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Dosage and evaluation of the antiradical activity of alkaloids of the root barks of *Oulotricha* Le Thomas: isolation of Liriodenine

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Abstract
Oulotricha Le Thomas, a subspecies of *Annona senegalensis* (Annonaceae), is a plant present in all the Congolese savannahs whose mature fruits are widely consumed by the said population and sometimes edible. It is used traditionally to treat infections and some illness. Today, there are more than 160 alkaloids characterized in Annonaceae but any isoquinolin are not isolated of this plant. As a result, an extraction of the alkaloids confirmed by TLC after revelation with KI-Iodine, followed by a spectral scanning assay these alkaloids were made. Subsequently, this extract was fractionated until the isolation one compound and evaluated for anti-radical activity. On an extraction yield of 0.3% of the alkaloids was obtained 0.02% of isolated Liriodenine. The dosage of alkaloids at wavelengths between 254.5 and 360.5 nm detected three peaks of characteristic absorbance whose the maximum absorbance was observed at 320 nm. This extract exhibited a good anti-radical activity.

Keywords: Oulotricha le Thomas, Annonaceae, alkaloids, Liriodenine, antiradical activity

1. Introduction
Annonaceae are one of the most natural and homogeneous families, both in morphology and habitat. There are about 128 kinds and more than 2050 species in this family, widespread in the tropics, most of which are in low-lying humid dense forest. The kind Annona is among the most widespread and consists of about 110 species which we find of *Annona senegalensis* Pers, a species more present in Africa. However, in the Congo, there is a subspecies of *Annona senegalensis* Pers called *Oulotricha* Le Thomas, a plant found in all the Congolese savannas. The mature fruits of this plant are yellow-orange frozen sweet and sometimes edible [1, 2, 3]. Traditionally, it is used against stomach upset, diarrhea, epilepsy, gonorrhea and for treat infections [1, 3, 4].
Previous work in this subspecies has shown the presence of terpenes and sterols, phytosterols, flavonoids, alkaloids and fatty acids [5, 6].
However, today, there are more than 160 alkaloids characterized in Annonaceae whose derivatives of isoquinoleine appear as the major alkaloids of this family. Indeed, these compounds are endowed with significant pharmacological properties [7-11].
The object of this study is to measure and evaluate the anti-radical activity of the total alkaloids as well as to isolate an alkaloid in the root bark of *Oulotricha* Le Thomas from Republic of Congo.

2. Material and Methods
2.1. Plant material
Bark roots samples from *Oulotricha* Le Thomas were harvested south of Brazzaville (Congo). A specimen was identified at the Botanical Laboratory of Plant Resource Center (CERVE) and deposited at the National herbarium of Brazzaville (IEC, Nkounkoun ° 1, 1bis, 1ter).

2.2. Extraction of total alkaloids
500 g of root bark powder of *Oulotricha* Le Thomas was macerated for 24 hours in cyclohexane (2 x 1.5 L). The marc was taken up in MeOH / NH_4OH (9: 1 v / v, 2 x 1.5 L) maceration for 48 hours. After filtration of the mixture, a small amount of distilled water was added to the resulting filtrate and then concentrated under reduced pressure. Half of the methanolic solution thus obtained (the filtrate) was acidified with the aqueous solution 5 % of

hydrochloric acid, and a liquid-liquid partition was carried out with the acidified aqueous solution. The acidified aqueous solution was then basified with an aqueous solution of NaOH (1M). A liquid-liquid partition is carried out between the basified solution and the dichloromethane. The dichloromethane phase (total alkaloids) is dried with anhydrous Na_2SO_4 and then evaporated to dryness under reduced pressure. A mass of 1.5 g of dichloromethane extract was obtained. Then, a Mayer test was performed on the extract to confirm the presence of total alkaloids.

2.3. Screening of alkaloids by thin layer chromatography (TLC)

The analyses on TLC were performed on the silica gel 60 F_{254} aluminum plates (Merck). The plates were developed in the saturated glass vessel of the appropriate eluent (mobile phase).

The mobile phase consisted of a tertiary mixture of the various separation solvents (butanol/acetic acid/water: V/V/V: 6/1/1). The plates were observed in visible light and in ultraviolet light (λ = 254 nm and 365 nm) after KI-iodine exposure [12, 13].

2.4. Dosage by spectral scanning of total alkaloids

The UV spectrums of methanolic extract of the total alkaloids and witness (caffeine) was obtained using a UV-Visible double-beam spectrophotometer (4211/50 Zuzi). Absorbance measurements of these solutions were performed by spectral scanning by fixing the wavelengths in the range between 220 nm and 370 nm [14].

2.5. Fractionation and isolation of the compound 1

The dicholoromethane extract containing total alkaloids was subjected to column chromatographic separation using the absorbent (silica gel) and eluents cyclohexane/EtOAc (V/V: 9/1) then CH_2Cl_2/MeOH (V/V: 8/2).

The fractions collected were grouped according to their chromatographic similarity on TLC. The revelation and observation of these constituents were made by an ethanolic solution 10 % of phosphomolybdic acid and ultraviolet.

Eight (8) fractions were collected and noted fractions 1 to 8 respectively at 14.32 mg, 454.88 mg, 76.71 mg, 30.23 mg, 8.06 mg, 80.75 mg, 144.99 mg and 17.29 mg.

Fraction 6 (80.75 mg) obtained in an elution system CH_2Cl_2/MeOH (V/V: 8/2) was fractioned on silica gel with the same polarity elution solvents (V/V: 95/5). The subfractions obtained were grouped according to their chromatographic similarity. A total, 5 subfractions were obtained and noted f6-1 to f6-5.

The purification of the yellow powder (f6-2: 10.17 mg) noted Compound 1 having a purity level of 95.5% was observed by C18 HPLC using a water-methanol mobile phase.

2.6. Structural analyzes compound 1

The ^1H and ^{13}C NMR spectrums were recorded on a Bruker type apparatus at 400 MHz, using $CDCl_3$ as the solvent. The internal standard is the TMS. COSY ^1H-^1H and HSQC ^1H-^{13}C data were acquired on the invbt microprogram (J = 145 and 9 Hz respectively). The mass spectrum was recorded on a Hewlett-Packard 5890 Series II apparatus, and the C18 HLPC was performed at wavelengths of 254.5 and 360.5 nm.

2.7. Evaluation test of the anti-radical activity of the alkaloids

The evaluation of the anti-radical activity is carried out with a spectrophotometric apparatus whose procedure is described as follows:

The various volumes of 100, 200, 400 and 1000 µL at a concentration of 5 mg/mL of the total alkaloid extract were added to 2 mL of the methanol solution of DPPH° (0.025 g/L) freshly prepared. The absorbances at 517 nm were measured at different time intervals until the plateau was obtained. After incubation in the dark at room temperature, the absorbances were read using a spectrophotometer [15].

The concentration of DPPH° in g/L in the reaction medium was calculated from a calibration curve made at various concentrations $[DPPH°]_t$ by an equation of linear regression (1) where t is the time, a represents the slope and b represents the intersection of the curve with the y-axis. The percentage of DPPH° residual (%DPPHH°res) was calculated by an equation (2) where $[DPPH°]_{t=0}$ is an initial concentration of stable radical without extract and $[DPPH°]_t$ is an concentration of DPPH° remaining in the reaction at the plateau.

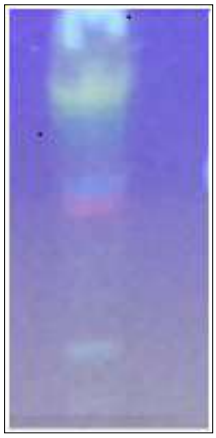
$$A_{517nm} = a * [DPPH^{\circ}]_t + b \quad (1)$$

$$\%DPPH°_{res} = [DPPH°]_t / [DPPH°]_{t=0} \quad (2)$$

3. Results and discussion

3.1. Determination of alkaloids by TLC

The chromatograms 1 and 2 showed respectively the spots of colors violet, red, blue and yellow and brownish, respectively observed at UV 365 nm and visible, before and after revelation with KI-Iode. The staining of these spots justifies the presence of alkaloids in this extract [12, 13].



Chromatogram 1
Before revelation,
Obs. at UV 365 nm

Chromatogram 2
After revelation KI-Iode,
obs. visible

Eluent: Butanol/acetic acid/water

3.2. Dosage by spectral scanning of total alkaloids

The UV absorption spectrum of this extract of total alkaloids in methanolic solution, obtained at wavelengths between 254.5 and 360.5 nm, made it possible to detect three peaks of characteristic absorbance at 260, 320 and 400 nm whose maximum absorbance is observed at 320 nm (Fig. 1). The maximum absorbance peak of caffeine (control) is detected at 300 nm (Fig. 2). According to the bibliographic data, the length range of alkaloids including caffeine is absorbed from 240 to 320 nm [16].

These data are close to our results and may confirm the presence of total alkaloids in our extract.

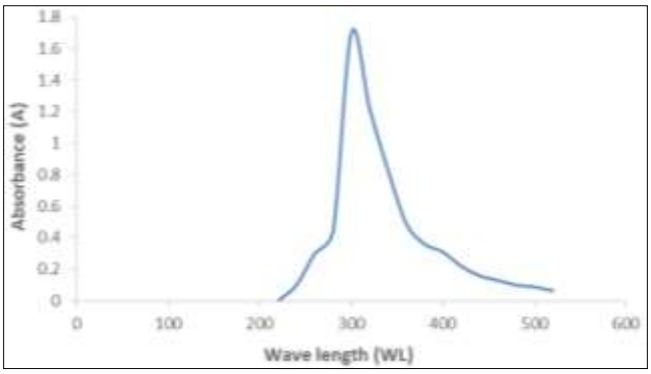


Fig 1: UV absorption spectrum of the extract of total alkaloids of *Oulotricha* Le Thomas

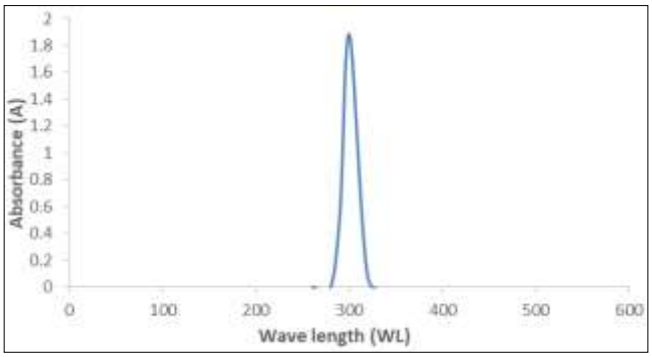


Fig 2: UV absorption spectrum of caffeine.

3.3. Structural analysis of compound 1

The isolated compound represents 0.02 % of the plant material for a total alkaloid yield of 0.3 %.

Electron impact mass spectrometry (70 eV) analysis revealed a molecular ion of m/z = 275 to deduce the formula: $C_{17}H_9NO_3$. The values of m/z at 247, 217 and 189 corresponding to $[M\text{-}CO]^+$, $[M\text{-}CO\text{-}CH_2O]^+$ and $[M\text{-}CH_2O\text{-}2CO]^+$ ions were also observed.

The spectral data of analyzes of compound 1 are shown in Table 1.

The ^1H NMR spectrum (400 MHz, $CDCl_3$) revealed the presence of eight protons: two singlets, one at 6.41 ppm (OCH_2O) and the other at 7.22 ppm (H-3); two doublets, one at 7.82 ppm (H-4, J = 5.30 Hz) and the other at 8.92 ppm (H-5, J = 5.30 Hz); two doublets of duplicates, one of which 8.59 ppm (H-8) and 8.68 ppm (H-11); and two triplets located respectively at 7.60 ppm (H-9, J = 7.80 Hz) and 7.78 ppm (H-10, J = 8.16 Hz).

However, the COSY ^1H-^1H spectrum showed correlations between protons H-4 and H-5, protons H-8 and H-9, H-8 and H-10, H-9 and H-11; which denotes the existence of a conjugation in the molecule.

Analysis of the ^{13}C NMR spectrum *(J*-mod) made it possible to demonstrate the existence of 17 carbons whose chemical shifts and the position of the signals made it possible to differentiate 9 quaternary carbons (from 110.2 to 182.98 ppm); one of which belongs to a conjugated ketone (C-7: 182.98 ppm), 7 aromatic CHs from 102.88 to 144.53 ppm and a methylene group $O\text{-}CH_2\text{-}O$ at 102.18 ppm. This result corroborates with the ^1H-NMR data. The presence of 6 aromatic quaternary carbons involved in ring junctions as well as the comparison of these ^1H-NMR data provided by the bibliography reveal an oxo-aporphine type structure [17, 18].

The correlations observed on the HSQC spectrum of compound 1 indicated the chemical shifts of the different protons bound to its carbons. The analysis of the correlations made it possible to confirm the presence of the methylene group (H-1': 6.41 ppm) in singlet form integrating for two protons with the chemical shift of the carbon (C-1': 102.18 ppm). The HSQC spectrum has also allowed the attribution of each aromatic proton to each of the carbons that carry them.

On the basis of the spectral data described below (Table 1), the structure of compound 1 corresponds to Liriodenine (Fig. 3). This aporphine alkaloid derived from isoquinoline is isolated for the first time in the root bark of *Oulotricha* Le Thomas. However, the works of You and *al*. isolated this molecule in the leaves of *Annona senegaleensis* [19].

The works of Chacón *et al*., Michael *et al*. and Zhiahen *et al*., have shown that the Liriodenine isolated of annonaceae family has significant antimicrobial activity and acts on carcinogenic cells [20-22]. However, the Congolese population traditionally uses the various organs of this plant in the treatment of microbial diseases [1, 4]. The presence of total alkaloids and liriodenin in this plant may justify this use.

Table 1: ^1H and ^{13}C NMR Chemical Displacements, HSQC (500MZ) and COSY (500MZ) Correlations in $CDCl_3$ of Liriodenin

Position	^{13}C (ppm)	^1H (ppm)	Correlation HSQC (^1H-^{13}C)	Correlation COSY (^1H-^1H)
1'(-CH_2-)	102,18	6,41 (2H, s OCH_2O)	C1'	
1	147,8			
2	152,13			
3	102,88	7,22 (1H, s)	C3	
3a	144,34			
4	124,9	7,82 (1H, d, J= 5,30 Hz)	C4	H5
5	144,53	8,92 (1H, d, J= 5,30)	C5	H4
6a	135,98			
7	182,98			
7a	130,8			
8	127,15	8,59 (1H, dd, J= 8,1, 0,9 Hz)	C9	H9
9	128,9	7,60 (1H, dt J= 8,1, 0,9 Hz,)	C8, C10	H8, H10
10	134,14	7,78 (1H, dt J = 8,1, 0,9 Hz)	C9, C11	H9, H11,
11	127,6	8,68 (1H, dd, J= 8,1, 0,9 Hz	C11	H10
11a	134,4			
11b	110,2			
11c	123,66			

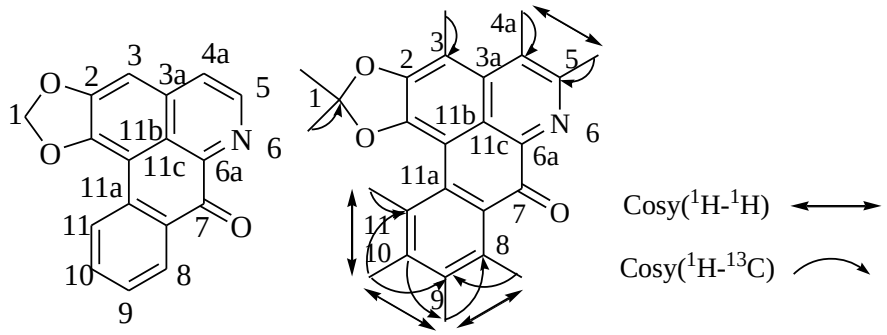


Fig 3: Structure of Liriodenine

3.4. Evaluation of anti-radical activity of total alkaloids

The extract of the total alkaloids of different volumes at concentration of 5 mg/mL. It decreased the absorbance of DPPH° as a function of time as shown in the curves of fig. 4.

It has been noticed that at volumes of 100 µL, 200 µL and 400µL respectively, the absorbance decreases slowly, but at 1000 µL (1 mL) of the same concentration, there was a significant decrease with the appearance of a good plateau at 600 seconds (10 minutes), which justifies the almost reduction of DPPH° in its non-radical form.

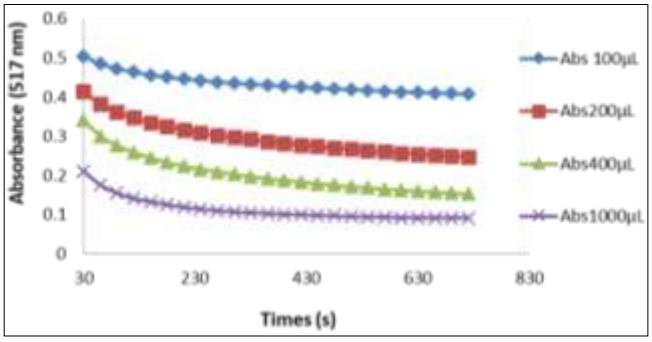


Fig 4: Evaluation curve of the anti-radical activity of the total alkaloids by reduction of the absorbance of DPPH° as a function of time

These results obtained by reducing the absorbance of DPPH° by total alkaloids as a function of time did not make it possible to calculate the anti-radical efficiency. Thus, it was necessary to transform these data to residual fractions of DPPH° as a function of time by determining the calibration curve at 257 nm (Fig. 5) whose absorbance is defined by the following linear regression equation (3):

$$A_{517nm} = 21.107 * [DPPH°]_t + 0.0157/R^2 = 0.997 \quad (3)$$

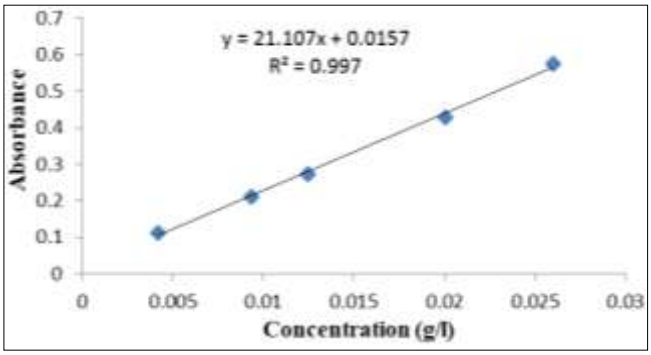


Fig 5: Residual DPPH ° calibration curve

The curve illustrated below (Fig. 6), has shown that the total alkaloid extract tested is capable of neutralizing the DPPH° over time and depending on their concentrations, this results in the decrease of the initial concentration of this radical (0.025 g/L). Similarly, the percentage of residual DPPH° depends on the volumes and concentrations of the extract used. The amount of DPPH° residual was lower (14 %) in the concentration of 5 mg/mL to 1000 µL compared to other volumes. This made it possible to emphasize a good anti-radical efficiency of DPPH° reduction.

Although this reduction is not significant enough, but it gives the extract of the total alkaloids of the plant, the trapping power of the radical DPPH°.

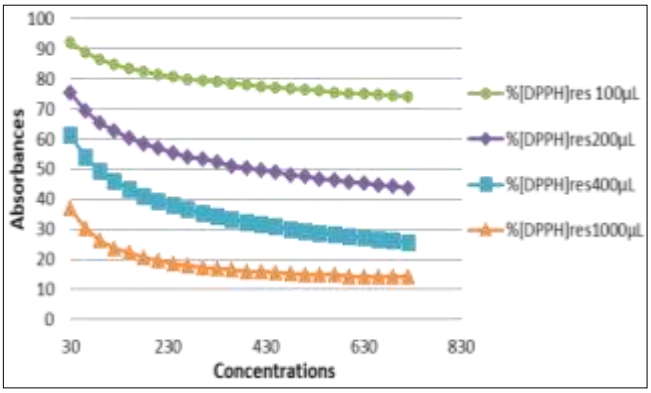


Fig 6: Residual DPPH° calibration curve for concentrations

4. Conclusion

The extraction of total alkaloids from the root bark of *Oulotricha* Le Thomas has isolated an alkaloid called Liriodenine. This aporphine alkaloid derived from isoquinoline endowed with significant antimicrobial activity and acting on carcinogenic cells was isolated for the first time in these organs of this Congolese plant species. The dosage performed at wavelengths between 254.5 and 360.5 nm of this extract allowed the confirmation of the alkaloids by detection of the three peaks of characteristic absorbance at 260, 320 and 400 nm whose maximum absorbance is observed at 320 nm. This extract exhibited a good anti-radical activity with a decrease in the absorbance of DPPH° as a function of time.

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