design of effective, non-toxic phytomedicines accessible to poor populations remains one of the main objectives of African researchers. Numerous laboratory studies have confirmed the pharmacological activity of certain traditionally used plant species,[3] including *Erytrophleum suaveolens*.[4,5] This plant species is used in western Côte d'Ivoire to treat Buruli ulcer.[6] In order to contribute to the valorization of this plant and to search for new natural remedies with fewer side effects, we set ourselves the objective of isolating molecules and evaluating the anti-parasitic activity of the methanolic extract and the molecules isolated from it's leaves against *Trypanosoma brucei brucei*. To our knowledge, these different studies on leaves of *Erythrophleum suaveolens* are the first to be carried out in Côte d'Ivoire.

2. MATERIALS AND METHODS
2.1. General experimental procedures
Optical rotations were measured at 25 °C on a Polaar 32 Polarimeter. IR spectra were recorded with a Bruker Vector 22. The NMR spectra were acquired on Bruker AM-300 (300 MHz), and AM-400 (400 MHz) using

CD_3OD as solvent. Solvent residual signal was used for calibration. A Sunfire® preparative C18 column (150 × 19 mm, i.d. 5 µm, Waters) was used for preparative HPLC separation using a Waters Delta Prep fitted with a binary pump (Waters 2525) and a photodiode array detector (190-600 nm, Waters 2996). A silica 24 g Grace cartridge was used for flash chromatography using an Armen Instrument spot liquid chromatography flash device. Open column chromatographies were carried out on silica gel 60 (40-63 µm) or with Sephadex® LH-20 (25-100 µm) (Pharmacia Fine Chemicals, USA). TLC analyses were carried out on precoated silica gel 60 F254 (Merck, Darmstadt, Germany), and spots were visualized by spraying the plates with Dragendorff reagent, 10% H_2SO_4 solution or phosphomolybdic acid followed by heating.

2.2. Plant material

Leaves of *E. suaveolens* were collected in Toumodi province (Côte d'Ivoire) in December 2014 and authenticated by Pr. IPOU IPOU Joseph. Voucher specimens (n° 10 KABLAN ES-2014) were deposited in the herbarium of Centre National de Floristique (CNF), Université Félix HOUPHOUET-BOIGNY, Abidjan (Côte d'Ivoire).

2.3. Extraction and isolation

The air-dried powdered leaves (600 g) was extracted three times with methanol (7 L, 24 h) at room temperature. The extracts were combined and evaporated to dryness (21.3 g) under reduced pressure. A 13 g aliquot of this extract was mixed with a mixture of MeOH and CH_2Cl_2 (1:1; 50 mL), alkalinized with a few drops of NH_4OH (6 M). This solution was extracted with a solution of 1 M sulfuric acid (50 mL). The acid/aqueous phase was then extracted twice with CH_2Cl_2 (100 mL each). The remnant aqueous phase was alkalinized using 6 M NH_4OH (pH 10) and extracted with EtOAc (4 × 100 mL) to yield 2.5 g of ethyl acetate extract. The extract was subjected to flash chromatography using a silica 24 g Grace cartridge with a gradient of CH_2Cl_2-MeOH (1:0 to 0:1) at 24 mL/min to afford seven fractions (F1-7), according to their TLC profiles. Fraction F4 (108 mg) was further fractionated on a Sephadex® LH-20 column using an isocratic regime of CH_2Cl_2/MeOH (1:1), that resulted in three subfractions (F41-F43). Fraction F42 (32 mg) was selected and taken through preparative HPLC separation using a gradient of CH_3CN-H_2O with 0.1% formic acid (95:5 to 50:50) to afford **1** (7.9 mg, RT : 14.25). Fraction F5 (132 mg) was fractionated on a column of Sephadex® LH-20 using CH_2Cl_2/MeOH (1:1) mobile phase to give five fractions (F51-F55). Further preparative HPLC fractionation of subfraction F55 (37 mg) yielded compound **2** (7.4 mg, RT : 19.94).

2.4. *In vitro* trypanocidal activity

The tests were carried out at the Antiparasitic Chemotherapy Laboratory of the Faculty of Pharmacy of Châtenay-Malabry UMR CNRS 8076 BioCIS, using the GVR 35 strain (Glascow Veterinary Research) of *Trypanosoma brucei brucei*, frozen in liquid nitrogen. The parasites were adequately diluted using culture medium to obtain 200,000 trypomastigotes per mL. The circulating forms of the parasite were cultured *in vitro* without loss of their infectivity, for 24 hours at 37°C in an air atmosphere containing 5% CO_2. The culture medium was composed of β-mercaptoethanol (0.2 mM): 1.4 µL; hypoxanthine: 1.36 mg; thymidine: 0.387 mg; sodium pyruvate: 22 mg; Hepes: 650 mg; glucose: 100 mg; $NaHCO_3$: 220 mg; complemented horse serum: 15 mL; gentamicin: 5 mg; "MEM (Minimum Essential Medium) non essential amino acids": 1 mL; "MEM with Earle's salts" and L-glutamine: qsp 100 mL. The parasites were distributed in a 96-well plate of 200 µL at a rate of 2,105 per mL. Then, 5 µL of the appropriate dilution of the compounds in DMSO are added, each concentration being tested in triplicate. The control wells only received DMSO (5 µL, ie 2.5%). After 24 hours of incubation, the viability of the trypanosomes was estimated by direct observation using an optical microscope. Experiments were performed in triplicate and repeated three times.[1] The test results were expressed as a lethal concentration killing all the parasites in the wells after 24 hours (LC_{100}, Lethal Concentration) after microscope observation. Melarsoprol was used as reference drug.

3. RESULTS AND DISCUSSION

3.1. Chemical study

Compound 1 (Fig. 1) was obtained as an amorphous solid, soluble in methanol. Its HRESI-QTOF-MS showed peak of the pseudo-molecular ion $[M+H]^+$ at *m/z* 420.2714, corresponding to molecular weight of 419.2636 g/mol. This mass corresponds to molecular formula $C_{24}H_{37}NO_5$ (calculated mass 419.2672). IR spectrum, recorded in $CHCl_3$, showed absorption bands at υ_{max} 1726; 1702 and 1648 cm^{-1} which are characteristic of carbonyl groups. Absorption bands of methyl (CH_3) and methylene (CH_2) groups were also observed at υ_{max} 2991; 2944 and 2931 cm^{-1}. ^1H and ^{13}C NMR spectra of **1** were performed in deuterated methanol (CD_3OD). The ^1H NMR spectrum (Table 1) showed two methyl singlets at δ_H 1.19 (H-18) and 0.84 (H-20). The over singlets observed at δ_H 3.67, 5.81 and 2.74 were respectively attributed to the protons of a methoxyl group (CH_3-O, H-24), an alkenic proton (C=CH, H-15) and protons of a N-methyl amine (N-CH_3, H-23). The ^{13}C NMR spectrum (Table 1) confirmed the presence of ester groups with the peaks at δ_C 178.6 (C-19) and 169.5 (C-16). A ketone group (C=O) was observed at δ_C 211.9 (C-7) and sp^2 carbons at δ_C 167.3 (C=C; C-13) and 112.8 (=CH-, C-15). The N-methylamine signal was observed at δ_C 33.9 (N-CH_3). Analysis of the HMBC spectrum revealed $^2J_{CH}$ correlations between the protons at δ_H 2.95 (H-6), 2.65 (H-6) and 2.36 (H-8), and the carbonyl at δ_C 211.9 (C-7, ketone). A $^3J_{C\text{-}H}$ correlation was also observed between the proton at δ_H 1.58 (H-5) and C-7. The NOESY spectrum showed correlations between H-5, H-6, H-9, H-

17 and H-18 protons. Thus, on the basis of these spectral data, the structure of compound **1** was established as Norcassamine (Fig. 1). This molecule is known as it was previously isolated from *E. chlorostachys* by Loder et al,[7] from the stem and root barks of *Erythrophleum suaveolens*.[8,9] It is the first isolation from the leaves of this species.

Table 1 : ^{1}H and ^{13}C NMR spectral data for compounds 1 and 2 (in CD_3OD)

	1		2	
Position	**δ_H, m (*J* in Hz)**	**δ_C**	**δ_H, m (*J* in Hz)**	**δ_C**
1	1.09, m ; 1.94, m	40.1	1.15, m ; 1.86, m	39.8
2	1.55, m ; 2.04, m	20.4	1.52, m ; 1.84, m	20.4
3	1.19, m ; 2.26, m	38.8	1.11, m ; 2.07 m	38.8
4	-	45.0	-	45.1
5	1.58, dd (3.4, 15.1)	54.7	1.57, dd (2.8, 13.8)	60.3
6	2.65, m ; 2.95, m	41.5	2.62, m ; 2.89, m	40.2
7	-	211.9	-	212.5
8	2.36, dd (3.9, 12.8)	54.7	2.33, dd (3.4, 12.6)	54.7
9	1.78, dt (7.0, 17.0)	48.6	1.70, m	48.4
10	-	37.8	-	37.8
11	1.16, m ; 2.06, m	28.3	1.23, m ; 1.96, m	28.0
12	2.09, m ; 3.78, m	25.1	2.07, m ; 2.75, m	26.3
13	-	167.3	-	155.9
14	3.01, m	40.1	3.09, m	39.6
15	5.81, s	112.8	5.88, s	116.6
16	-	169.5	-	171.4
17	1.11, d (6.8)	15.4	1.07, d (6.8)	15.2
18	1.19, s	28.5	1.16, s	28.6
19	-	178.6	-	178.6
20	0.84, s	12.5	0.83, s	12.5
21	4.36, m	60.0	3.52, m	50.9
22	3.67, m	49.1	3.66, m	60.6
23	2.74, s	33.9	2.97, s	33.7
24	3.67, s	52.0	3.66, s	52.0

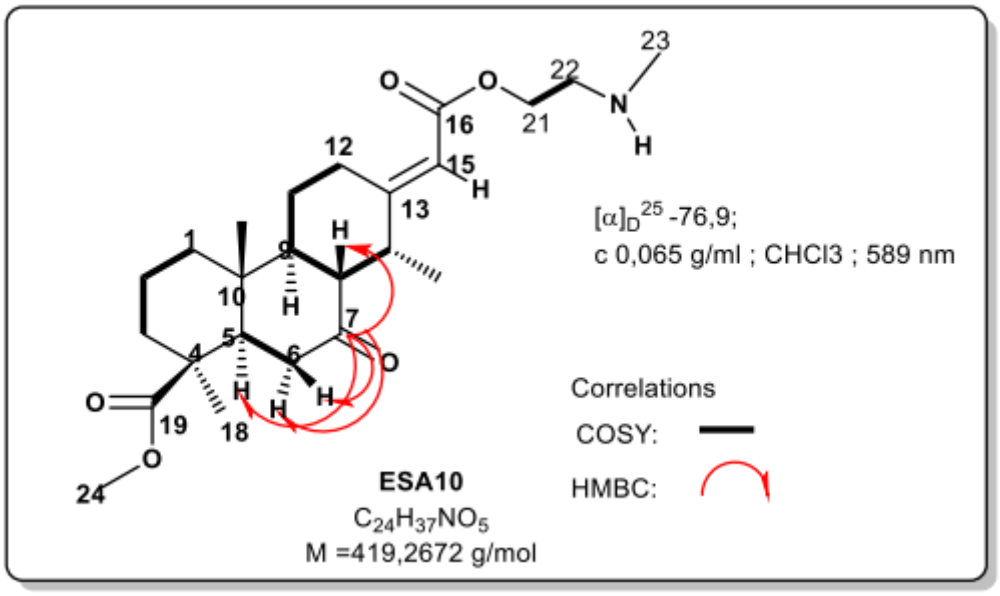

Figure 1: Norcassamine isolated from the leaves of *Erythrophleum suaveolens.*

Compound 2 (Fig. 2) was obtained as a yellowish amorphous solid, soluble in methanol. Its HRESI-QTOF-MS showed the peak of the pseudo-molecular ion $[M+H]^+$ at *m/z* 420.2714, giving a molecular weight of 419.2636 g/mol. This mass allowed us to propose the formula $C_{24}H_{37}NO_5$ (calculated mass 419.2672). IR spectrum showed absorption bands at υ_{max} 1723; 1701 and 1650 cm^{-1}, characteristic of carbonyl groups

absorptions. The majority of signals in ^{1}H NMR spectrum of this compound are observed in the high fields region (Table 1). This implies the presence of aliphatic protons. Also, the proton and carbon spectra of compound **2** are similar to those of 6α-hydroxy-norcassamide.[10] The difference is observed in the peak at δ_C 76.9 (C-6, HC-OH) which is present in the spectrum of 6α-hydroxy-norcassamide and absent in that of **2**. Indeed, the two compounds are distinguished by the absence of a hydroxyl group at C-6 position in compound **2**. On the HMBC spectrum, a $^{2}J_{C\text{-}H}$ correlation was observed between the protons at δ_H 2.62 (H-6), 2.89 (H-6) and 2.33 (H-8), and the carbonyl of ketone function at δ_C 212.5 (C-7) ; in addition a $^{3}J_{CH}$ correlation was observed between the proton H-5 (δ_H 1.57) and C-7. The NOESY spectrum showed that this compound is also in the same spatial arrangement as **1**. Thus, the structure of **2** was formally identified as Norcassamide (Fig. 2). This compound has already been isolated from the stem barks of *E. fordii*,[11] the roots and the stem barks of *E. suaveolens*.[8] Norcassamine and Norcassamide are two classical molecules of the genus *Erythrophleum*. These compounds already isolated from stem barks and roots[9] are isolated for the first time from the leaves of this species.

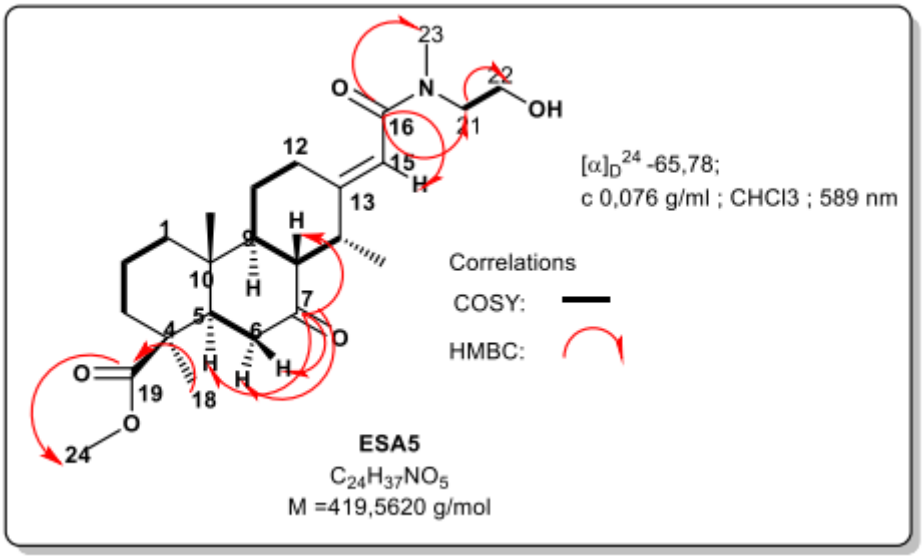

Figure 2: Norcassamide isolated from the leaves of *Erythrophleum suaveolens.*

3.2. Anti-trypanocidal activity

For biological activities, we evaluated the cold and reflux methanol extracts of leaves, stem bark and roots bark. We also evaluated 4 compounds, 2 isolated from leaves (Norcassamine and Norcassamide) and 2 others isolated in our previous work from stem bark and root bark (6α-hydroxy-*nor*-cassamine and 6α-hydroxy-*nor*-erythrophlamide). The results of the biological activity test, in LC_{100}, Lethal Concentration (causing 100% death), are expressed in mg/L or in µM (pure products). The values recorded for extracts and compounds range from 12.5 to over 125 µg/mL. This work indicates that leaves have the best pest control activity compared to stem and root barks. It can be seen that the cold methanolic extract is more active than the hot methanolic extract on *Trypanosoma brucei brucei* (Table 2). This could be due to the fact that the increase in temperature results in high heat which will cause the destruction of the molecules. These results are in line with the literature. Indeed, work carried out by Kablan[12] showed that trunk bark had trypanocidal activities. Also, Norcassamine (**1**) is more active than Norcassamide (**2**). Therefore, the amine function could increase the activity contrary to the amide function. Indeed, the different results are in agreement with the data in the literature.[9] The genus *Erythrophleum* contains various classes of compounds, including alkaloids, terpenoids, phytosterols, saponins, flavonoids and their derived glycosides. In particular, *E. suaveolens* is a species rich in nitrogenous compounds, the cassane-type diterpenoids. These are chemotaxonomic markers of this species. Numerous *in vitro* and *in vivo* pharmacological results have also revealed that the species possesses cytotoxic, antioxidant, antibacterial, antiparasitic, anticonvulsant, anti-inflammatory, anticancer, anti-angiogenic, sedative and cardiac activities.[13,14]

Table 2: Trypanocidal activity of methanolic extracts and isolated compounds from *E. suaveolens* against *Trypanosama brucei brucei.*

		LC_{100}	
		mg/L	**µM**
Leaves	Cold	12.5 ± 0.1	-
	Reflux	> 50.00	-
Stem bark	Cold	> 50.00	-
	Reflux	> 50.00	-
Root bark	Cold	> 50.00	-
	Reflux	> 50.00	-
Norcassamine		62.5 ± 0.1	149.16 ± 0.1
6α-hydroxy-*nor*-cassamine		62.5 ± 0.1	143.68 ± 0.1
6α-hydroxy-*nor*-erythrophlamide		> 125	> 299.76
Norcassamide		> 125	> 298.33
Melarsoprol		0.4 ± 0.1	0.2 ± 0.1

4. CONCLUSION

The present work is a contribution to the valorization of *Erythrophleum suaveolens*, a plant widely used in traditional Ivorian medicine. Phytochemical and biological studies on the methanolic extract from the leaves of this plant made it possible to isolate two diterpenoids, one with an amine function (Norcassamine) and the other with an amide function (Norcassamide). The biological tests carried out showed that the cold methanolic extract has a trypanocidal activity on *Trypanosoma brucei brucei* even if this remains moderate compared to that of the reference molecule, Melarsoprol. The results showed that the cold methanolic extract has a better activity than the hot methanolic extract. Norcassamine also showed better activity compared to Norcassamide. To the best of our knowledge, the evaluation of the trypanocidal activity of the extracts from *Erythrophleum suaveolens* leaves was carried out here for the first time.

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6. FUNDING

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7. COMPETING INTERESTS

Authors declared that no competing interests exist.

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