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ISOLATION AND STRUCTURAL ELUCIDATION OF THREE NEW COMPOUNDS CARISSININDOLE, SPININDOLE-A AND SPININDOLE-B FROM THE LEAVES OF *CARISSA SPINARIUM* (APOCYNACEAE) ORIGINATED FROM MADAGASCAR

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ABSTRACT

From the leaves of *Carissa spinarium* (Apocynaceae), a medicinal plant species endemic to Madagascar, three new compounds typical of Indolomonoterpenic alkaloids compounds named Carissinindole, Spinindole-A and Spinindole-B containing three membered rings in its side chain were isolated by repeated silica gel column chromatography. Their structures were determined by 1D and 2D NMR spectroscopy and spectroscopy High-resolution MS-IES. The results of the present research work is reported for the first time and revealed that a comparative study of the Apocynaceae family will help to understand the origin and maintenance of diversity among this family and their implication at Pharmacognosy level (biotaxonomy).

KEYWORDS: Carissinindole, Spinindole-A, Spinindole-B, Carissa spinarium, Spectroscopic techniques, Phytochemical markers, Biotaxonomy.

1. INTRODUCTION

Medicinal plants play an important role in human life to control disease[1,2] and as a valuable source of new drugs. The World Health Organization (WHO) has estimated that up to 80% of people still rely on herbal remedies for their health care.[3,4] As stated by the International Union for Conservation of Nature and the World Wide Fund, around 50,000–80,000 flowering plant species are used worldwide for therapeutic properties. Due to the low cost and easy availability of traditional drugs, the WHO also encouraging usage of herbal drugs for various human diseases.[5-6]

Madagascar is the poorest country in the world because their economic growth is very low, the insufficiencies of medical infrastructure, the lack of access to care hinder the effective management of hypertension because of very low purchasing power and those which leads to a marked increase in the frequency of this disease.[7-10] This is why Malagasy patients turn to traditional medicine to treat them.[11] The practice of traditional medicine holds an important place in Malagasy society[12] to heal and keep human beings in good health because of their custom, their very low purchasing power and the inadequacies of the infrastructure of the modern medical. In addition, Madagascar constitutes one of the most important biodiversity hotspots worldwide with more than 90% of its plant species being endemic.[20] This rate of endemism is besides raised on all the taxonomic levels, eight families are regarded as being entirely endemic of the island.[13-16]

During an ethno-botanical survey conducted in the South –west part of Madagascar, the majority of traditional healer interviewed reported that the aerial part of *Carissa spinarium* (Apocynaceae) known under vernacular name of Relefo [Malagasy name] issued by the local communities to treat conditions assumed to be hypertension. Extensive phytochemical studies have shown that *Carissa spinarium* is rich in a variety of alkaloids compounds especially indolomonoterpenic.[9]

According to available literature, no phytochemical research investigation has been carried out on this plant species. As part of our phytochemical work on Madagascar medicinal plants, we investigated leaves part of this plant. In the present paper, we report for the first time the isolation and structure elucidation of three new compounds from Carissa *spinarium* which have been

trivially named Carissinindole, Spinindole-A and Spinindole-B.

2. MATERIALS AND METHODS

2.1. General

Silica gel 60 and 100, and TLC precoated plates were purchased from Merck, Darmstadt, Germany, Analytical HPLC was performed on a Waters system (Millenium 32 workstation, 2690 separation module, 996 photodiode array detector) equipped with HiChrom Lips 100-5-250D column (4.6 x 250nm: LiChrospher Phase 5 µm, Si 100). A Perkin-Elmer 241 polarimeter was used for measurement of optical rotation. The 1D and 2D vasoconstrictive, hypertensive, and cardiac stimulant action [Beyaoui, et al, 2012][6] and can act as an allergenic substance [Nofal, 2004; Assarehzadegan, 2009].[21,4] Salsola species have antioxidant and anti-inflammatory properties [Ahlam & Fatma, 2007].[2] Alkaloid experiments were performed at 600 MHz and 400MHz for 1 H and 13Crespectively, on a Bruker Avance 600MHz instrument equipped with an Ultra Shield Plus magnet and triplet resonance cryoprobe with gradient unit. Sample temperatures were stabilized at 298 K. The deuteriomethyl13C signal and the residual 1 H signal of the solvent (DMSO-D6) were used as secondary references (δ39.6and δ2.49 from tetramethylsilane, respectively). The exact molecular mass (HRMS) of molecular ion [M-H]+ and fragment F+ were determined with a Micromass QTof-2 mass spectrometer equipped with an electrospray ion source and a Micromass QTof-2JOEL mass spectrometer equipped with a Direct Analysis in Real Time (DART) atmospheric pressure ion source.

2.2. Plant material

The leaves of *Carissa spinarium* (Apocynaceae) was collected in Ankamaroa forest at nearly 33 km from Anontsibe-Centre, Manja District, Menabe's Region, in the South-West part of Madagascar. The plant sample was identified by Mbola Versène Balzac, a botanist from of Faculty of Science, University of Toliara-Madagascar, and by comparison with reference specimens available at the Department of Botany, Tsimbazaza Zoological and Botanical Park, Antananarivo. Voucher specimens with assigned sample number CSA-001 was deposited at the Herbarium of the Laboratory of Applied Chemistry, Layflaylle Street, University of Toliara, Madagascar.

2.3. Phytochemical screening

A Chemical screening has been done on this plant using a aqueous or organic fraction following an established protocol.[22]

Detection of phenols (Ferric Chloride Test)

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicated indicates the presence of phenols.

Detection of flavonoids

The ethanol extract (5 ml) was added to a concentrated sulphuric acid (1 ml) and 0.5g of Mg. A pink or red coloration that disappear on standing (3 min) indicates the presence of flavonoids.

Detection of anthocyanosids

The presence of anthocyanosids is revealed by a color change as a function on pH due to titration of the acidic aqueous solution with a solution of NaOH.

If the solution turns a red color, the pH is less than 3, if against a blue color; the pH is between 4 and 6.

Detection of tannins

Two methods were used to test for tannins. First, about 1 ml of the ethanol extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration. Second, 2 ml of the aqueous extract was added to 2 ml of water, a 1 to 2 drops of diluted ferric chloride solution was added. A dark green or blue green coloration indicates the presence of tannins.

Detection of leucoanthocyanins

To 2 ml of aqueous extract was added few drop of Shinoda regeant in a test tube and then boiled. A red purple coloration in the supernatant indicates the presence of leucoanthocyanins.

Detection of saponins

To 1 ml of aqueous extract was added few volume of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth for 20 min.

Detection of alkaloids

Five ml of the extract was added to 2 ml of HCl. To this acidic medium, 1 ml of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

Detection of coumarins

Evaporate 5 ml of ethanolic solution, dissolve the residue in 1-2 ml of hot distilled water and divide the volume into two parts. Take half the volume as a witness and to add another volume of 0.5 ml 10% NH_4OH. Put two spots on filter paper and examined under UV light. Intense fluorescence indicates the presence of coumarins.

Detection of free quinones

To 1 ml of organic extract was added few drops of Borntrager reagent (NaOH 10% ou 10% NH_4OH) in a test tube. The solution was and then shaken vigorously. A sharp red or orange coloration indicates the presence of free quinones.

2.4. Extraction

1 kg of the plant sample powder was macerated in petroleum ether for 24 hours under continuous agitation and after filtration; the marc was spread in a fume hood ventilated at 30°C to eliminate the hexane solvent. If it was dry, the marc was soaked in ammonia and then macerated in chloroform for four hours under continuous agitation. This operation was carried out twice. After filtration, the obtained solutions were dried using anhydrous Na_2CO_3 and then evaporated to dryness. The crude extract obtained was redissolved in acidified water solution (10% concentrated hydrochloric acid) and then filtered. The soluble part in this solution was alkalinized using ammonia until pH=10, and the resulting basic solution was partitioned with chloroform, then shaken for five minutes and allowed to settle in the separatory funnel to form two phases. After decantation, the organic phase was recovered, dried with anhydrous Na_2CO_3, and then evaporated to dryness, yielding the total alkaloids from the aerial part of CSA-03.

2.5. Isolation

Five (5) grams of the alkaloids crude extract was first subjected to seperation using LH-20 Sephadex gel column chromatography eluted with a mixture of methylene chloride/methanol/ammoniac-sell ($CH_2Cl_2/CH_3OH/NH_4OH$: 6/4/5µl) and the column was in isocratic regime and at the end, it resulted into six fractions (F1-F6). These fractions were selected for the following steps. These fractions were checked for purity by analytical TLC, and the zone was detected with a UV lamp at 254 and 365 nm and by spraying with Dragendorff solution of reactive, followed by heating at 120 °C for 1-3 min. F5 and F6 were combined on the basis of TLC profile similarity and resubmitted to fractionation by silica gel column chromatography eluting with a mixture of diethyl ether/chloroform/methanol (2/7.5/0.5) and into appending of 5µl NH_4OH for preparation each use; the column was in isocratic regime and at the end, it resulted into five fractions and 180 mg of F53 was subjected to further purification using a silica gel column chromatography, with chloroform and a gradient of methanol for elution. The latter provided three pure compounds. The purity of Carissinindole, Spinindole-A and Spinindole-B was then detected by analytical HLPC with the mixture of chloroform and methanol 1:1 (v/v) as mobile phase, and the chromatography was performed with isocratic regime during 25 min. The eluted compound was detected based on its UV absorption in the wavelength range from 200 nm to 400 nm.

3. RESULTS

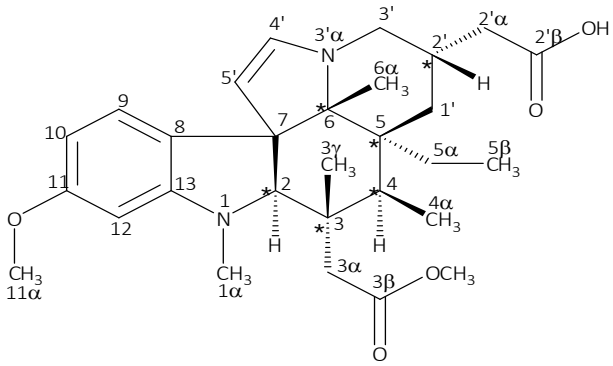
The results of chemical screening of the leaves extract of *Carissa spinarium* (Apocynaceae) revealed the presence the alkaloids, flavonoids, steroids, diterpenes and triterpenoids. However, chemical group such as saponins, coumarins, free quinones, anthocyanosids, tannins, leucoanthocyanins and phenols, were not found in the leaves extract of *Carissa spinarium*. The bioassay-guided fractionation of the alkaloid crude extract of the leaves of *Carissa spinarium* using repeated silica gel column chromatography resulted in the isolation of three pure compounds as evidenced by analytical TLC and HPLC analysis. These three pure compounds named CS-01, CS-02 and CS-03.

The isolation compound CS-01, showed a quasi-molecular ion at m/z = 497.01731 $[M+H]^+$, in the ESI.TOF-HRSM, spectrum which to the molecular formula $C_{29}H_{40}N_2O_5$. Its ^{1}H-NMR spectrum exhibited their six signals protons at δ0.96 (d), δ1.04(s), and δ1.24 (s) characteristic attributed to three methyl groups, two singulet between δ3.68 and δ 3.83 (s) characteristic to the signals of méthoxy groups and one singulet at δ2.70 attributed to the characteristic of the methyl amine (N-CH_3). Eight (8) signals doublet-doublet between δ1.26, δ1.51, δ2.00, δ2.04, δ2.25, δ2.61, δ2.83 and δ2.96 attributed to their signals of genime, protons of different chemical environments of the methylene groups. Three alkenes protons between δ7.10 (d), δ6.25 (d), and δ6.23 (s) attributed to the characteristic of signals alcens protons typical for benzene skeleton and the one signal of labile proton at δ11.79 attributed to hydroxyl proton characteristic acid. Three peak between δ1.57 (q), δ1.82 (m), and δ2.96 (s) characteristic attributed of the methine groups (CH) in a cycle and one signal at δ1.25 (q) unambiguously attributed to methylene (-CH_2-) protons to tie with the methyl, and at the end the two signals of alcens protons between δ4.58 (d), δ5.68 (d), indicate that these alcens protons signals are characteristic the presence of alcens protons in the pyrroline cycle. The 1D ^{13}C (broad-band) NMR spectrum contained twenty-nine (29) signals including the carbonyls between δC 173.1(C-3β) and δC 178.9(C-2'β). Examination of the 1D ^{13}C (DEPT), and the 2D (HSQC) spectra data of the compound-1 (CS-01) revealed the presence of eight (8) signals correspond carbons of typical indole skeleton whose: six (6) alkene carbons (C=C) double bonds indicating three (3) shielded aromatic methine groups at δ95.6 (C-12), δ103.5 (C-10) and δ124.3 (C-9), three (3) quaternary carbons of which the characteristic are attributed to the typical carbons of benzene skeleton at δ130.1 (C-8), δ158.7 (C-11) and δ152.3 (C-13), two signals carbons between δ144.9 (C-4') and δ108.6 (C-5') characteristic attributed of the carbons (CH) in the pyrroline cycle, seven carbons characteristic attributed to the alkyl groups in the cycle between two methylene groups at δ41.1(C-1'), δ50.2(C-3'), two methine groups at δ22.2 (C-2') and δ34.9 (C-3) and three quaternary carbons at δ27.5 (C-3), δ39.8(C-5) and δ69.6 (C-6).

In addition to the examination of the 1D ^{13}C and the 2D HSQC spectrum that permitted to reveal the presence of the six methyl groups at δ13.7 (CH_3), δ15.8 (4-CH_3), δ19.1 (6-CH_3), δ 20.5 (3-CH_3), δ38.4 (N-CH_3), δ51.9 (3β-OCH_3), δ55.8 (11-OCH_3) at the end two signals carbons attributed of methylene between δ23.7 (C-5α) and δ37.9 (C-3α). The ^{1} H and ^{13}C chemical shift values

of individual spin system were determined by correlation in the 2D HSQC spectrum.

The individual ^{1}H and ^{13}C chemical shift assigned by ^{1}H-^{1}H COSY spectrum and 2D HSQC an HMBC correlation spectra are shown respectively in table 1. To the best of our knowledge; this is the first time that a membered ring occurs in the side chain of a typical indolomonoterpenic (fig.1).



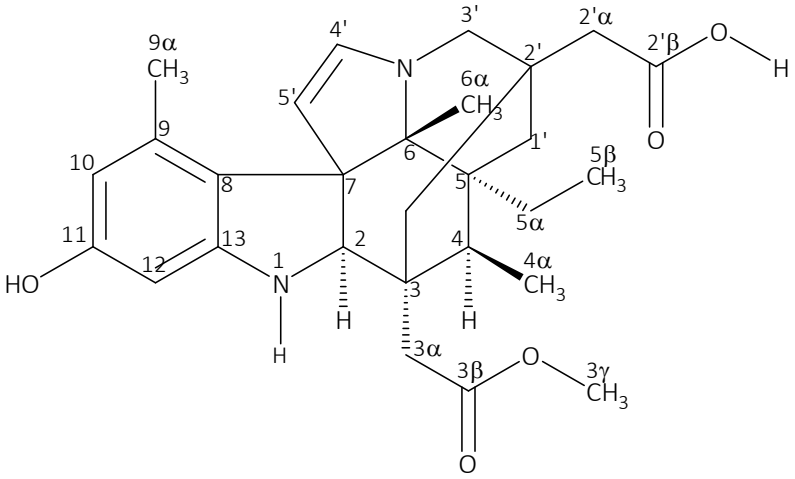
Compound 1: Carissinindole

Figure 1: Structure of Compound.[1]

Table 1: ^{1}H and ^{13}C chemical shift, the correlation ^{1}H-^{1}H (COSY) and important HMBC correlation of Carissinindole.

Position		1D-NMR experiments		2D-NMR experiments		
N°	Types	δC	δH	COSY	Multiplicity	HMBC
1	N	-	-	-	-	-
1α	CH_3	38.4	2.70	-	s	C-13
2	CH	63.8	2.96	-	s	C-7, C-3, C-3α' and C-3α
3α'	CH_3	20.5	1.04	-	s	C-3
3	Cq	27.5	-	-	-	-
3α	CH_2	37.9	2.04	H-3α_2	d	C-3 and C-3β
			2.96	H-3α_1	d	C-3 and C-3β
3β	C=O	173.1	-		-	-
3ϒ	CH_3	51.9	3.68	-	s	C-3β
4	CH	34.9	1.57	H-4α	q	C-3, C-4α and C-5
4α	CH_3	15.8	0.96	H-4	d	C-4
5	Cq	39.8	-		-	-
5α	CH_2	23.7	1.25	H-5β	m	C-5 and C-5β
5β	CH_3	13.7	0.90	H-5α	t	C-5α
6	Cq	69.6	-		-	-
6α	CH_3	19.1	1.24	-	s	C-5, C-6 and C-7
7	Cq	53.8	-	-	-	-
8	Cq	130.1	-	-	-	-
9	CH	124.3	7.10	H-10	d	C-13, C-11 and C-7
10	CH	103.5	6.25	H-9	d	C-12 and C-8
11	Cq	158.7	-	-	-	-
11α	O-CH_3	55.8	3.83	-	s	C-11
12	CH	95.6	6.23	-	s	C-10 and C-8
13	Cq	152.3	-	-	-	-
1'	CH_2	41.1	1.26	H-1'b and H-2'	dd	C-5 and C-2'
			1.51	H-1'a and H-2'	dd	C-5 and C-2'
2'	CH	22.2	1.82	H-1', H-3' and H-2'α	mm	C-1', C-2'α and C-3'
2'α	CH_2	40.0	2.00	H-2' and H-2'α_2	dd	C-2' and C-2'β
			2.25	H-2' and H-2'α_1	dd	C-2' and C-2'β
2'β	CO_2H	178.9	11.79	-	bosse	-
3'	CH_2	50.2	2.61	H-2' and H-3'b	dd	C-2'
			2.83	H-2' and H-3'a	dd	C-2'
4'	CH	144.9	5.68	H-5'	d	C-5' and C-3'
5'	CH	108.6	4.58	H-4'	d	C-7, C-6 and C-4'

Compound 2 (CS-02) had as molecular formula $C_{28}H_{36}N_2O_5$from ESI.TOF.HRMS (m/z= 481, 3965 $[M+H]^+$ calculated). The ^{1}H-NMR spectrum showed characteristic singlet attributed to four methyl groups δ0.85, δ0.94, δ2.24 and δ2.34, one singulet between δ3.24 characteristic to the signals of méthoxy group, two protons δ6.21(s), δ5.84(d) attributed to characteristic of signals alcens proton typical for benzenes skeleton, and two signals protons between δ5.84 (s) and δ6.21 (s), attributed to characteristic of alcens protons of the pyrroline cycle , and thereafter others singulets at δ12.18 and δ9.43 characteristic attributed to the labile protons hydroxyls typical for a phenol and an acid respectively. Ten (10) signals doublet-doublet between δ (1.16/, 1.43), δ (1.41/ 1.61), δ (2.04/2.27), δ (2.14/2.29) and δ (2.61/2.80) attributed to their signals of genime, protons of different chemical environments of the methylene groups, two peak between δ1.57 (q), and δ2.96 (s) characteristic attributed of the methine groups (CH) in a cycle and one signal at δ5.66 (s) unambiguously attributed of halogen proton to tie with the azote and at the end one signal proton at δ1.25 (q), typical for methylene (-CH_2-) protons to tie with the methyl. Examination of the 13 C NMR (broad brand and DEPT), and the HSQC spectra data of the pure compound revealed the presence of a two carbonyls carbons at δC 174.4 (C-3β) and δC 177.3 (C-3'β) and eight (8) signals correspond to the carbons of typical indole skeleton at δC54.8 (C-2); δC 56.1(C-7); δC 121.9(C-8); δC 136.1(C-9); δC 105.6(C-10); δC 156.4(C-11); δC 94.6(C-12), δC 150.7 (C-13) and two signals carbons between δ144.9 (C-4') and δ108.6 (C-5') , characteristic indicating of the carbons (CH) in one pyrroline cycle, eight signals carbons characteristic attributed to the alkyl groups in the cycle between three methylene groups at δ35.1(C-1'), δ52.9(C-3') and δ34.1 (C-3α'), one methine group at δ28.7 (C-4) and four quaternary carbons at δ21.9 (C-2'), δ31.0 (C-3), δ40.1 (C-5) and δ70.8 (C-6), and six signals carbons for typical methyl groups at δ12.4 (5α-CH_3), δ13.1 (4-CH_3), δ18.1 (6-CH_3), δ 23.3 (9-CH_3) and δ53.7 (3β-OCH_3) and at the end two signals carbons attributed of methylene between δ23.7 (C-5α) and δ33.2 (C-3α). The ^{1}H and ^{13}C chemical shift values of individual spin systems were determined by correlation in the 2D HSQC spectrum. The individual ^{1}H and ^{13}C chemical shift assigned by the ^{1}H-^{1}H COSY spectrum and 2D HSQC and HMBC correlation spectra, respectively (Table 2) and the structure of compound-2 (CS-02) to present in the figure.2.



Compound.2: Spinindole A

Figure 2: Structure of compound.2

Table 2: ^{1}H and ^{13}C chemical shift, the correlation ^{1}H-^{1}H (COSY) and important HMBC correlation of Spinindole A.

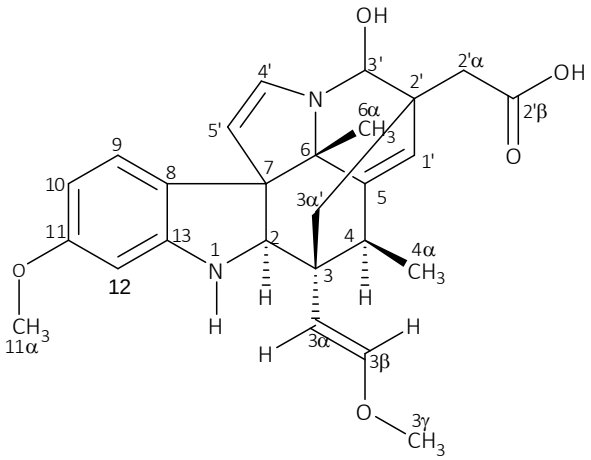
Position		1D-NMR experiments		2D-NMR experiments		
N°	Types	δC	δH	COSY	Multiplicity	HMBC
1	NH		5.66	-	s	C-13 and C-2
2	CH	54.8	2.96	-	s	C-7, C-3 and C-3α
3	Cq	31.0	-	-		
3α	CH_2	33.2	2.29	H-3α_2	d	C-3 and C-3β
			2.14	H-3α_1	d	
3β	C=O	174.3	-			
3Ɣ	O-CH_3	53.7	3.24		s	C-3β
3α'	CH_2	34.1	1.66	H-3α'$_2$	d	C-2 and C-3
			1.41	H-3α_1'	d	
4	CH	28.7	1.57	H-4α	q	C-3, C-5 and C-4α
4α	CH_3	13.1	0.94	H-4	d	C-4

5	Cq	40.1	-			
5α	CH_2	23.7	1.25	H-5β	q	C-5 and C-5β
5β	CH_3	12.4	0.85	H-5α	t	C-5α
6	Cq	70.8	-	-	-	-
6α	CH_3	18.1	2.24	-	s	C-6
7	Cq	54.3	-	-	-	
8	Cq	121.9	-	-	-	
9	Cq	136.1	-	-	-	
9α	CH_3	23.3	2.34	-	s	C-9
10	CH	105.6	6.21	-	s	C-8 and C-12
11	C-OH	156.4	9.43	-	Bosse	C-10 and C-12
12	CH	94.6	5.84	-	s	C-8 and C-10
13	Cq	150.7	-	-	-	-
1’	CH_2	35.1	1.43	H-1’b	d	C-2’ and C-5
			1.16	H-1‘a	d	
2’	Cq	21.9	-	-	-	-
2’α	CH_2	40.1	2.27	H-2’α_2	d	C-2’, C3’ and C-2’β
			2.04	H-2’α_1	d	C3’ and C-2’β
2’β	CO_2H	177.3	12.18	-	Bosse	C-2’α
3’	CH_2	52.9	2.61	H-3’b	d	C-2’, C-2’α
			2.80	H-3’a	d	C-2’
4’	CH	144.9	5.68	H-5’	d	C-5’
5’	CH	108.6	4.58	H-4’	d	C-4’ and C-7

The molecular formula of compound 3 (CS-O3) was determined to be $C_{27}H_{35}N_2O_5$ by ESI.TOF-HRSM and NMR experiments. The ^{1}H-NMR spectrum showed singlet characteristic attributed to two methyl groups δ1.18, δ1.36 and three méthoxy δ3.68, δ3.80, δ3.83 and three (3) signals protons between δ6.23 (d), δ6.28 (d) and δ6.07 (s) attributed to characteristic of signals alcens proton typical for benzenes skeleton, four doublet-doublet between δ1.45, δ1.78, δ2.09 and δ2.34, characteristic attributed to the signals of genime, protons of different chemical environments of the methylene group.

Four doublet characteristic attributed to two protons δ5.09 and δ5.73 attributed to characteristic of signals alcens proton typical for pyrroline skeleton, two peak at δ4.49, δ6.19 characteristic attributed to signals protons typical for linear chain of alkene groups between one ethylene group, and at the end two signals between δ2.15 (s), δ3.04(s) attributed to characteristic of the methyne groups and one signal at δ5.66 (s) attributed to characteristic of halogen proton labile to tie with the azote.

The 1D ^{13}C broad band-NMR spectrum contained 27 signals of the carbons indicating eight (8) signals correspond to the carbons of typical indole skeleton, one peak carbon of the carbonyl group between δ177.3 and two signals carbons attributed to the linear chain of alcens groups are present in the compound 3. Examination of the 1D ^{13}C (DEPT), and the 2D HSQC spectra data of the compound 3 revealed the presence of about two (2) alcens carbons (C=C) double bond typical for pyrroline skeleton at δC146.9 and δC 118.6, seven carbons characteristic attributed to the alkyl groups in the cycle between two methylene groups at δ32.3 (C-3α’), δ41.4 (C-2’α), two methine groups at δ129.3 (C-1’) and δ91.1 (C-3’) and four quaternary carbons at δ30.5 (C-2’), δ39.4 (C-3), δ149.0(C-5) and δ58.7 (C-6). In addition to the examination of the 1D ^{13}C and the 2D HSQC spectrum that permitted to reveal the presence of the five methyl groups at δ12.4 (4-CH_3), δ28.6 (6-CH_3), δ55.9 (3β-OCH_3), δ55.8 (11-OCH_3). The ^{1}H and ^{13}C chemical shift values of individual spin systems were determined by correlation in the 2D HSQC spectrum. The individual ^{1}H and ^{13}C chemical shift assigned by the ^{1}H-^{1}H COSY spectrum and 2D HSQC and HMBC correlation spectra, respectively (Table 3) and the structure of compound-3(CS-03) to present in the figure.3.



Compound.3: Spinindole B

Figure 3: Structure of compound.[3]

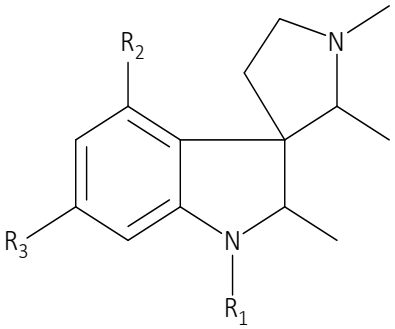
Table 3: ^{1}H and ^{13}C chemical shift, the correlation ^{1}H-^{1}H (COSY) and important HMBC correlation of Spinindole B.

Position		1D-NMR experiments		2D-NMR experiments		
N°	Types	δC	δH	COSY	Multiplicity	HMBC
1	NH	-	5.66	-	s	C-2 and C-13
2	CH	62.7	3.04	-	s	C-7, C-3, C-3α' and C-3α
3	Cq	39.4	-	-	-	-
3α'	CH_2	32.3	1.78	H-3α'$_2$	d	C-3 and C-2'
			1.45	H-3α'$_1$	d	
3α	Cq	107.5	4.49	H-3β	d	C-3 e and C-3β
3β	CH	150.6	6.19	H-3α	d	
3ϒ	O-CH_3	55.9	3.80	-	s	C-3β
4	CH	37.4	2.15	H-4α	q	C-3, C-4α and C-5
4α	CH_3	12.4	1.18	H-4	d	C-4
5	Cq	149.0	-	-	-	-
6	Cq	58.7	-	-	-	-
6α	CH_3	28.6	1.38	-	s	C-6
7	Cq	64.3	-	-	-	-
8	Cq	123.9	-	-	-	-
9	CH	125.8	6.28	H-10	d	C-13, C-11 and C-7
10	CH	104.5	6.23	H-9	d	C-12 and C-8
11	Cq	153.6	-	-	-	-
11α	CH_3	55.8	3.83	-	s	C-11
12	CH	96.4	6.07	-	s	C-10 and C-8
13	Cq	152.0	-	-	-	-
1'	CH	129.3	5.50	-	s	C-4, C-5, C-6 and C-2'
2'	Cq	30.5	-		-	-
2'α	CH_2	41.4	2.34	H-2'α$_2$	d	C-2' and C-2'β
			2.09	H-2'α$_1$	d	
2'β	CO_2H	177.3	-	-	-	-
3'	HCOH	91.1	2.87	s	s	C-2'
			5.03	-	TR	
4'	CH	146.9	5.73	H-5'	d	C-5' and C-6
5'	CH	118.6	5.09	H-4'	d	C-7, C-6 and C-4'

TR: trouble resolute

DISCUSSION

Carissa spinarium (Apocynaceae), known by the vernacular name "Relefo," is among the endemic plant species in the South and Southwest regions of Madagascar[17-20], and it is very important in these areas due to its therapeutic virtues. According to ethnobotanical data, this plant has been used in traditional medicine to treat several diseases such as malaria, bacterial infections, and hypertension.[9] The results of the phytochemical screenings conducted on the leaf extracts of C. spinarium show that it was rich in alkaloids and terpenes. This screening result was similar to chemotaxonomy.[21] The application of the bio-guided fractionation method using chromatography analysis technique allowed the isolation and identification of three new indolomonoterpenic alkaloid molecules called Cassinindole, Spinindole A, and Spinindole B. The results of the structural analyses using 2D NMR showed that these three compounds share a common basic structure. (fig.4).



	Cassinindole	Spinindole A	Spinindole B
R_1	-CH_3	H	H
R_2	H	CH_3	H
R_3	O-CH_3	OH	O-CH_3

Figure 4: Structure of basic of this three molecules.

After-wards, the study of the stereochemistry of these molecules shows that the CS-03 molecule exhibits two enantiomers, α and β. (figure. 5). This conformation was identified from the asymmetric carbon C-3, as this carbon is composed of a 3-oxa-but-1-ene group. The two (2) forms α and β were determined based on the δ bond between C3 and C3α: if the bond is behind, we have the β form, and if it is in front, we have the α form. The HPLC analysis result revealed that the form (R [α, E])-Spinindole B was predominant, representing 64% of the mixture, while the form (R [β, E])-Spinindole B constituted 36%.

This result indicates that the mixture is not racemic. Moreover, it is likely that the form (R [α, E]) is more active than the form (R [β, E]), although definitive confirmation requires the separation of the enantiomers.

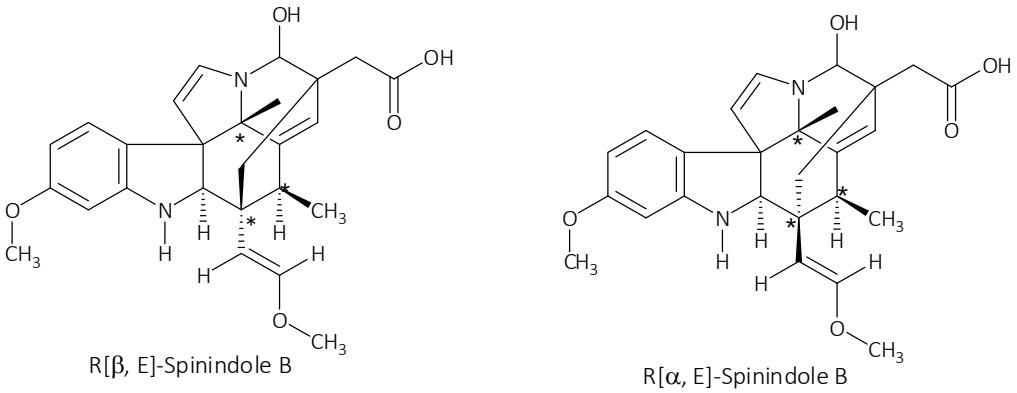


Figure 5: The enantiomers of Spinindole B.

According to Jean Le Men, indolomonoterpenic alkaloids originate from the same biosynthetic pathway and are divided into three main groups based on the numbering of the corresponding terpenic skeletons (fig.6).[22]

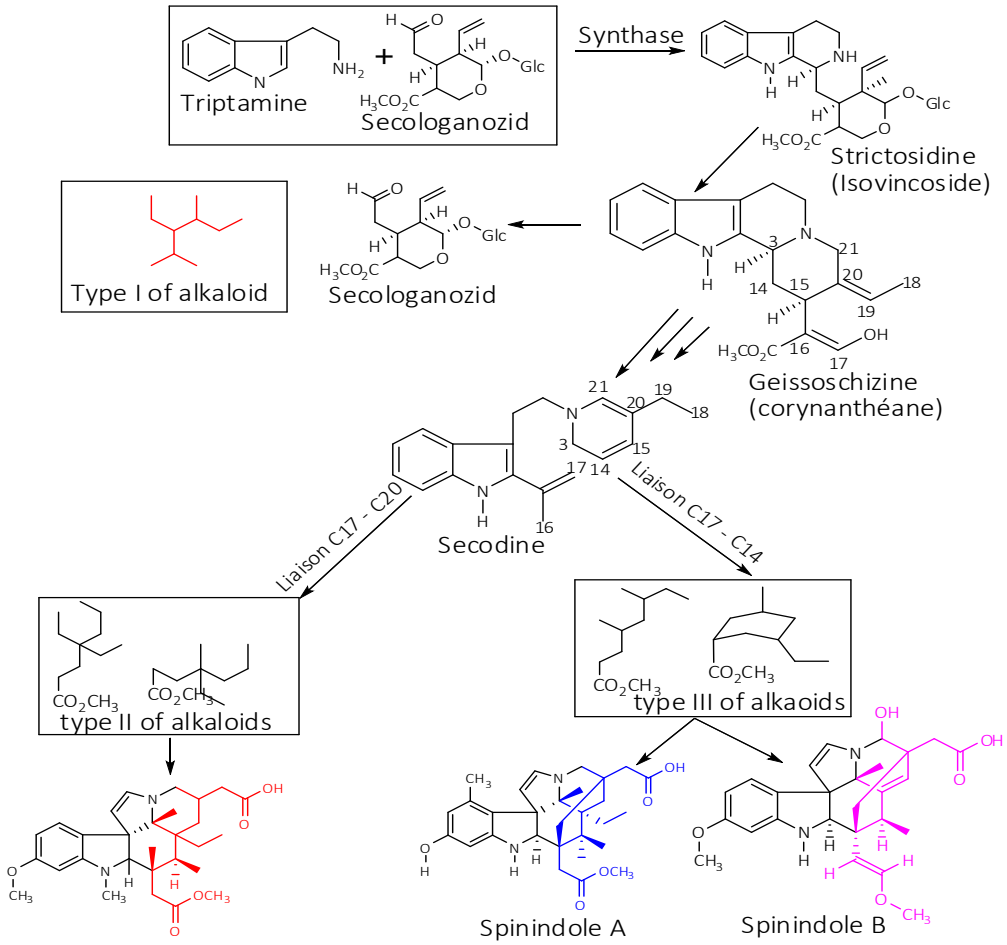


Figure 6: Possible biogenesis pathways of the three new pure compounds.

Figure 6 shows that the biosynthetic formation mechanisms of these three molecules are different. Indeed, carissinindole belongs to the group of type II indolomonoterpenic alkaloids, resulting from the cyclization reaction of the C17-C20 bond. Spinindoles A and B, on the other hand, belong to the group of type III indolomonoterpenic alkaloids, resulting from the cyclization reaction of the C17-C14 bond.

The bibliographic research conducted on these three molecules revealed that they were not described in the literature, neither their chemical structures nor the biological studies. Moreover, the chemotaxonomy of the Apocynaceae family has revealed that type III indolomonoterpenic alkaloids are very rare in the Apocynaceae.[22-29] This chemotaxonomy allows us to prove our result because the three isolated products (Cassinindole, Spinindole A, and Spinindole B) are all new molecules and, moreover, it is the first time they have been found in the Apocynaceae family.

CONCLUSION

Carissa spinarium (Apocynaceae) is endemic to the southwestern region of Madagascar, known by vernacular name "Relefo".

Three new indolomonoterpenic alkaloids were isolated from the leaves extracts of this plant thank to the application of fractionation methods based on chromatographic analysis techniques. The chemical structures of these isolated compounds were elucidated using spectroscopic analysis methods. (1D, 2D –NMR, HREIS-MS). They were respectively attributed to: Cassinindole, Spinindole A, and Spinindole B. This is the first time type III indolomonoterpenic alkaloid have been isolated from endemic plant (Apocynaceae) of Madagascar.

All these results have led to the conclusion that the endemic plants of the South and Southwest of Madagascar constitute excellent substrates for the research of new bioactive molecules.

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