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Characterization of Chemical Constituents of *Luffa operculata* (Cucurbitaceae)

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Abstract

A mixture of new ceramides (**1**, **2**, **3**, **4** and **5**) together with a binary mixture of ceramides with long chain alkyl (**6** and **7**), triterpenoid (**10**) and steroids (**11** and **12**) have been isolated from bark of the fruits and of the stems of *Luffa operculata* (Cucurbitaceae). The structures were elucidated by comprehensive spectroscopic analysis including ^{1}H and ^{13}C NMR, DEPT (distortionless enhancement by polarization transfer), COSY (correlated spectroscopy), HMQC (heteronuclear multiple quantum coherence), HMBC (heteronuclear multiple bond connectivity), IR (infrared), HR-ESI-MS (electrospray ionization-high resolution mass spectra) and LR-MS (low resolution electron ionization mass spectra) experiments. All the ceramides are reported for the first time in Cucurbitaceae and this is the first report of the rare triterpene **10** isolated from *Luffa operculata*. The ceramides **6** and **7** showed a high acetylcholine esterase inhibitory effect.

Keywords: Cucurbitaceae, Ceramides, Triterpenes, Spectroscopic Data

1. Introduction

As a part of our continuing chemical studies on plants of Cucurbitaceae family, we have investigated the bark of the fruits and the stems of *Luffa operculata* specie. *L. operculata* Cogn. (Cucurbitaceae), locally known as "cabacinha", a perennial shrub widely distributed in Northeastern Brazil where an aqueous solution from its fruits has been used in popular medicine for the treatment of sinusitis [1]. In the previous paper [2], we reported the isolation and structure elucidation of triperpenes cucurbitane type from these fruits. In this paper, we report the isolation and structure elucidation of ceramides (**1**-**5**, **6** and **7**), triterpene oleanane type (**10**) and steroids (**11** and **12**) from the bark of the fruits and stems of this plant. In plants, recent studies indicate that ceramides may be involved in signal transduction, membrane stability, host-pathogen interactions, and stress responses [3].The compound **6** and **7**, as well as the steroids mixture (**12**), showed an acetylcholine esterase inhibitory effect. Inhibition of acetylcholinesterase (AchE) is used as a strategy for the treatment of Alzheimer's disease (AD), a neurodegenerative malady characterized by cognitive impairment and personality changes. One of the most promising approaches for treating this disease is to enhance the acetycholine level in rain using acetylcholine esterase (AChE) plant-derived inhibitors [4]. In this work we report an evaluation of the cholinesterase inhibition effect of the ceramides **6** and **7** following the methodology of Elmann, adapted by Rhee [5] for the layer chromatography (TLC).

2. Materials and Method

2.1. General Procedures

^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 300 and DRX 500 spectrometers in $CDCl_3$, with TMS as an internal standard. DEPT and all 2D experiments (COSY, HMQC and HMBC) with standard Bruker pulse sequence; IR spectra were carried out on Perkin-Elmer 2000 series FT-IR; electrospray ionization mass spectra

(HR-ESI-MS) obtained in mass spectrometer model LCMS-IT-TOF (225-07100-34, Shimadzu) and on a QP5050 (Shimadzu) instrument at 70 eV for low resolution; melting point were measured on Mettler Toledo FP90 apparatus, uncorrected; the spots were visualized by spraying with a mixture of vanillin-perchloric acid ethanol.

2.2. Extraction and Isolation of Constituents

Luffa operculata stems were collected in Acarape County, Brazil and identified in the Departamento de Biologia do Centro de Ciências da Universidade Federal do Ceará (UFC). A voucher specimen (N° 43.056) was deposited at Departamento de Biologia (UFC) Prisco Bezerra Herbarium.The air-dried stems (935 g) were powdered and extracted at room temperature with hexane and EtOH. The hexane extract (4.1 g) was subjected to column chromatography (CC) on silica gel (Si gel) 60 (230 - 400 mesh) using hexane, CH_2Cl_2, EtOAc and MeOH as solvents. The CH_2Cl_2 fraction (2.56 g) was further subjected to CC on Si gel 60 (230 - 400 mesh) to yield a material (17.5 mg) white greasy (**1 - 5**) and **11** (102 mg). The AcOEt fraction (4.95 g) of EtOH extract (22.5 g) was successively chromatographed on Si gel column to afford **10** (7.5 mg) as white powder. The air-dried bark of fruits (195.8 g) were powdered and extracted at room temperature with hexane and EtOH. The EtOH extract (10.5 g) was subjected to CC on silica gel 60 (230 - 400 mesh) using CH_2Cl_2, EtOAc and EtOH as solvents. The CH_2Cl_2 fraction (0.29 g) was successively chromatographed on Si gel column to afford **12** (21 mg) as a white powder, while the AcOEt fraction (0.59 g) after successively chromatographed on Si gel column afforded **6** and **7** (24.5 mg) a white solid.

3. Results and Discussion

The CH_2Cl_2 fraction of the hexane extract of the stems of *L. operculata* was chromatographed on silica gel column to yield a white greasy material. Its IR spectrum disclosed bands due to methylene and methyl (γ_{max} 2923/2853 cm^{-1} and δ_{max} 1462/1380 cm^{-1}), carbonyl (γ_{max} 1737 cm^{-1}) groups, as well as bands of C - O/C - N (γ_{max} 1172 cm^{-1}) bounds. The LR-MS displayed a cluster of four 14-amu-apart ion peaks at m/z 311, 297, 283, 269 and 255 indicative of a mixture of homologous compounds (**Scheme 1**). In agreement, the NMR data (**Table 1**) revealed signals due to methylene groups [intense and broad signal at δ_H 1.29 - 1.34; several peaks at δ_C 23.46 - 34.89 (very high peak at δ_C 29.85)], as well as signals to one primary methyl group (δ_H 0,89, t, 6.7 Hz; δ_C 14.80) all characteristic of a long alkyl chain. The methylene hydrogens at δ_H 2.39 [t, 7.3 Hz; δ_C 34.89 (methylene carbon *alfa* to carbonyl)] showed ^2J and ^3J HMBC correlations with the carbons at δ_C 174.20 (C = O), 32.64 (methylene carbon *beta* to carbonyl) and 30.26 (methylene carbon *gama* to carbonyl) and allowed to establish the partial structure **I**.

$$CH_3\text{-}(CH_2)_n\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}C(=O)\text{-}$$

I

In addition, the ^1H and ^{13}C spectra exhibited signals due to two other methylene groups (δ_H 4.38, t, 4.9 Hz, 2H; δ_C 64.67 and δ_H 3.66, t, 5.2 Hz, 2H; δ_C 66.76) and to a secondary *gem*-dimethyl group (δ_H 1.12, d, 6.0 Hz, 6H; δ_C 22.71 and δ_H 3.57, m, 1H; δ_C 72.40) and allowed to suggest the partial structure **II**.

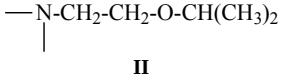
	n	+•M	*m/z*	Fragmentos	m
	14	397	311		18
	13	383	297		17
	12	369	283		16
	11	355	269		15
	10	341	255		14

m/z **86** *m/z* **73** *m/z* **43**

Scheme 1. Structures for the amides 1-5.

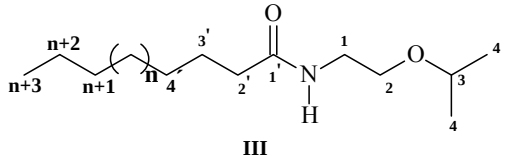
Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) data of compounds 1 - 5 in pyridine-d_5, δ in ppm, J in Hz and multiplicities, in parenthesis.

No.	1 - 5		
C	δ_C	δ_H	$^{2,3}J_{CH}$
1'	174.20	-	H-1; H-2'; H-3'
CH			
3	72.40	3.57 (m)	H-4; H-2
CH_2			
1	64.67	4.38 (t, 4.9)	H-2
2	66.76	3.66 (t, 5.2)	H-1
2'	34.89	2.39 (t, 7.3)	H-3´
3'	25.81	1.67 (m)	H-2´
4'	30.26	1.29 - 1.34 (m)	
5'-n	29.85 - 30.51	1.29 - 1.34 (m)	-
n + 1	32.64	1.29 - 1.34 (m)	3H-n + 3
n + 2	23.46	1.29 - 1.34 (m)	-
CH_3			
4	22.71	1.12 (d, 6.0)	-
n + 3	14.80	0.89 (t, 6.7)	H-2; H-4



II

In the ^{1}H - ^{1}H COSY spectrum, the mutual correlations between the signals at δ_H 4.38 and 3.66, as well as between the signals at δ_H 1.12 and 3.57, supported the fragment **II**. The linkage of theses partial structures (**I** and **II**) to each other was based on additional long-range connectivities observed between the hydrogens at δ_H 4.38 (-NCH_2-) and the carbon atom in δ_C 174.20 (C = O) in the ^{1}H-^{13}C HMBC spectrum and resulted in the general structure **III**, corresponding to amides mixture. Others correlations in the HMBC spectrum were assigned in the **Table 1**.



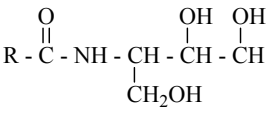
III

Finally, the fragments in the mass spectrum due to the peaks at *m/z* 311, 297, 283, 269 and 255 obtained by McLafferty rearranjement from molecular ion peaks at *m/z* 397, 383, 369, 355 and 341 (observed at 395, 381, 367, 353 and 339, respectively), respectively, allowed the possible structures for the amides **1** - **5** (**Scheme 1**), unknown ceramides up to date. Others important peaks as *m/z* 86 (100%), 73 and 43 all are in agreement with the proposed structures (**Scheme 1**).

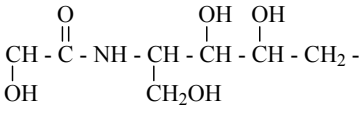
1	n = 14	$M^{+\bullet}$ 397	N-(2-isopropoxy-ethyl)eicosamide
2	n = 13	$M^{+\bullet}$ 383	N-(2-isopropoxy-ethyl)nonadecanamide
3	n = 12	$M^{+\bullet}$ 369	N-(2-isopropoxy-ethyl)octadacanamide
4	n = 11	$M^{+\bullet}$ 355	N-(2-isopropoxy-ethyl)heptadacanamide
5	n = 10	$M^{+\bullet}$ 341	N-(2-isopropoxy-ethyl)hexadecanamide

The AcOEt fraction of the EtOH extract from barc fruit of *L. operculata* was chromatographed on silica gel column to afford a white solid whose high-resolution high-resolution ESI mass spectrometry in the negative mode displayed two 14-amu-apart quasimolecular ion peaks $[M-H]^-$ at *m/z* 736.5277 and 722.3396, indicative of a binary mixture of homologous compounds. The IR spectrum of this solid disclosed bands at 3336/3218, 2918/2849 and 1621 cm^{-1} suggestive of OH and/or NH, CH_3/CH_2 and C = O groups, respectively, as well as bands at 1070/1025 cm^{-1} of C-O/C-N bound; further

bands at 1544, 1466 and 750 cm^{-1} were attributed to NH, CH_3/CH_2 and CH_2 groups, respectively. The ^{13}C and DEPT NMR spectra (**Table 2**) showed several aliphatic methylenes (δ_C 23.28 - 36.17) and methyl terminal signal (δ_C 14.55) which constructed a long alkane chain. These spectra also revealed the presence of six methine [δ_C 53.42; three oxygenated (δ_C 72.75, 73.26 and 77.32) and two olefinic (δ_C 131.16 and 131.04)] carbons. In addition, signals at δ_C 62.36 and 175.64 indicated an oxymethylene carbon and an ester or amide carbonyl, respectively. The ^1H NMR spectrum also revealed characteristic signals for long alkyl chains (δ_H 1.27 - 1.33) as well as a signal at δ_H 8.61 compatible with hydrogen of secondary amide (RCONHR') which, was further substantiated by its ^{13}C NMR (δ_C 175.64) and IR (1621 and 1544 cm^{-1}) spectra. In the ^1H-^1H COSY spectrum, the amide hydrogen with resonance at δ_H 8.61 coupled to a methine hydrogen at δ_H 5.13 (δ_C 53.42) which in turn revealed coupling to a methyne carbinolic hydrogen at δ_H 4.38 (δ_C 77.32) and to a diastereotopic methylene group observed at δ_H 4.45 and 4.53 (δ_C 62.36). On the other hand, in the HMBC spectrum, the hydrogen resonance at δ_H 4.38 showed correlation to the δ_C 53.42 (CH), 62.38 (CH_2), 73.26 (CH) and 34.55 (CH_2). The HMQC spectrum established the association of the methyne carbon at δ_C 73.26 with the carbinolic hydrogen at δ_H 4.32. This analysis, based on amide function (RCONHR'), allowed to establish the partial structure **IV**.

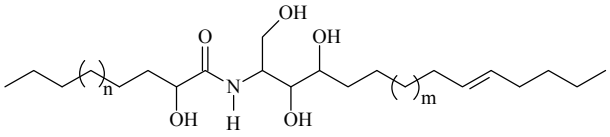


IV



V

The third oxygenate methine carbon at δ_C 72.75 was associated to hydrogen in δ_H 4.64 by HMQC experiment. In addition, the HMBC spectrum showed that this hydrogen was correlated with carbonyl carbon and with the methylene carbons at δ_C 36.17 and 26.19, *beta* and *gama* carbons, respectively, to carbonyl function. Thus, a partial structure **IV** was expanded to **V**. Based on the above spectral analysis and by comparison with spectral data [IR, NMR (^1H and ^{13}C) and MS] of the literature [3,6-8] the sample was identified as a ceramides mixture with general structure **VI**.

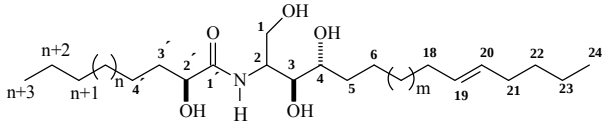


VI

The position of the double bond at C-19 was indicated by strong peaks corresponding to *m/z* 97 ($^+CH_2CHCHCH_2CH_2CH_2CH_3$), 57 ($^+CH_2CH_2CH_2CH_3$) and 43 ($^+CH_2CH_2CH_3$). The *E* stereochemistry of double bond was determined on the basis of ^{13}C NMR chemical shift of the methylene carbons adjacent to the olefinic carbons, which is observed at $\delta_C \approx 27.00$ in *Z* isomers and at $\delta_C \approx 32.00$ in *E* isomers [3,6].

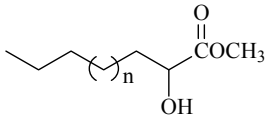
After comparison with analogous compounds [3,7-11] the relative stereochemistry inferred for the sterocenters 2, 3, 4 and 2' was presumed to be S^*, S^*, R^* and R^*, respectively. On the basis of the above mentioned data, the structures of compounds **6** and **7** were established as *rel*-(2*S*,3*S*,4*R*,19*E*)-2-[(2'*R*)-2'-hydroxydocosanoylamino]-tetracosadec-19-ene-1,3,4-triol (**6**) and *rel*-(2*S*,3*S*,4*R*,19*E*)-2-[(2'*R*)-2'-hydroxyhenicosanoylamino]-tetracosadec-19-ene-1,3,4-triol (**7**).

These data support the structures **6** and **7** proposed for ceramides:



6 $[M-H]^-$ 736.5277 n = 15, m = 11
7 $[M-H]^-$ 722.3396 n = 14, m = 11

The structures of acyl chains were confirmed by analysis of the mixture of products (**8** and **9**) resulting from methanolysis of **6** and **7**. The CG-MS of **8** and **9** was in agreement with structures of **6** and **7**, showing the presence of two constituents, which were identified as methyl-2-hydroxydocosanoato (*m/z* 370 [M^+]) and methyl-2-hydroxyhenicosanoato (*m/z* 356 [M^+]).



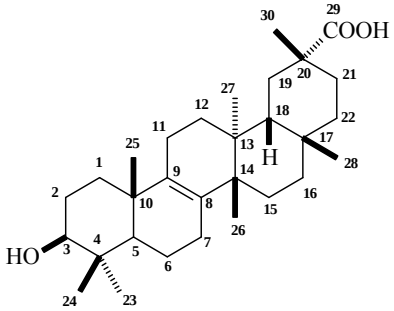
8 M^+ 370 n = 16
9 M^+ 356 n = 15

The AcOEt fraction of the EtOH extract of the stems of *L. operculata* was successively chromatographed on silica gel column to afford **10** as white powder, mp 262°C - 263°C. The ^{13}C NMR spectrum of **10** exhibited thirty signals divided by DEPT spectra in nine quarternary carbons, three CH, eleven CH_2 and seven CH_3

Table 2. ^{13}C (125 MHz) and ^{1}H (500 MHz) data of compounds 6 and 7 in pyridin-d_5, δ in ppm, J in Hz and multiplicities, in parenthesis.

No.	6 and 7		
C	δ_C	δ_H	$^{2,3}J_{CH}$
1'	175.64	-	NH-1'
CH			
2	53.42	5.13 (m)	NH-1'; H-1; H-3
3	77.32	4.38 (m)	H-1; H-2; H-5
4	73.26	4.32 (m)	H-3; H-5
19	131.16	5.53 (m)	-
20	131.04	5.53 (m)	-
2'	72.75	4.64 (m)	-
CH_2			
1	62.36	4.45; 4.53 (m)	H-2; H-3
5	34.55	1.95; 2.30 (m)	H-3
6	27.00	1.71; 1.80 (m)	H-5
7 - 17	30.25 - 30.53	1.27 - 1.33 (m)	-
18	33.31	2.05	H-19; H-20
21	34.23	2.00; 2.30 (m)	-
22	32.43	1.27 - 1.33 (m)	-
23	23.28	1.27 - 1.33 (m)	H-24
3'	36.17	2.05; 2.25	H-2'
4'	26.19	1.71; 1.80	H-2'
5'-n	30.25 - 30.53	1.27 - 1.33 (m)	-
n + 1	32.43	1.27 - 1.33 (m)	-
n + 2	23.28	1.27 - 1.33 (m)	-
CH_3			
n + 3	14.55	0.89 (t, 6.4)	-
24	14.55	0.89 (t, 6.4)	-

groups. In the ^{1}H and ^{13}C NMR spectra of **10** characteristic feature can be identified: methyl groups (δ_H 0.99, 1.04, 1.05, 1.08, 1.22, 1.28 and 1.42; δ_C 20.43, 16.82, 22.69, 31.44, 28.93, 18.28 and 33.64) all bonded to the quarternary carbons; one carbinolic methyne carbon (δ_H 3.38, dd, J = 10.0 and 5.0; δ_C 78.34); one tetrasubstituted double bond (δ_C 134.89 and 134.45) and one carboxylic carbon (δ_C 181.68). Together, these data were consistent with a molecular formula of $C_{30}H_{48}O_3$, including one -OH and one -CO_2H groups. Based on this NMR data (**Table 3**), the seven degrees of unsaturation could be attributed to one carbon-carbon double bond, one carbonyl group, and five ring systems. Compound **10** was distinct from oleanolic acid by two remarks: the double bond was located at Δ^8 based on the long range connectivities between two methyl signals at δ_H 0.99 (3H-25) and 1.05 (3H-26) and the olefinic carbon signals at δ_C 134.89 (C-8) and 134.45 (C-9), respectively; the long range coupling between the methyl signal at δ_H 1.42 (3H-30) and carbon carboxylic signal at δ_C 181.68 (C-29). Thus, based on the above spectral analysis and by comparison with spectral data [IR, NMR (^{1}H and ^{13}C) of literature [12,13] the structure was confirmed as 3β-hydroxy-D:C-friedoolean-8-en-29-oic acid, known as bryonolic acid, a triterpenoid rare in nature.



The steroids were identified as 24α-etil-5α-colest-7,*trans*-22-dien-3β-ol [**11** (spinasterol)] and a mixture of 24α-ethyl-5α-colest-7,*trans*-22-dien-3β-ol (**11**) and 24β-ethyl-5α-colest-7,*trans*-22,25-trien-3β-ol (**12**) from their spectral analysis and by comparison of their physical and spectral data with literature [14,15] values.

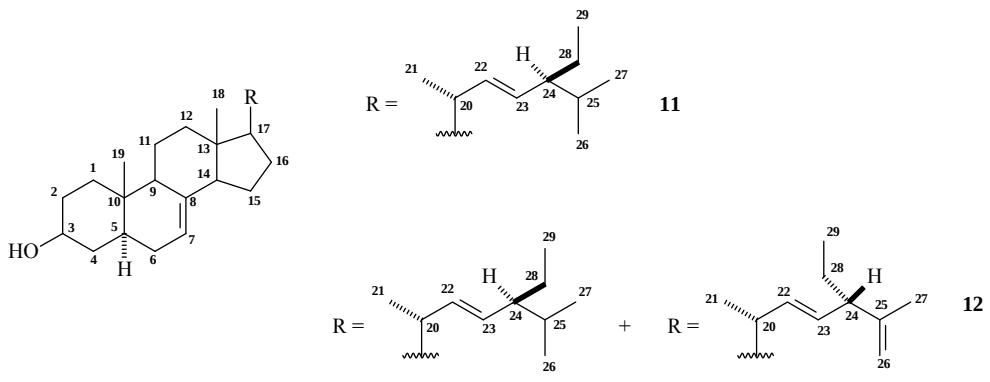


Table 3. ^{13}C (125 MHz) and ^{1}H (500 MHz) data of compound 10 in pyridin-d_5, δ in ppm, J in Hz and multiplicities, in parenthesis.

No.			
C	δ_C	δ_H	$^{2,3}J_{CH}$
4	39.71	-	3H-23; 3H-24
8	134.89	-	3H-26
9	134.45	-	3H-25
10	38.12	-	3H-25
13	37.99		3H-27; 3H-26
14	42.44	-	3H-26; 3H-27
17	31.60	-	H-19a
20	40.91	-	H-19a; 3H-30
29	181.68	-	H-19a; H-19b; 3H-30
CH		-	
3	78.34	3.38 (dd, 10.0; 5.0)	3H-23; 3H-24; 3H-25
5	51.25	1.08	3H-23; 3H-24; 3H-25
18	45.46	1.57	3H-28
CH_2			3H-25
1	35.82	1.63; 1.84	-
2	28.27	1.84; 2.11	-
6	19.91	1.42; 1.72	-
7	28.97	1.86; 2.59	-
11	21.37	1.90; 1.94	-
12	30.82	1.22; 1.49	3H-27
15	25.77	1.37; 1.72	-
16	37.81	1.39; 2.75	3H-28
19	31.74	1.70; 2.73	-
21	30.98	1.46; 1.84	3H-30
22	35.37	1.03; 2.45	3H-28
CH_3			
23	28.93	1.22 (s)	H-3; 3H-23
24	16.82	1.04 (s)	-
25	20.43	0.99 (s)	-
26	22.69	1.05 (s)	-
27	18.28	1.28 (s)	-
28	31.44	1.08 (s)	-
30	33.64	1.42 (s)	

Table 4. Cholinesterase inhibition of constituents from *L. operculata.*

Substance[a]	Zone of inhibition (mm)
6 and **7**	12
11	N[b]
12	8
Physostigmine[a,c]	9

[a]Concentration = 2mg/mL; [b]N = No effect; [c]Positive control.

In the anticholine esterase activity test, fisostgmine was used as positive control (with an inhibition zone of 9 mm) since it is a drug that binds and activates the acetylcholine receptor. Acetylcholine esterase (AChE) hydrolyzes the neurotransmitter acetylcholine at one of the highest known wnzymatic rates. Therefore the anticholine esterase activity of the ceramides (**6** and **7**) (with aninhibition zone of 12 mm) is relevant as the results below (**Table 4**).

4. Conclusions

Many previous studies showed that *Luffa operculata* is rich in triterpenes cucurbitano type, as expected for a Cucurbitaceae. Although almost all of these metabolites were found only in their fruits, this study showed that the stems and bark of the fruits of this plant are bioproductors of ceramides as well as steroids and triterpenes of another type (oleanane). According to the analysis of spectral data, the mixture of long chain ceramides seems to involve more than two components, requiring a further thorough study about the subject.

5. Acknowledgements

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