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Evaluation of antioxidant activities of methanol extracts and cassane diterpenoids from *Erythrophleum suaveolens* [(Guill. et Perr.) Brenan] (Fabaceae)

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Abstract
Erythrophleum suaveolens (Fabaceae), a medicinal plant from Côte d'Ivoire, was investigated for its antioxidant constituents. This led to the isolation of two cassane diterpenoid amides, 6α-hydroxy-*nor*-cassamide (1) and *nor*-cassamide (2), from the root barks. Their structures were established according to their spectroscopic analyzes (1D and 2D NMR, HR-ESIMS, IR). The antioxidant capacity of methanol extracts and isolated compounds were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the Ferric Reducing Antioxidant Potential (FRAP). A significantly increased DPPH and FRAP activities were observed for all samples. But, the best activity was obtained with methanol crud extract of the root barks from *E. suaveolens* (ESER).

Keywords: Fabaceae, *Erythrophleum suaveolens*, antioxidant, nor-cassamide, DPPH, FRAP

1. Introduction
Antioxidants are compounds that delay or inhibit the oxidation process by blocking the initiation or propagation of oxidizing chain reaction due to oxidative stress [Shahidi F., *et al*., 2015] [15]. Antioxidants were known as free radical scavengers, chelators of pro-oxidant metals, reducing agents, or as quenchers of singlet oxygen. Described as an imbalance between the body's ability and free radicals to detoxify these molecules or repair the resulting damage, oxidative stress causes extensive damage to biological molecules such as DNA, lipids and proteins [Lichtenberg *et al*., 2015; Sies *et al*., 1997] [8, 17]. Oxidative stress seems to be the main cause of several diseases [Mates *et al*., 2000] [9]. Oxidative stress results from the increase of free radicals in mitochondrial multiplication [Girodon *et al*., 1997; Sohal *et al*., 2002] [5, 18], which potentiates the appearance of multifactorial affections: Alzheimer's disease, rheumatism, diabetes, cardiovascular diseases, etc [Sergeant *et al*., 1998] [10]. Given the diversity and severity of diseases caused by oxidative stress, several researches have been initiated to discover new antioxidants in order to limit this aggression of the cellular constituents and the associated pathologies. In order to contribute to the search for new antioxidants from natural sources; we investigated the genus *Erythrophleum* (Fabaceae), a tropical woody genus [Jerome *et al*., 2013]. Three species are well represented in Côte d'Ivoire: *Erythrophleum africanum* (Harms), *Erythrophleum ivorense* (A. Chev.) and *Erythrophleum suaveolens* (Guill. & Perr.) Brenan [Wakeel *et al*., 2014]. *Erythrophleum* is considered to be a rich source of cassane diterpenoids [Kablan *et al*., 2014; Dade *et al*., 2015] [7, 3], compounds that possess antioxidant activities [Dickson *et al*., 2007] [4]. In this study, we are interested by *Erythrophleum suaveolens* (Guill. & Perr.) Brenan [Dade J., *et al*., 2015] [3], a plant, used in Ivorian traditional medicine for the treatment of Buruli ulcer [Kablan A., *et al*., 2014] [7]. In Cameroon and Democratic Republic of Congo, the stem bark extract is used for the treatment of dermatie, convulsions, cardiac problems, headaches and migraines, rheumatism, edema, and respiratory disorders (asthma) [Dade J., *et al*., 2015] [3]. In our previous study, we have investigated the methanol extract of the stem barks from this species. This led to the isolation of cassane diterpnoids [Dade J., *et al*., 2015] [3]. Present study was focus on the isolation of antioxidant constituents from the root barks of *E. suaveolens*. Therefore, the antioxidant activities of methanol extracts from stem barks and the root barks were evaluated.

We report here, the isolation and structure determination of two cassane diterpenoids amides from the root barks and the antioxydant activity Measurement.

2. Materials and Methods

2.1. Plant material

The stem barks and the root barks from *E. suaveolens* (Guill. et Perr.) Brenan (Fabaceae) were collected in the region of Toumodi (Côte d'Ivoire), in December 2014. The plant was identified and authenticated by Pr. Joseph Ipou Ipou, herbarium of the National Flower Center of University Félix Houphouet-Boigny (Côte d'Ivoire). A Voucher specimen KABLAN ES-2014 (for stem bark) and KABLAN ER-2014 (for root bark) were deposited in this herbarium.

2.2. Extraction and isolation

2.2.1. Preparation of methanol extract from the stem barks of *E. suaveolens*

The dried and powdered stem barks of *E. suaveolens* (500 g) were extracted three times with MeOH (6 L) to afford the methanol extract (ESET).

2.2.2. Preparation and purification of methanol extract from the root barks of *E. suaveolens*

The dried and powdered root barks from *E. suaveolens* (700 g) were extracted three times with MeOH (7 L). The solvent was evaporated to dryness to yield the methanol extract (ESER). A part of this extract (15.0 g) was dissolved in 50 ml of MeOH, alkalinized with a few drops of NH_4OH then, 50 ml of CH_2Cl_2 were added to heighten the dissolution. Finally, an acid solution (H_2SO_4, 1N) was added. The organic phase (CH_2Cl_2) was removed and, acid aqueous phase was extracted twice with CH_2Cl_2 (2x100 ml). The dichloromethane extracts were put together and evaporated to dryness. The acid aqueous phase was alkalinized (pH 10) with a solution of NH_4OH then, alkaloids were extracted four times with AcOEt (4x 100 ml) to yield ethyl acetate extract (EA), after evaporation under reduced pressure. This extract (EA) was then subjected to flash chromatography using silica gel (12 g Grace Cartridge), with a gradient at 18 ml/min. Seven fractions were obtained according to their TLC profiles. The fraction F2 (120.0 mg) was purified twice on preparative HPLC, using the mixture H_2O+0.1% HCOOH/ACN (20:50) as mobile phase, following a gradient of elution to afforded compounds 1 and 2.

2.3. Antioxidant activities

2.3.1. Determination of DPPH Radical Scavenging Capacity

Principle: DPPH is characterized by its ability to produce stable free radicals. This stability is due to the delocalization of free electrons within the molecule. The presence of these DPPH radicals • gives rise to a dark purple coloration of the solution. The reduction of DPPH radicals • with an antioxidant causes a discoloration of the solution [Molyneux, 2004] [11]. The color change can be followed by spectrophotometry at 517 nm and in this way the antioxidant potential of a substance or a plant extract can be determined [Popovici *et al*., 2010, Molyneux, 2004] [13, 11].

Protocol: The *in vitro* antiradical activity of extracts (ESET and ESET) from *E. suaveolens* was measured by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) test, according to the method of Parejo *et al*. (2002) with some modifications. Slowly, 2 ml of a hydromethanol solution (70/30) containing DPPH (100 µM) was mixed with 2 ml of different dilutions of the extracts (0 -1 mg/ml). A range of concentrations (0-200 µg/ml) for vitamin C was used as reference. The resulting mixture is then kept away from light at room temperature for 30 minutes. The absorbance is then measured at 517 nm against a control consisting of 2 ml of the DPPH solution and 2 ml of the hydromethalolic solution. Samples and references are prepared under the same operating conditions. The decrease in absorbance is measured spectrophotometrically and the Percent of inhibition (% PI) is calculated according to the formula below:

$$PI = \frac{(A_0 - A_1)x100}{A_0}$$

PI (%): inhibition power in%
A_0: absorbance of the DPPH solution in the absence of the extract (white)
A_1: absorbance of the DPPH solution in the presence of the extract (test)

The Concentration of extracts, isolated compounds or vitamin C, that is responsible of the inhibition of 50% (IC_{50}) of DPPH radicals, was determined by projection from 50 % on the graph representing the percentage inhibition of DPPH, depending on the concentrations of the extracts and vitamin C. (% Inhibition DPPH = f (extract concentrations).

2.3.2. Ferric Reducing Antioxidant Power (FRAP)

Principle: The FRAP method is based on the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). This method evaluates the reducing power of compounds [Ou *et al*., 2001]. The presence of reducing agents (HA) in plant extracts causes the reduction of Fe^{3+}/ ferricyanide complex with ferrous form. Therefore, Fe^{2+} can be evaluated in measuring and monitoring the increase in the density of the cyan blue color in the medium at 700 nm [Chung *et al*., 2002] [2]. This method measures the reducing power of antioxidants present in a mixture by their ability to reduce ferric tripyridyltriazine (Fe^{3+} -TPTZ) to ferrous ion (Fe^{2+} -TPTZ) at acidic pH.

Protocol: The FRAP test was carried out according to the method described by Pulido *et al*. (2000) [14]. A fresh solution of FRAP reagent (10 mM) was prepared by mixing 2.5 ml of TPTZ solution (10 mM in 40 mM HCl) with 2.5 ml of $FeCl_3$,$6H_2O$ (20 mM) and 25 ml of buffer acetate (300 mM sodium acetate, pH led to 3.6 with acetic acid). Then, 2500 µl of the FRAP reagent was added to 100 µl of the test compounds dissolved in a hydromethanol solution (70:30). After 30 min of incubation in the dark, the absorbance was read at 593 nm. Trolox was used as a dosage control. A calibration line was made with the following Trolox concentrations: 1, 0.5, 0.25, 0.125, 0.0625, 0.031 mg/ml.

3. Results and Discussion

3.1. Extraction and isolation

The preparation of the methanol extract from the stem barks of *E. suaveolens* led to 17.21 g of crud extract (ESET). This extract (ESET) was investigated in our previous study and cassane diterpenoids were isolated, among which 6α-hydroxy-norcassamide and *nor*-cassamide [Dade J., *et al*., 2015] [3]. The preparation of the methanol extract from the root barks of *E. suaveolens* afforded 32.3 g of crud extract (ESER). Only ESER was investigated here, for it chemical composition, because of its high antioxidant activity. Thus, ESER was

extracted a first time with dichloromethane then with ethyl acetate. The fractionation and purification of the ethyl acetate extract (EA) afforded compounds 1 (50.0 mg) and 2 (11.0 mg).

Compound 1 (Figure 1) was obtained as an amorphous solid. The HR-ESIMS showed a peak at *m/z* 436.2709 $[M+H]^+$ corresponding to the molecular formula $C_{24}H_{37}NO_6$. The IR absorption bands at 1650, 1702 and 1723 cm^{-1} were due to carbonyl functional groups. The ^1H NMR spectrum (Table 3) showed two methyl singlets at δ_H 1.43 (H-18) and 0.91 (H-20), a methoxyl singlet at δ_H 3.70 (H-24), an olefinic proton singlet at δ_H 5.91 (H-15) and a secondary amine (N-CH_3) at δ_H 3.11 (H-23). The ^{13}C NMR spectrum (Table 1) revealed carbonyl signals at δ_C 171.3 (C-16) for amid, 179.1 (C-19) for ester α,β-unsaturated and 211.5 (C-7) for ketone. Two olefinic carbons were observed at δ_C 155.7 (C-13) and 116.7 (C-15). According to these spectral dada, this compound was identified as 6*α*-hydroxy-*nor*-cassamide (1), a molecule previously isolated from the stem barks of *E. suaveolens* [Dade J., *et al*., 2015] [3] and the stem bark of *E. fordii* Oliv. [Qu J., *et al*., 2006]. But, this compound is reported for the first time in the root barks of *E. suaveolens*.

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

	1		2	
N°	**δ_H (ppm), J (Hz); mult**	**δc (ppm)**	**δ_H (ppm), J (Hz); mult**	**δc (ppm)**
1	1.60 (1H, *m*), 1.88 (1H, *m*)	40.8	1.15 (1H, *m*), 1.86 (1H, *m*)	39.8
2	1.52 (1H, *m*), 1.71 (1H, *m*)	20.4	1.52 (1H, *m*), 1.84 (1H, *m*)	20.4
3	2.09 (1H, *m*), 2.23 (1H, *m*)	40.0	1.11 (1H, *m*), 2.07 (1H, *m*)	38.8
4	-	46.5	-	45.1
5	1.46 (1H, *m*)	59.7	1.57 (1H, *dd*, 2.8, 13.8)	60.3
6	4.76 (1H, *d*, 12.4)	76.9	2.62 (1H, m), 2.89 (1H, m)	40.2
7	-	211.5	-	212.5
8	2.50 (1H *dd*, 2.7, 12.6)	52.9	2.33 (1H, *d*, 3.4, 12.6)	54.7
9	1.74 (1H, *m*)	48.0	1.70 (1H, *m*)	48.4
10	-	37.9	-	37.8
11	1.29 (1H, *m*), 1.97 (1H, *m*)	28.2	1.23 (1H, *m*), 1.96 (1H, *m*)	28.0
12	2.11 (1H, *m*), 2.75 (1H, *m*)	26.1	2.07 (1H, *m*), 2.75 (1H, *m*)	26.3
13	-	155.7	-	155.9
14	2.97 (1H, *m*)	33.9	3.09 (1H, *m*)	39.6
15	5.91 (1H, *s*)	116.7	5.88 (1H, *s*)	116.6
16	-	171.3	-	171.4
17	1.14 (3H, *d*, 6.8)	15.3	1.07 (3H, *d*, 6.8)	15.2
18	1.43 (3H, *s*)	32.1	1.16 (3H, *s*)	28.6
19	-	179.1	-	178.6
20	0.91 (3H, *s*)	14.3	0.83 (3H, *s*)	12.5
21	3.52 (2H, *m*)	50.9	3.52 (2H, *m*)	50.9
22	3.61 (2H, *m*)	61.3	3.66 (2H, *m*)	60.6
23	3.11 (3H, *s*)	38.9	2.97 (3H, *s*)	33.7
24	3.70 (3H, *s*)	52.1	3.66 (3H, *s*)	52.0

Compound 2 (Figure 1) was obtained as an amorphous solid. Its HR-ESI-MS spectrum gave fragment of the molecular ion $[M+H]^+$ at *m/z* 419.2636. The molecular weight was deducted as 419.2672, corresponding to the molecular formula $C_{24}H_{37}NO_5$. The IR spectrum, recorded in $CHCl_3$, displayed absorption bands of carbonyl groups (C=O) at ν_{max} 1652, 1701 and 1722 cm^{-1}. The ^1H and ^{13}C NMR spectra were recorded in CD_3OD. The ^1H NMR spectrum (Table 1) showed two methyl singlets at δ_H 1.43 (H-18) and 0.91 (H-20). The over singlets observed at δ_H 3.70, 5.91 and 3.11 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 amide (N-CH_3, H-23). The ^{13}C NMR spectrum (Table 1) confirmed the presence of ester groups with the peaks at δ_C 179.1 (C-19). A ketone group (C=O) was observed at δ_C 212.5 (C-7) and sp^2 carbons at δ_C 155.9 (C=C; C-13) and 116.6 (=CH-, C-15). The N-methylamide signal was observed at δ_C 33.7 (N-CH_3). According to these spectral data, compound 2 was identified as *nor*-cassamide (2). This compound is known, because previously isolated from the stem barks of *E. suaveolens* [Dade J., *et al*., 2015] [3] and the stem bark of *E. fordii* Oliv. [Qu J., *et al*., 2006]. Compound **2** is also reported for the first time in the root barks of *E. suaveolens*. Both molecules are cassane diterpenoids type.

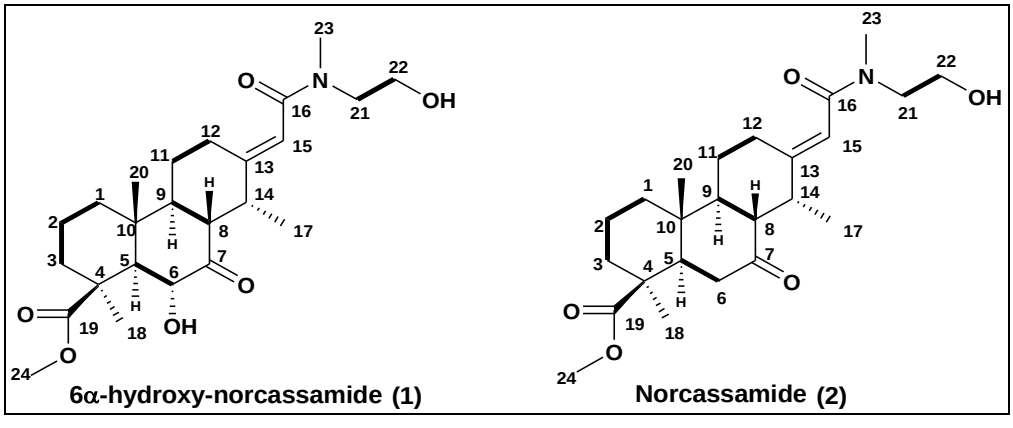

Fig 1: Cassane diterpenoids isolated from the roots of *E. suaveolens*

3.2 Antioxidant activities

3.2.1. Effect of scavenging DPPH radicals

In this experiment, the scavenging ability of extracts and cassane diterpenoids on DPPH free radical were examined in the concentration range of 50-500 µg/mL using the DPPH colorimetric assay. The results are given in Table 2. The scavenging ability of extracts and isolated compounds compared to those of vitamin C is shown in Figure 2. The results indicated that methanol extract of the stem barks (ESET), methanol extract of the root barks (ESER) and the isolated compounds (which are cassane diterpenoids type) exhibited very significantly radical scavenging. Among the extracts, the most active was ESER but, ESET (IC_{50}= 9.04 ± 1.12 and ESER (IC_{50}= 9.04 ± 1.12) were more active than isolated compounds 1 and 2 (Figure 2, Table 2). ESER (IC_{50}= 4.16 ± 0.57) was more active than vitamin C (IC_{50}= 6.065 ± 0.41) (Figure 3, Figure 4 and Table 2). Concerning the isolated compounds, 1 (IC_{50}= 105.4 ± 0.11) was more active than 2 (IC_{50}= 105.4 ± 0.11). The difference between compounds 1 and 2 was the presence of a hydroxyl group in position C-6 for 1. This could explain this difference of activity. The high antioxidant activity of ESER in comparison with that of ESET supposes that they have not the same chemical composition, in quantity.

Table 2: DPPH radical scavenging activities of MeOH extract of the root bark, the stem bark, and isolated compounds from *Erythrophleum suaveolens*.

Samples	Vitamine C	ES ER	ES ET	Compound 1	Compound 2
CI_{50} [µg/ml]	6.065 ± 0.41	4.16 ± 0.57	9.04 ± 1.12	105.4 ± 0.11	536.7 ± 11.2

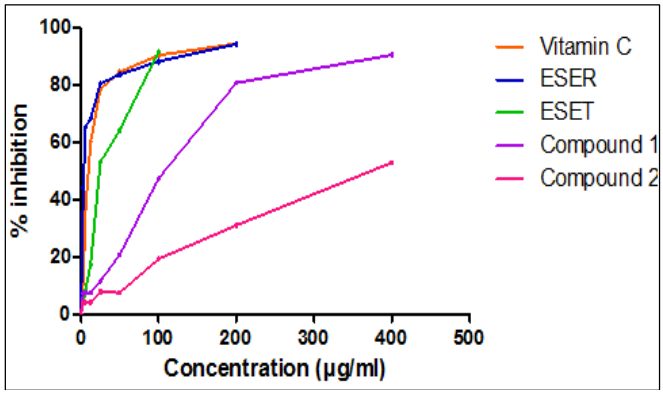

Fig 2: The scavenging effects of extracts, compounds 1, 2 and Vitamin C

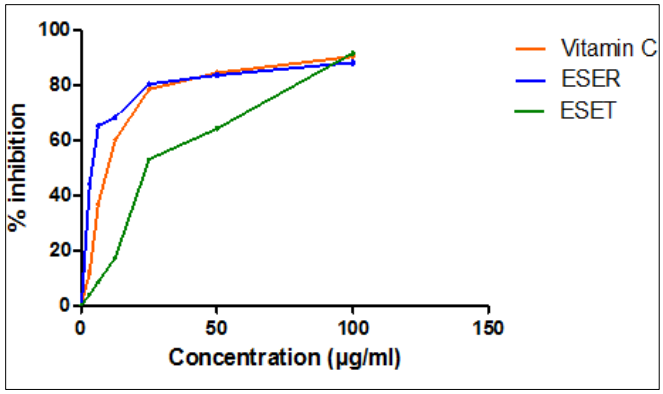

Fig 3: The scavenging effects of ESER, ESET and Vitamin C

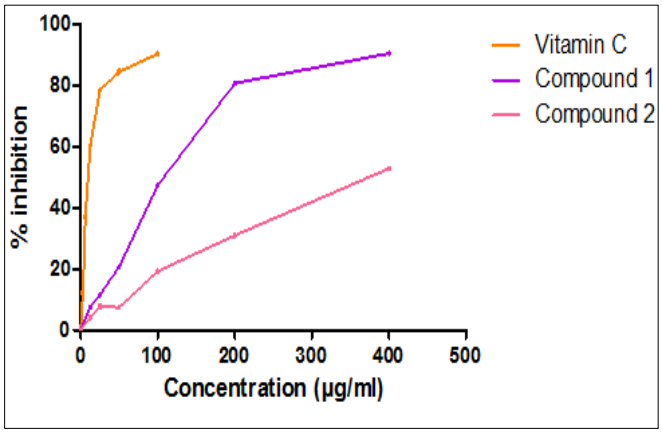

Fig 4: The scavenging effects of 1, 2 and Vitamin C on hydroxyl radicals

3.2.2. Evaluation of ferric reducing antioxidant power

The ability of compounds to reduce Fe^{3+} to Fe^{2+} was evaluated using the established FRAP method. The results (Table 3 and Figure 5) showed that 6α-hydroxy-*nor*-cassamide (1), *nor*-cassamide (2), ESER and ESET everyone reduce ion Fe^{3+} to Fe^{2+}. This method allowed us to confirm the antioxidant activity of these samples. In comparing their activities, we found that extracts ESET and ESER were more active than 6α-hydroxy-*nor*-cassamide (1) and *nor*-cassamide (2). These results of FRAP method were in confirming with those obtained with DPPH method.

Table 3: The Ferric Reducing Antioxidant Potential (FRAP) of MeOH extract of the root bark, the stem bark, and isolated compounds from *Erythrophleum suaveolens*

Sample	Compound 2	Compound 1	ES ET	ES ER
C (µM Eq trolox/g EXS	8,802 ± 1,07	39,92 ± 0,51	654,0 ±15,37	801,2 ±2,28

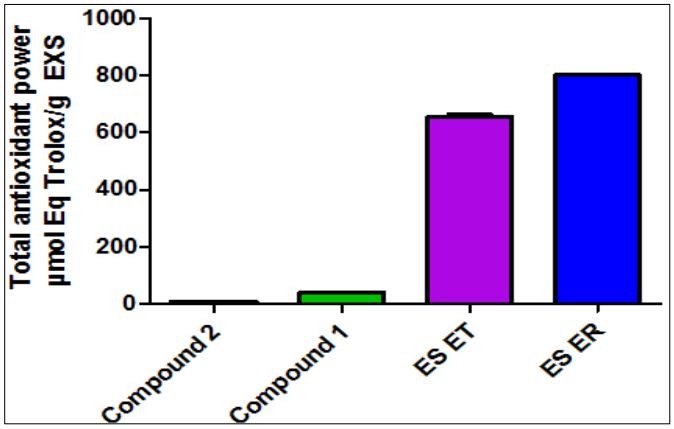

Fig 5: Antioxidant capacities of ESER, ESET, 1 and 2 by FRAP method.

4. Experimental Section

4.1. General experimental procedures

IR spectra were recorded with a PerkinElmer type 257 spectrometer. The NMR spectra were recorded on a Bruker AM-400 (400 MHz) NMR spectrometer, using CD_3OD as the solvent. The TMS signals were used as references. Silica 12 g Grace cartridges were used for flash chromatography using an Armen Instrument spot liquid chromatography flash apparatus. Sunfire® preparative C18 columns (150 × 19 mm, i.d. 5 µm and 150 × 30 mm, i.d. 5 µm; Waters) were used for preparative HPLC separations using a Waters Delta Prep equipped with a binary pump (Waters 2525) and a UV−visible diode array detector (190−600 nm, Waters 2996). Samples were analyzed using an Agilent LC-MS system comprising an Agilent 1260 Infinity HPLC coupled to an Agilent 6530 Q-TOF-MS equipped with an ESI source operating with a positive polarity. Chemicals and solvents were purchased from Sigma-Aldrich.

5. Conclusion

The antioxidant activities of methanol extract of the root barks and methanol extract of the stem barks from *E. suaveolens* were evaluated. The purification of the methanol extract of the root barks (ESER) using HPLC method, led to the isolation of two cassane diterpenoid amides: 6α-hydroxy-*nor*-cassamide (1) and *nor*-cassamide (2). Their complete structures were established according to spectroscopic data (NMR, IR and SM). The antioxidant activities of extracts and isolated compounds were measured using DPPH and FRAP methods, vitamin C was used as reference molecule. The best antioxidant activity was obtained with methanol extract of the root barks (ESER).

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