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Ximenia americana: Chemical and Spectral Studies of Extracts of Seeds. Analysis of Trimethylsilyl Derivatives by Gas Chromatography and Mass Spectrometry

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Abstract

Among the numerous botanical species found in the Brazilian plant biome, *Ximenia americana* is widespread in the Northeast of Brazil, especially in the state of Ceará, where it is known as bush plum. Its bark, leaves and roots are used in popular medicine for the treatment of skin infections, hemorrhoids, stomach ulcers, gastric pain, among others. In this work, hexane and ethanolic extracts of *X. americana* seeds were subjected to silylation reactions followed by analysis of the silylated derivatives by gas chromatography-mass spectrometry (GC/MS). In the hexane extract, 18 substances were identified, where the most abundant and most important components were the Octadec-9-enoic (38.14%), (9Z,12Z)-Octadec-9,12-dienoic (18.83%), Ethanedioic (8.21%) and (Z,Z,Z)-9,12,15-Octadecatrienoic (7.22%) acids. From the ethanol extract 18 substances were also identified having D-Sucrose (29.36%), L-Sorbose (9.19%), syllo-Inositol (8.34%) and D-Glucose (7.45%) as the main components. In addition, the presence of a steroid in hexane extract and triterpenes in ethanol extract was recorded. The constituents were identified using chromatographic and spectrometric methods, especially gas chromatography-mass spectrometry. To our knowledge, this is the first study of this nature from the oil of the *X. american* seeds.

Keywords

Ximenia americana, Seeds, TMS Carbohydrates Derivatives, GC/MS

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1. Introduction

Brazil is considered the country with the greatest biological diversity. It houses about 14% of the world's plant diversity distributed in different biomes, mainly the Amazon region, the Atlantic Forest and Caatinga [1]. The "caatinga" is an exclusively Brazilian biome. It reaches an area of 850 square kilometers, occupying about 10% of Brazilian territory and is present mainly in the interior of Northeast Brazil and northern Minas Gerais state [2]. Basically is formed by xerófilas plants or adapted to dry conditions (found in semiarid northeast). It has been recorded about a thousand plant species in the "caatinga". According to experts, there may be double that amount. Importantly, the investigation in natural products assumed a great sense of urgency in response to the expanding human population and its consequent demand for food and good health. However, while many countries increase their investments in chemistry and pharmacology of natural products derived from plants, investments in Brazil are still quite insufficient in this area. The Ximenia genus (Olacaceae) comprises about 8 species [3], *Ximenia americana* being the most common. It is popularly known as "ameixa do mato", "ameixa de espinho" and "ameixa da Bahia" and is widely distributed in northeast Brazil. A tea obtained from its barks has been used in popular medicine as cicatizing, adstringent and as an agent against excessive menstruation. As a powder, it treats stomach ulcers and the seeds are purgative [4] [5]. In previous phytochemical study [6], the stem and roots of this plant were found to contain saponins, glicosydes, flavonoids, tannins, phenolics, alkaloids, quinones and terpenoids types. In addition, the plant is potentially rich in fatty acids and glycerides and the seeds contain derivatives cyanide. Concerning the fatty acids, in addition to common C_{16}, C_{18} and C_{22}, a distinctive feature is the presence of acetylenic fatty acids. Our continuing interest in the chemistry of *X. americana* led us to investigate its seeds, still little studied from the chemical and pharmacological aspects. The hexanic and ethanolic extracts of *X. americana* were found to contain besides triglycerides, various fatty acids, furanoses/ pyranoses carbohydrates, steroids and triterpenoids. The identification of components was made through chemical and spectral studies.

2. Experimental Part

2.1. General

GC/MS analysis were performed on Shimadzu GCMS model QP2010SE Plus using a (5%-phenyl)-dimethylpolysiloxane Rtx®-5MS capillary column (30 m × 0.25 mm × 0.25 µm) with film thickness 0.1 µm; the temperatures of the injector and detector were 260˚C and 300˚C, respectively; column conditions: 60˚C (0.5 min) to 260˚C (5 min) at 6˚C/min, then 12˚C/min to 300˚C (10 min) using He as carrier gas, flow rate 1.7 mL/min with split mode. The analysis with the mass detector was performed in the scan mode with analysis time of 52.21 minutes; EIMS data were recorded with electron impact ionization at 70 eV (1.5 KV voltage, analyzer quadrupole and ion source 200˚C), in the range 47 - 600 Da. ^{1}H- and ^{13}C-NMR spectra were recorded on Brucker DRX 500 spectrometer in C_5D_5N and are reported in ppm relative to using tetramethylsilane (TMS) as internal standard; IR spectra were recorded on Perkin-Elmer model Spectrum 100 FT-IR using a Universal Attenuated Total Reflectance (UATR). All solvents and reagents were of analytical grade and obtained from commercial sources (Synth and Merck). Silica gel 70 - 230 mesh (Merck) was used for column chromatography; silica gel Merck Kieselgel 60 (2 - 25 mm silica gel) with fluorescent indicator on aluminum support paper (Merck F254) was used for thin-layer chromatography (TLC) and UV light (254 and 365 nm) and vanillin/ perchloric acid/EtOH solution under heating, as developing agents. Mp's were determined on digital Mettler Toledo, model FP62 apparatus and are uncorrected.

2.2. Plant Material

Seeds of *X. americana* were collected in Marrecas site, near the city of Acarape, Ceará, Brazil in 2014. A voucher specimen (N˚ 040411) is deposited at the Herbarium Prisco Bezerra of the Department of Biology, Federal University of Ceará, Fortaleza, Brazil.

2.3. Isolation of Triterpenoids

On concentration in vacuo, the crude EtOH extract afforded a greenish-white precipitate, which was filtered. The precipitate after successive washes with MeOH resulted in a white solid (60 mg) amorphous soluble in pyridine, which was characterized as a triterpenes mixture: mp 209˚C - 212˚C.

2.4. Isolation of Triglycerides

The hexane fraction (2.1 g) of hexane extract was subjected to column chromatography on silica gel, eluted with increasing concentrations of EtOAc in hexane. After thin layer chromatography, the fractions 17 - 20 eluted with hexane-EtOAc 4:3, was rechromatographed on silica gel column eluted with increasing concentrations of CH_2Cl_2 in hexane. Thin layer chromatography brought together all fractions 16 - 44 (eluted with hexane-dichloromethane 1:1), which were then characterized as triglycerides.

2.5. Synthesis of Trimethylsilyl Derivatives

The extracts were submitted to a silylation reaction according to a known methodology [7] with slight modifications. Hexane extract (2 mg) was treated with BSTFA (100 µL) and TMCS (1%) and the mixture was stirred for 1 h at 60˚C, followed by analysis by GC/MS.

3. Results and Discussion

In separate experiments, hexane and ethanol crude extracts of *X. anericana* seeds were successively partitioned into hexane, dichloromethane, ethyl acetate and methanol. Considering one of the main objectives of this study, namely the identification of oil components of the seed *X. americana*, using mainly gas chromatography coupled to mass spectrometry (GC/MS) and, without knowing the types of compounds present (hydrocarbons, alcohols, organic acids, esters of fatty acids, steroids, sugars, among others), preparation of volatile derivatives has become a basic requirement. Thus, the crude hexane was subjected to silylation reaction with BSTFA/ TMCS followed by obtaining a chromatogram (GC) of the crude reaction product. The structures of the components corresponding to 18 peaks in the chromatogram of total ion were identified by GC/MS from the fragmentation pattern observed in their mass spectra, and by the comparison to the mass spectra reported in the literature.

Among the identified compounds (**Table 1**), most are fatty acids, especially the hexadecanoic (palmitic acid), (Z,Z,Z)-9,12,15-octadecatrienoic (α-linolenic acid), (9Z,12Z)-octadec-9,12-dienoic (linoleic acid) and octadec-9-enoic (oleic acid) acids, the latter as the major component. It is also worth reporting the presence of the steroid *β*-Sitosterol (3.4%).

The MS of the TMS derivatives of these acids exhibit a characteristic profile fragmentation. Thus, their MS in addition to peaks due to $[M]^+$ show peaks at *m/z* 73, 117, 132, 145 and 313 corresponding to fragments $[CH_3)_3Si]^+$, $[CH_3)_3SiCO_2]^+$, $[CH_3)_3SiCO_2CH_3]^+$, $[CH_3)_3SiCO_2C_2H_4]^+$ and $[CH_3)_2SiCO_2C_{15}H_{31}]^+$, respectively. In general, at 70 eV eims of these trimethylsilyl derivatives, m/z 73 $[TMS]^+$ is registered as the base peak. Taken as an example, the mass spectrum (**Figure 1**) of the component with retention time 24.0 (**Table 1**) which was identified as the palmitic acid (RCO_2TMS, $R = C_{15}H_{31}$) showed a molecular peak at *m/z* 328.

The loss of methyl radical to give the ion at *m/z* 313 (M-15), occurs mainly from the silyl group [8] because at this point, the cation is more stable (**Scheme 1**). The ions at *m/z* 145 and 132 were explained by the fission of the bond β,γ (relative to the carbonyl group) of the chain R with possible transfer of hydrogen via a mechanism involving four centers, and through the expected McLafferty rearrangement [9] [10], respectively.

Also, the mass spectra of silylated fatty acids containing a double bond showed virtually the same behavior. Even those containing more than one double bond, but showing differences, exhibited a similar profile. However, the peaks corresponding to the fragments at *m/z* 117, 132 and 145 in these latter cases were much less intense.
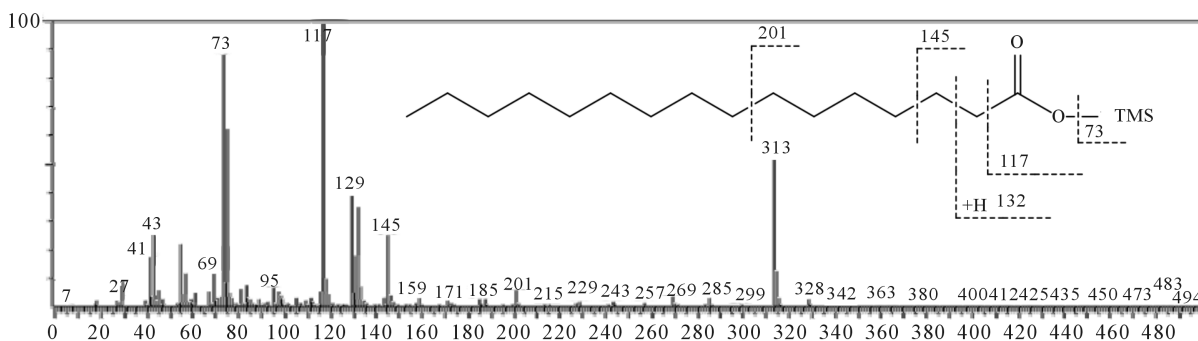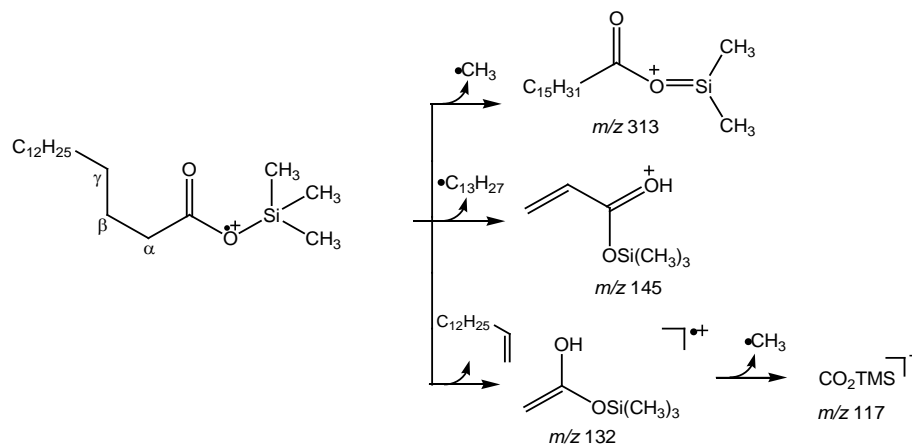

**Figure 1.** Mass spectrum of the TMS-hexadecanoic acid (palmitic acid).

Scheme 1. Mass spectral fragmentation observed for TMS-hexadecanoic acid (palmitic acid).

Table 1. Components of the hexane extract of the seeds of *X. americana*.

Peak	Compounds	RT	%	Mass spectra, *m/z* (rel. intensity)
1	Ethanedioic acid	6186	8.21	73(29), 75(39), 90(28), 147(100)
2	Disiloxane, hexamethyl-	7100	5.10	66(12), 73(22), 147(100), 148(17)
3	Pentasiloxane, dodecamethyl-	8732	0.69	73(81), 147(100), 281(91), 369(18)
4	Glycerol	10,957	0.76	73(100), 103(18), 117(19), 147(49), 205(22), 218(14)
5	n.i.	12,230	1.89	73(66), 146(100), 205(62), 279(51), 367(100)
6	Nonadioic acid	20,403	0.97	55(46), 73(100), 75(76), 117(28), 129(30), 317(21)
7	Hexadecanoic acid	24,023	4.17	73(89), 75(62), 117(100), 129(40), 132(35), 145(26), 313(52), 328(5)
8	9,12-Octadecadienoic acid (*Z,Z*)-	26,297	18.83	73(100), 75(98), 117(20), 129(27), 132(4), 145(8), 337(39), 352(4)
9	cis-9-Octadecenoic acid	26,358	38.14	73(100), 75(85), 117(65), 129(70), 132(19), 145(33), 339(47), 354(6)
10	trans-9-Octadecenoic acid	26,440	1.70	73(100), 75(80), 117(80), 129(49), 132(15), 145(40), 339(67), 354(7)
11	Octadecanoic acid	26,666	2.17	73(79), 75(58), 117(100), 129(38), 132(37), 145(27), 341(50), 356(4)
12	Linolenic acid	27,887	7.22	73(80), 75(83), 79(100), 117(22), 129(19), 132(4), 145(10), 335(7), 350(3)
13	9,12-Octadecadiynoic acid	28,907	2.52	73(95), 75(100), 91(82), 105(84), 117(50), 119(58), 129(29), 132(7), 145(12), 148(57), 333(8), 348(4)
14	trans-Octadec-11-en-9-ynoic acid, 8-trimethylsilyloxy-, methyl ester	29,526	1.24	73(100), 147(13), 217(12), 237(90), 365(4)
15	1-Monooleoylglycerol	32,741	0.90	55(55), 73(94), 103(48), 117(18), 129(100), 147(71), 201(20), 203(28), 205(12), 265(9), 397(40), 410(5), 485(6), 500(3)
16	Tetracosanoic acid	33,449	0.58	73(100), 75(75), 117(79), 129(30), 132(34), 145(33), 201(12), 425(40), 440(8)
17	cis-15-Tetracosenoic acid	37,198	1.13	73(100), 75(82), 117(62), 129(68), 132(22), 145(34), 423(32), 438(8)
18	*β*-Sitosterol	39,343	3.04	43(58), 73(52), 129(100), 255(14), 357(42), 381(22), 396(40), 471(9), 486(14)
19	Lupeol	43,736	0.74	73(50), 189(100), 203(52), 218(38), 279(10), 369(12), 393(9), 408(5), 483(4), 498(9)

n.i.: no identified.

Steroids in general, exhibit a very similar fragmentation pattern. The mass spectrum of the constituent with retention time 39.343 in the chromatogram showed a molecular peak at *m/z* 486 and was identified as *β*-Sitosterol. The peaks at *m/z* 471, 396, 381, 357, 213 and 129, are the most representative, since they correspond to fragments of higher mass and are relatively more prominent peaks. The ion at *m/z* 471 (M-15) can occur in two paths (**Scheme 2**): fission of the C-Si bond (a) or the C_{10}-C_{19} bond (b). Studies [11] from similar compounds demonstrated that the most attractive site of methyl cleavage is at the C–Si bond (a), a type of fragmentation very common in the mass spectral fragmentation of TMS ethers and esters. The ion at *m/z* 396 (M-90) was justified due to the loss of trimethylsilanol (TMS-OH) with the formation of ionized diene (c). Studies with 2,2,4,4-d_4 steroids suggests that some of the migrant hydrogen comes from C-4 and on the basis of stereochemical considerations it seems most likely the 4β hydrogen is probably migrant [11] (**Scheme 2**). In addition,
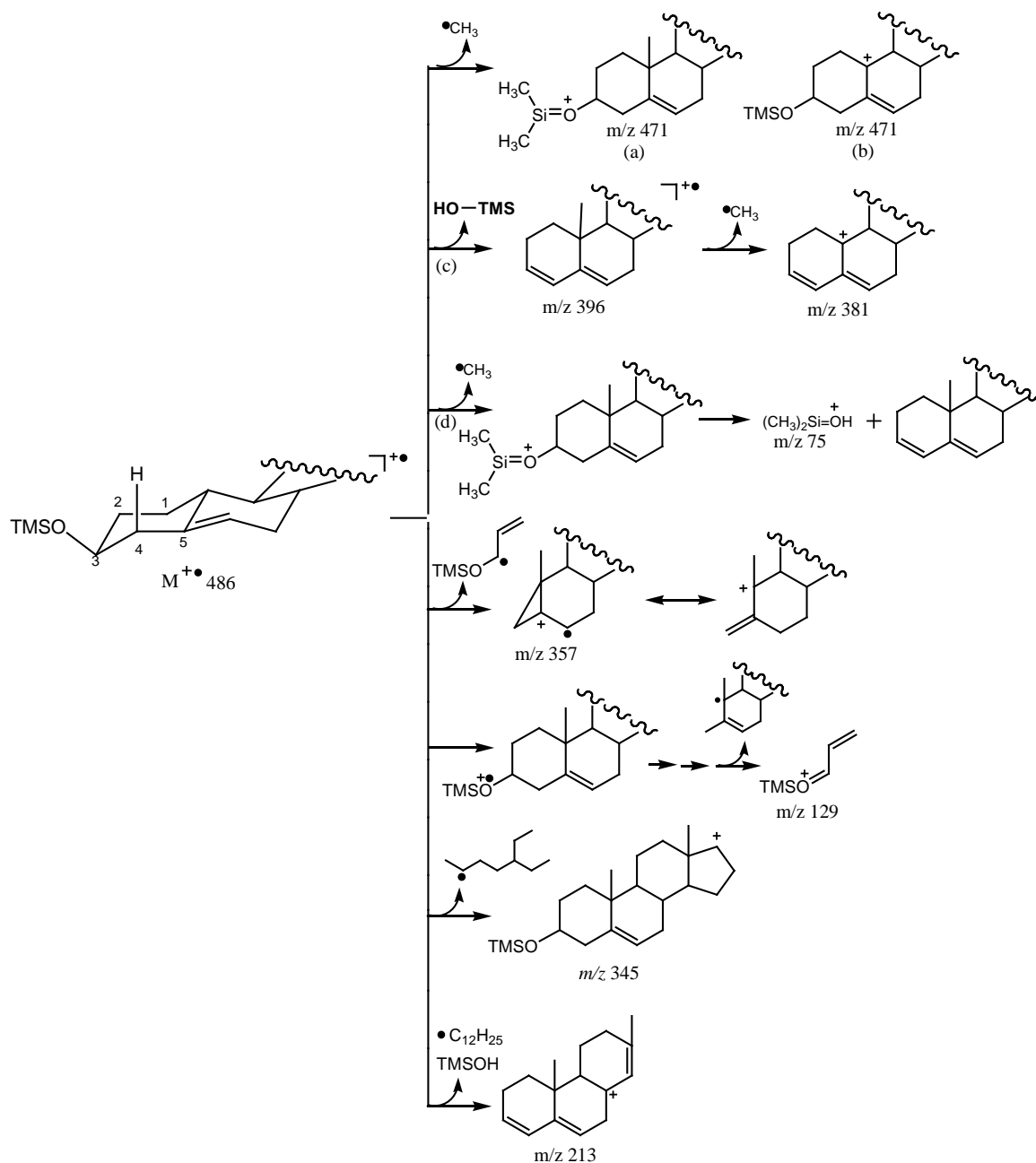
Scheme 2. Mass spectral fragmentation observed for TMS-*β*-Sitosterol.

another fragmentation (d) related to the M–90 results in one fragment at *m/z* 75, for which high-resolution mass measurements [11] indicated the composition $(CH_3)_2SiOH$. This fragment results from cleavage of a methyl radical from the TMS function followed by loss of the dimethylsilanol moiety. Deuterium labeling and methyl substitution at C-4 reveal a situation identical with the hydrogen transfer involved in the M-90 fragmentation, namely, that only some (40%) of the hydrogen originates from the C-4 position and that it is probably the axial 4β hydrogen atom that is implicated [11]. In turn, the fragment at *m/z* 396 loses methyl radical to yield the *m/z* 381. According to the literature [10], in the formation of *m/z* 357, the hydrogen atom H-2 it is transferred to C-6 carbon through six-atom system, followed by cleavage of the allylic bond C-3-C-4 and rearrangement of the ring A. Other typical ions (*m/z* 303, 275, 213, 184, 121, 95, 57, 43), resulting from the cleavage of the steroidal skeleton and of the side chain were observed [9] [11] [12]. The base peak at *m/z* 129 was justified by a complex fragmentation occurred in ring A. Labeling studies [11]-[13] with deuterium showed that the C_4 carbon is not part of ion *m/z* 129 (**Scheme 2**).

The crude EtOH extract was subjected to reaction silylation with BSTFA/TMCS followed by obtaining a chromatogram GC/MS of the crude reaction product. The structures of the components corresponding to 18 peaks in the chromatogram of total ion were identified by GC/MS from the fragmentation pattern observed in their mass spectra, and by the comparison to the mass spectra reported in the literature. Among the identified substances (**Table 2**), most are represented by furanosides and pyranosides carbohydrates and the main constituents were D-(+)-Sucrose (29.36%), L(-)-Sorbose (9.19%), scyllo-Inositol (8.34%) and D-Glucose (7.45%).

Table 2. Components of the EtOH extract of the seeds of *X. americana*.

| Peak	Compounds	RT	%	Mass spectra, m/z (rel. intesity
1	1,2-Bis(trimethylsiloxy)ethane	6181	2.13	147(100), 73(58), 191(23)
2	Disiloxane, hexamethyl-	7097	1.46	66(12), 73(22), 147(100), 148(14)
3	Glycerol	10,958	5.58	73(100), 103(18), 117(19), 147(80), 205(38), 218(15)
4	n.i.	14,239	1.41	172(100), 82(94), 73(22)
5	beta.-D-Galactofuranoside	20,404	3.04	73(100), 217(82), 147(33)
6	D-Fructose	20,856	1.48	73(100), 217(55), 147(25)
7	Mannonic acid, lactone	20,982	3.08	73(100), 217(65), 147(27)
8	Sorbopyranose, L-	21,091	9.19	73(100), 204(68), 217(33)
9	D-Galactose	21,182	1.54	73(82), 204(100), 217(28), 147(51)
10	Inositol, scyllo-	21,305	8.34	305(20), 318(32), 217(40), 147(42), 191(22), 204(12)
11	alpha.-D-Glucopyranose	22,212	5.15	204(100), 73(82), 191(40)
12	D-Mannitol	22,916	6.27	73(100), 147(51), 319(42)
13	D-Glucose	23,521	7.45	204(100), 191(41), 217(18)
14	Inositol, myo-	25,082	1.62	305(56), 318(30), 217(62), 147(51), 191(30), 204(25)
15	9,12-Octadecadienoic acid (Z,Z)-	26,297	1.91	73(94), 75(100), 129(25), 337(34)
16	trans-9-Octadecenoic acid	26,353	3.31	73(96), 75(100), 117(79), 129(68), 145(32), 339(42)
17	D(+)-Sucrose	31,944	29.36	361(100), 73(80), 217(32), 147(23), 103(20), 437(12), 540
18	14,17-Nor-3,21-dioxo-.beta.-amyrin, 17,18-didehydro-3-dehydroxy-	43,022	1.79	73(100), 75(90), 117(55)
19	Silane, (9,19-cyclo-9.beta.-lanost-24-en-3. beta.-yloxy)trimethyl-	43,309	5.89	73(100), 117(62), 75(57)

n.i.: no identified.

The identification of these substances as trimethylsilyl derivatives by GC/MS can be made in view of some mechanistic proposals for characteristic fragments, according to published studies [14]-[16]. For example, the fragments *m/z* 204 and 217 are relatively common from mass spectra of trimethylsilyl derivatives of carbohydrates. Both peaks can be seen in mass spectra from TMS ethers, however, the peak at *m/z* 204 is generally the dominant from TMS ethers with pyranose ring, as observed in the spectrum (**Figure 2**) of the component with retention time 21.091 (**Table 2**). Its origin can occur by different mechanisms. One them, from the open-chain carbohydrate involves the TMS group via McLafferty type rearrangement [17] [18] the fragment being formed by the C-1–C-2 carbon (**Scheme 3**).

This two-carbon fragment, *m/z* 204, a common dominant peak of pyranose derivatives (aldo-, keto-, and glycosides), originates the most part, from C-2–C-3 and C-3–C-4 (aldopyranoses) [19] or must contain carbons C-3–C-4 and C-4–C-5 (ketopyranoses) [20] which may be formed from the molecular ion or from *m/z* 437, both by cyclic fragmentation [20] (**Scheme 4**). In turn, the ion at *m/z* 437, typical of ketoses [21], results from the loss of C-1 and its substituent by fisson of the C-1–C-2 bond [19] [20].

Thus, by comparing with published data [14] [15], the mass spectrum of the component with RT 20.856 (**Table 2**) allow the assignment of a pyranose structure to the TMS ether derivative, which was identified as L-Sorbose in pyranose form. On the other hand, the relative intensity of the peak at *m/z* 204, which is most intense peak in the mass spectra of glucopyranosides (**Figure 2**) is drastically reduced in the mass spectra of glycofuranosides (**Figure 3**).

The peak at *m/z* 217 (**Scheme 5**) is characteristic of monosaccharides with furanose ring, which it is usually a very intense peak and, sometimes, the base peak. The relatively high intensity of this peak was observed in the mass spectrum of the component with retention time 20.856 (**Table 2**). Importantly, the fragment at *m/z* 217, according to the literature, is formed by carbon atoms C-2, C-3 and C-4 from aldopyranoses [19] [21] or from other carbons from ketofuranoses [18], but without a specific proposal for fragmentation. On the other hand, its formation generally can occur from other parts of the molecule via rearrangement [19]. One possible mechanism
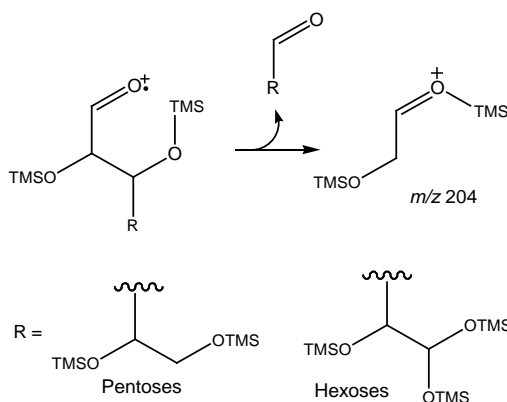
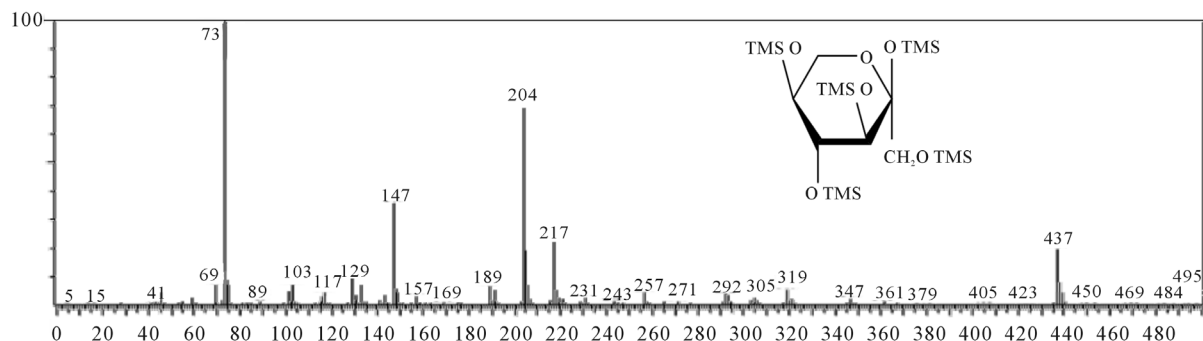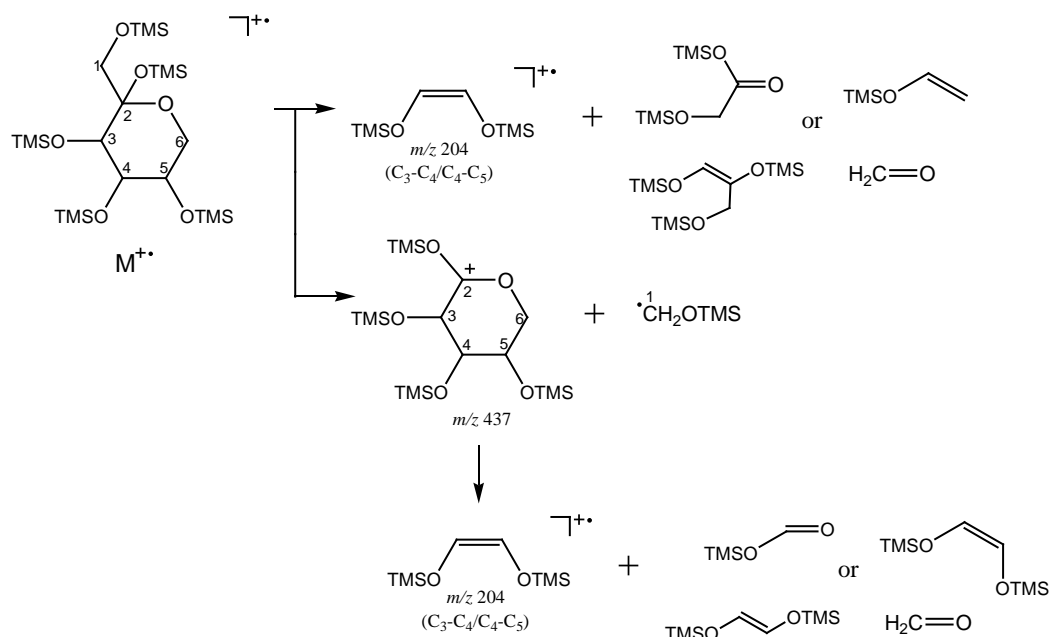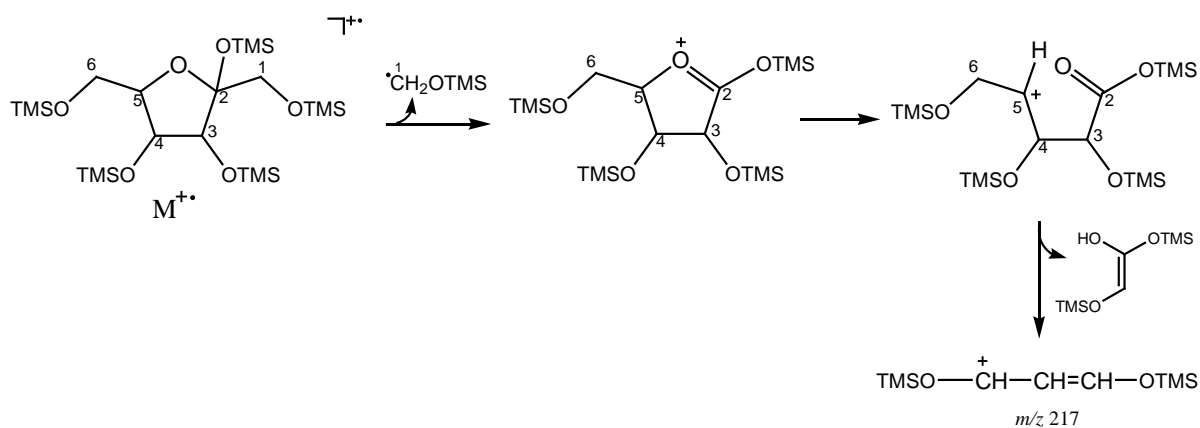**Scheme 3.** Formation of fragment *m/z* 204 via McLafferty type rearrangement.

Figure 2. Mass spectrum of TMS-L-Sorbose.

Scheme 4. Formation of fragment at *m/z* 204 via the alternative paths.

Scheme 5. Formation of fragment *m/z* 217 in furanose monosaccharides.
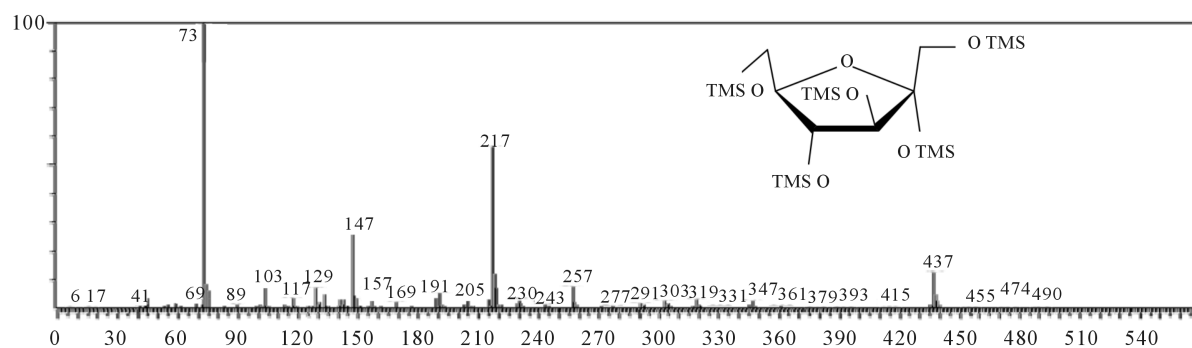

Figure 3. Mass spectrum of TMS-D-Frutose.

involving rearrangement is seen in **Scheme 5**. In the spectrum (**Figure 3**) was not observed the molecular ion peak ([M]$^{+\bullet}$ 540), but was recorded peak at *m/z* 437 (M-CH$_2$OTMS), characteristic of 2-ketohexoses [20] [21].

Thus, by comparing the mass spectra obtained in this study with published data [14] [15], the component (RT 20.856) was identified as the TMS ether of D-Fructose in furanose form.

Finally, by comparing the mass spectrum (**Figure 4**) component at RT 31.944 with the mass spectra of the literature [16], allowed to identify this component as the disaccharide Sucrose. It is noteworthy peaks at m/z 437 (M-CH$_2$OTMS), 361, 319, 217 and 204 (very low intensity) consistent with the pyranose and furanose structures. The other compounds of **Table 2** were identified by comparison of retention times and mass spectral data reported in the literature.

An intense peak at m/z 306 which is generally observed in the mass spectra of carbohydrates in their open forms [21] [22] is not registered in the mass spectra analyzed in the present study. This observation is in agreement with the carbohydrates identified in their cyclic forms.

At the concentration of the ethanolic extract of the seeds gave a precipitate which was recrystallized from MeOH. The ^{1}H NMR spectrum of this recrystallized (60 mg) showed that it was still mixture of two or three closely related compounds: IR spectrum γ max 3230 (OH), 2918/2910 (C$_{sp3}$-H), 1683 (C=O), 1029 (C–O) cm^{-1}. The ^{1}H NMR spectrum revealed the presence of various methyl groups, one of which (one singlet at 1.80 ppm) bonded to sp^2 carbon; the spectrum also showed signs as broad triplet at 5.72 (1H), broad singlet and 5.51 (1H) and two broad singlet at 4.96 (1H) and 4.78 (1H) due to hydrogens mutually coupled. The first two were attributed to olefinic hydrogens and consistent with the presence of triterpenes of oleanane/ursano types [23], while the last two were correlated to vinyl hydrogens in terminal double bond, characteristic of triterpene lupane type [24]. In turn, the ^{13}C NMR spectrum in accordance with the ^{1}H NMR spectrum recorded signals to lupane [δ_C 151.68 (C-20) and 110.32 (C-29)], as well as signs consistent with oleanane triterpenes [δ_C 122.92 (C-12), 56.10 (C-5), 42.36 (C-18), 46.83 (C-19), 33.54 (C-21) and 32.20 (C-22)] [23]-[25]; the spectrum also showed signs at δ_C 78,44 (due to oxygenated sp^3 carbon C-3) and 19,12 correlated to methyl carbon C-30 in lupane triterpenoids [24]. In addition, the signals δ_C 180.56 and 179.23 characteristic of carbons of carboxylic acids with relative intensities of 1:2, respectively. The DEPT experiment confirmed the presence of hydrogenated carbon, especially oxygenated CH (δ_C 78.44), CH$_2$ sp^2 (δ_C 110.32), CH sp^2 (δ_C 122.92), CH sp^3 (δ_C 56.10 and 42.36) and CH$_2$ sp^3 (46.83, 33.54 and 32.20). Based on the spectral comparison with the literature data (also considering the excessive number of signals in the ^{13}C NMR spectrum in the region between 14.00 and 56.00 ppm), it was concluded that the mixture was formed by triterpenes betulinic acid (56%), oleanolic acid (35%), and further a third component, also triterpenic nature in minor proportion (9%). The percentages were calculated from the integrations of signals at δ_H 3.56, 3.47 and 3.31 in the ^{1}H NMR spectrum, which were correlated with carbinolic hydrogens of the components of the mixture.

4. Conclusion

Carbohydrate analysis has proven to be a powerful technique in the understanding of biological processes. In addition to the possibility of studying the source of carbohydrates and their rotation in the environment, analysis of specific stable compounds may be used to trace microbial biomass and to detect food adulteration or in studies
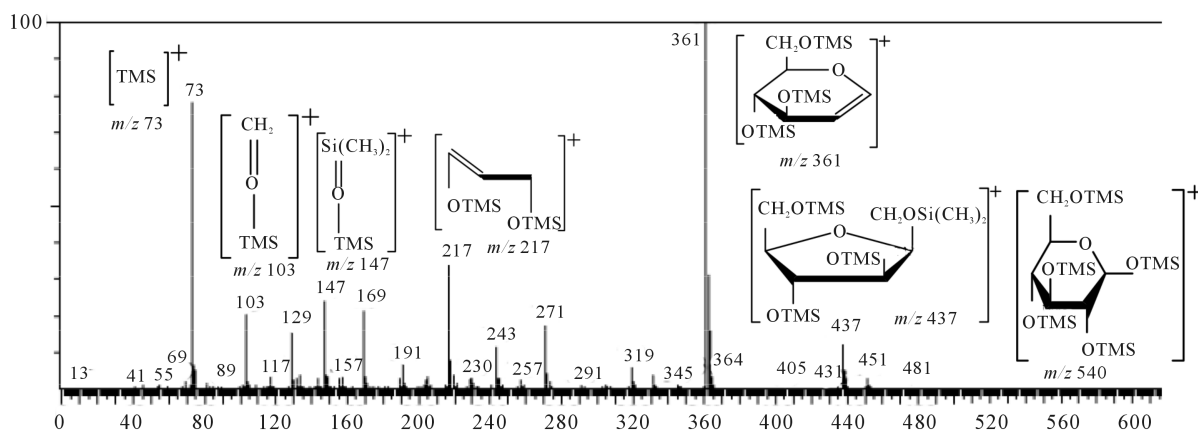

Figure 4. Mass spectrum of TMS-Sucrose.

nutrition. The techniques used in the analysis have proved very useful, therefore, allowed the identification of various chemical constituents, mainly carbohydrates, from both crude extracts. To our knowledge, this is the first study from hexane and ethanol extracts of the seeds of *X. ameriacana* using silylated derivatives by gas chromatography-mass spectrometry. This study, the first with oil from the seeds of *X. americana*, enabled a possible use of this oil as human or animal nutraceutical ingredient.

Acknowledgements

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