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Chemical Characterization of Metabolites from the Husk of *Theobroma cacao* by GC-MS in Cuba

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Abstract

An ethanolic extract at 90% made by Soxhlet was analyzed using a Shimadzu spectrometer coupled to gas chromatography (GCMS-QP2010 Ultra) after derivatization with BSTFA from the husk of *Theobroma cacao*. The drug powdered was evaluated through a phytochemical

screening to get information about the chemical components present in the sample. Ethereal, ethanolic and aqueous extract were analyzed by the same GCMS technique. Gas Chromatography Mass Spectrometry (GC-MS) analysis revealed the presence of 74 metabolites from this husk. According to NIST21 and NIST107 Libraries Databases some of the phyto-compounds screened were Caffeine, Theobromine, Catechine, Uridine, Stigmasterol, Cholesterol and Androstane-11,17-dione,3-[(trimethylsilyl)oxy]-,17-[O-(phenyl methyl)oxime]. Phytochemical screening revealed the possible presence of fats, alkaloids, triterpenes and steroids, catechins, reductants sugars, saponins, phenolic compounds, aminoacids or amines, quinones, anthocyanidins, flavonoids and bitter or astringent principles.

Keywords: *Theobroma cacao*, GC-MS, Phytochemical screening, Metabolites, Derivatization

I. Introduction

Cacao, (*Theobroma cacao*), also called cocoa, tropical evergreen tree (family Malvaceae, formerly Sterculiaceae) grown for its edible seeds, whose scientific name means “food of the gods” in Greek. Native to lowland rainforests of the Amazon and Orinoco river basins, cacao is grown commercially in the New World tropics as well as western Africa and tropical Asia. Its seeds, called cocoa beans, are processed into cocoa powder, cocoa butter, and chocolate (Cook, 2018).

Cacao grows in the forest understory to a height of 6-12 meters (20-40 feet), usually remaining at the lower end of this range. It’s oblong leathery leaves measure up to 30 cm (12 inches) in length, and are periodically shed and replaced by new leaves that are strikingly red when young. Its flowers are either foul-smelling or odorless; they can be present at all times but appear in abundance twice a year. These flowers grow in clusters directly from the trunk and limbs and are about 1 cm (0.4 inch) in height and breadth. They can be white, rosy, pink, yellow, or bright red, depending on the variety, and are pollinated by tiny flies called midges in many areas (Ronse, 2010).

After four years the mature cacao tree produces fruit in the form of elongated pods; it may yield up to 70 such fruits annually. The pods, or cherelles, range in color from bright yellow to deep purple (Figure 1). They ripen in less than six months to a length up to 35 cm (14 inches) and a width at the center of 12 cm (4.7 inches). Each pod has numerous ridges running along its length and holds 20 to 60 seeds, or cocoa beans, arranged around the long axis of the pod. The oval seeds are about 2.5 cm (1 inch) long and are covered with a sweet sticky white pulp (Clement et al., 2010).

Figure 1. Cacao's elongated pods

Cacao thrives at altitudes of 30 to 300 meters (100 to 1,000 feet) above sea level in areas where temperatures do not range much below 20 °C (68 °F) or above 28 °C (82 °F). Rainfall requirements depend upon the frequency and distribution of rain and the degree of water retention by the soil; the minimum necessary rainfall is about 100 cm (39 inches) evenly distributed throughout the year, but 150-200 cm (59-79 inches) is optimal. Successful cultivation also requires deep well-drained soil that is porous and rich in humus. Protection against strong winds is necessary because of the tree's shallow root system (FAO, 2017).

In the cocoa bean industry, some by-products go underutilized. Some of these components could provide other innovative products, and such is the case with the husk of the cocoa bean. Previous studies have attributed the husk with a high antioxidant capacity, which added to its relative low cost, makes it an attractive ingredient for the production of infusions. However, prior to promoting it as such, its quality needs to be guaranteed (Sangronis et al., 2014). The aim of this study was to evaluate the chemical composition of the husk of cocoa bean that grows in Cuba which is much utilized in the Cuban chocolate industry.

2. Material and Methods

2.1 Plant Material

The sample was the husk of cocoa bean after its separation from the fruits. It was supplied in 2018 by the Chocolate Factory located in Baracoa, Guantanamo Province, Cuba. After the collection the husks were packet in nylon bags without elimination of foreign matters. The material was grounded in a high-speed hammer-mill. The sample keeps its brown color (Fig. 2) and a very nice chocolate's smell.

Figure 2. Husk of cocoa beans (after separation and powdered)

2.2 Phytochemical Screening

The bioactive compounds were screened to ascertain their presences in diethyl ether, ethanol and water according to Chhabra et al., 1989.

2.3 Extract Preparation

The extracts were prepared with the ground material (60 g) without screen extracted in a Soxhlet apparatus with 675 mL of ethanol at 90% during 20 hours. The ethanolic extracts were concentrated and evaporated under vacuum to 200 mL at 120 rpm, a temperature of 70 °C and 500 mbar.

2.4 Procedures, Instrumentation and Parameters

The sample were subjected to chromatographic analysis in equipment GC/MS, brand Shimadzu QP2010, equipped with a splitter split/splitless. With a BP5 (30 m × 0.25 mm× 0.25 microns) capillary column under the following chromatographic conditions: Helium gas carrier obtained by electron impact fragments to a power of 70 eV rate of 1.2 mL/min, 1:50 split flow and the volume of injected sample of 1 ul. Programmed oven temperature: initial temperature was 70 °C with a heating ramp of 10 °C/min to 300 °C and remained stable at this temperature for 10 minutes. Subsequently the temperature was increased at a rate of 10 °C/minute to 300 °C for a total time of 78 minutes with an injector temperature 250 °C and the interface temperature 300°C.The compounds were analyzed using GC/MS NIST21 and NIST107 library and having into account the results obtained after phytochemical screening according with Gómez (2017). Silylation agent was N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) CAS 25561-30-2 Lot: 0901-1 Macherey-Nagel GmbH & C. KG. Chromatographic running was done four times: ethanolic extract by Soxhlet at 90 % and with the ethereal, ethanolic and aqueous extracts by separated getting in the phytochemical screening.

3. Results and Discussion

3.1 Phytochemical Screening

Preliminary phytochemical screening showed the presence of fats and oils, alkaloids, tannins and/or phenolic compounds, flavonoids, catechins, triterpenes and/or steroids, reductants sugars, aminoacids and/or amines, quinones, anthocyanin, saponins and bitter principles. The

test for coumarins and lactones was not 100 % confident, although showed some turbidity (Table 1).

Table 1. Phytochemical screening of the cacao husk

Test for constituent groups	Ether	Ethanol	Water
Sudan	+		
Dragendorff	-	++	+
Wagner	-	++	+
Baljet	-	± (turbidity)	
Liebermann-Burchard	+	+	
Catechins		+	
Resins		-	
Fehling		+	+
Foam		+	+
$FeCl_3$		+ (dark green)	+ (dark green)
Ninhydrin		+	
Börntrager		+++ (red)	
Kedde		-	
Anthocyanin		+	
Shinoda		+	+
Bitter and astringent principles			bitter

3.2 GC/MS Analyses

From ethanolic extract at 90%, seventy-four different chemical components were characterized from the husk of *Theobroma cacao* L. by GC/MS experiments in Cuba for the first time. Among them 11 aminoacids, 35 different kind of acids, 13 different kind of sugars or related compounds, one aldehyde, 3 sterols or related compounds, two alkaloids, one catechine, one purine, one pyrimidine, one lactone, one alkane, 3,4-dihydroxyphenylethylamine, 2,6-deoxyfructosazine, 1-monooleoylglycerol trimethylsilyl ether and 1,3-dipalmitin trimethylsilyl ether (Table 2). According with Rohloff in 2015, all those kinds of metabolites can be found and characterized using derivatization techniques in combination with GC-MS-Based Metabolite Profiling.

Chromatographic profile of cacao husk showed the same amount of chemical components with few differences up to 46 minutes of retention times in the Soxhlet extraction, ethereal and hydrolacoholic extracts (Figure 3). According with chromatographic profiles aqueous extraction gets the fewer amounts of chemical compounds, indicating that the components have a low or medium polarity.

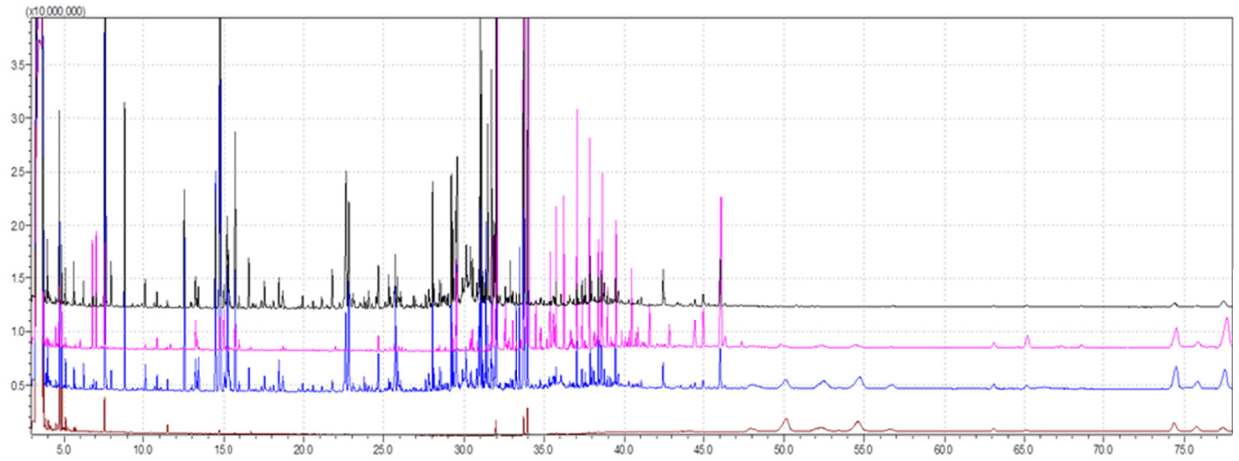

Figure 3. Chromatographic profiles of husk from *Theobroma cacao* in Cuba (a-Soxhlet; b-hydrolacoholic; c-ether, d-water).

Qualitative analysis indicated that caffeine is the majoritarian alkaloid. Theobromine was found in lower concentrations. Both results are according to Cuéllar et al., 2012, although in this case, theophylline was not found into ethanolic extract at 90%. Figure 4 show the magnified zone in which caffeine and theobromine appears. Table 2 lists the names, retention times, molecular masses, base peaks and chemical formulae of the identified compounds.

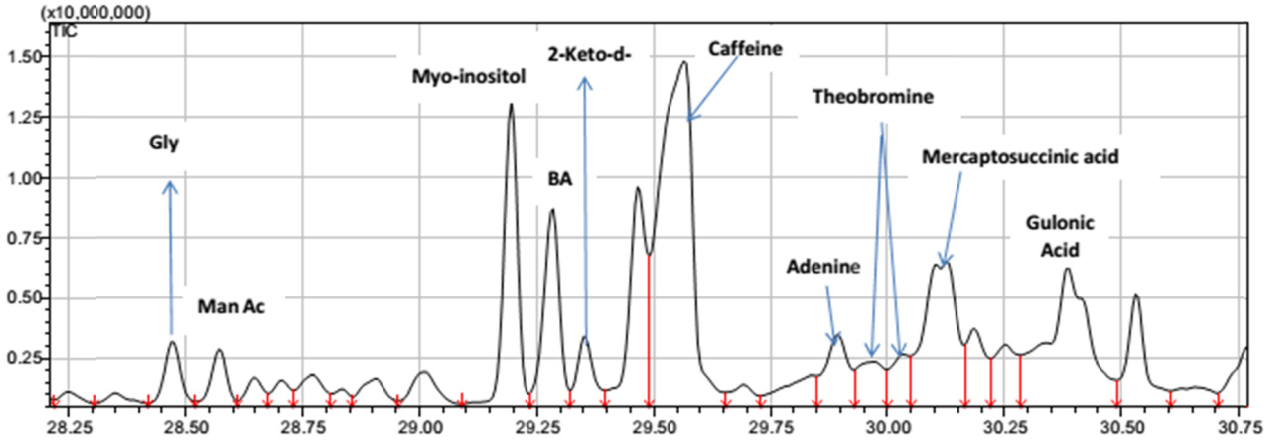

Figure 4. Magnified zone in which appear caffeine and theobromine (Gly: Glycine; Man Ac: Mannoonic acid; BA: 3, 4-hydroxy-Benzoic acid; 2-Keto-d-gluconic acid).

The results show the great variety of chemical components in this part of the plant that is in general, underutilized in all countries that produce cacao beans in the world, and as can see, may be can used to improve the health in human been or in the food industry to make new types of liquors or beverages or in the agricultural to supply animal food or as fertilizer (Sangronis et al., 2014; Cuéllar et al., 2012; Collazos, 2012). The beneficial activities of those compounds are known, in this sense, it is necessary to mention the important role of two of

those compounds identified in the sample like Dodecanoic and Hexadecanoic acid. Dodecanoic acid is useful as Antioxidant, Antibacterial, COX-1&COX-2 inhibitor, Antiviral, Hypocholesterolemic, and candidacies, while Hexadecanoic acid can act as Antioxidant, Flavor, Hypocholesterolemic, Nematicide, Pesticide, Lubricant, Antiandrogenic, Hemolytic and 5-Alpha reductase inhibitor (Rajkumar et al., 2016).

Table 2. Chemical compounds identified in *Theobroma cacao* husk by GC-MS

Peak No.	Retention time	Molecular Mass	Base peak	Compound Name	Molecular formula
1	7.643	234	73	Propanoic acid	$C_9H_{22}O_3Si_2$
2	7.935	220	73	Acetic acid	$C_8H_{20}O_3Si_2$
3	8.843	233	116	L-Alanine	$C_9H_{23}NO_2Si$
4	10.842	262	73	Butanoic acid	$C_{11}H_{26}O_3Si$
5	10.522	261	144	L-Valina	$C_{11}H_{27}NO_2$
6	13.224	276	73	Pentanoic acid	$C_{12}H_{28}O_3Si$
7	14.441	275	158	L-Leucine	$C_{12}H_{29}NO_2$
8	14.740	308	73	Glycerol	$C_{12}H_{32}O_3Si$
9	14.982	208	73	Benzenacetic acid	$C_{11}H_{16}O_2Si$
10	15.177	275	73	L-Isoleucine	$C_{12}H_{29}NO_2$
11	15.263	247	102	gamma-Amino butyric acid	$C_{10}H_{25}NO_2$
12	15.734	262	147	Butanedioic acid	$C_{10}H_{22}O_4Si$
13	17.542	321	73	L-Serine	$C_{12}H_{31}NO_3$
14	18.459	335	73	L-threonine	$C_{13}H_{33}NO_3$
15	18.690	276	73	Pentanedioic acid	$C_{11}H_{24}O_4Si$
16	19.934	336	73	3,4-Dihydroxybutanoic acid	$C_{13}H_{32}O_4Si$
17	21.776	350	73	Malic acid	$C_{13}H_{30}O_5Si$
18	22.624	273	156	L-Proline	$C_{11}H_{23}NO_3$
19	22.804	336	73	Butanal	$C_{13}H_{32}O_4Si$
20	24.038	424	73	2,3,4-Trihydroxybutiric acid	$C_{16}H_{40}O_5Si$
21	24.689	310	73	Benzenepropanoic acid	$C_{15}H_{26}O_3Si$
22	25.665	282	73	Benzoic acid	$C_{13}H_{22}O_3Si$
23	25.700	438	73	D-Arabinose	$C_{17}H_{42}O_5Si$
24	25.742	309	218	L-phenylalanine	$C_{15}H_{27}NO_2$
25	25.888	363	102	3,4-Dihydroxyphenylethylamine	$C_{17}H_{35}NO_2$

26	26.085	364	73	Arabinoic acid	$C_{14}H_{32}O_5Si$
27	26.873	318	73	Isocitric lactone	$C_{12}H_{22}O_6Si$
28	27.627	512	73	Ribitol	$C_{20}H_{52}O_5Si$
29	27.790	524	73	Myoinositol	$C_{21}H_{52}O_5Si$
30	27.47	512	73	Xylitol	$C_{20}H_{52}O_5Si$
31	28.467	287	73	Glycine	$C_{12}H_{25}NO_3$
32	28.587	452	73	2-Deoxy-galactose	$C_{18}H_{44}O_5Si$
33	28.709	333	75	L-Glutamic acid	$C_{13}H_{27}NO_5$
34	28.771	526	73	Ribonic acid	$C_{20}H_{50}O_6Si$
35	28.906	364	73	D-Arabinonic acid	$C_{14}H_{32}O_5Si$
36	29.289	370	73	3,4-hydroxy-Benzoic acid	$C_{16}H_{30}O_4Si$
37	29.355	554	73	2-Keto-d-gluconic acid	$C_{21}H_{50}O_7Si$
38	29.497	466	73	Mannoonic acid	$C_{18}H_{42}O_6Si_4$
39	29.5663	194	194	Caffeine	$C_8H_{10}N_4O_2$
40	29.899	279	264	9H-Purine-6-amine	$C_{11}H_{21}N_5Si$
41	30.029	180	180	Theobromine	$C_7H_8N_4O_2$
42	30.118	366	73	Mercaptosuccinic acid	$C_{13}H_{30}O_4Si$
43	30.388	466	73	Gulonic acid	$C_{13}H_{42}O_6Si$
44	30.910	397	218	L-Tyrosine	$C_{18}H_{35}NO_3$
45	30.986	410	73	1,2,3,4-tetrahydroxy-Butane	$C_{16}H_{42}O_4Si$
46	31.063	614	73	D-Mannitol	$C_{24}H_{62}O_6Si$
47	31.158	614	73	d-Glucitol	$C_{24}H_{62}O_6Si$
48	31.340	284	88	Palmitic acid	$C_{18}H_{36}O_2$
49	31.453	540	217	beta-D-Galactofuranose	$C_{21}H_{52}O_6Si_5$
50	31.585	540	73	D-Glucose	$C_{21}H_{52}O_6Si_5$
51	31.735	326	73	Palmitelaidic acid	$C_{19}H_{38}O_2Si$
52	31.833	438	217	D-Ribofuranose	C17H42O5Si
53	31.946	628	73	Galactonic acid	$C_{24}H_{60}O_7Si_6$
54	31.975	328	73	Hexadecanoic acid	$C_{19}H_{40}O_2Si$
55	32.183	612	73	Inositol	$C_{24}H_{60}O_6Si_6$
56	33.005	272	73	Dodecanoic acid	$C_{15}H_{32}O_2Si$
57	32.235	184	55	10-Undecenoic acid	$C_{11}H_{20}O_2$
58	33.479	326	88	Nonadecanoic acid, ethyl ester	$C_{21}H_{42}O_2$

59	33.702	352	75	9,12-Octadecadienoic acid	$C_{21}H_{40}O_2Si$
60	33.757	354	117	Oleic acid	$C_{21}H_{42}O_2Si$
61	33.987	356	73	Octadecanoic acid	$C_{21}H_{44}O_2Si$
62	35.753	384	73	Eicosanoic acid	$C_{23}H_{48}O_2Si$
63	36.056	460	73	Uridine	$C_{18}H_{36}N_2O_6Si_3$
64	37.578	846	73	D-Turanose	$C_{33}H_{78}O_{11}Si_7$
65	37.877	808	73	2,6-Deoxyfructosazine	$C_{33}H_{76}N_2O_7Si_7$
66	38.385	500	73	1-Monooleoylglycerol trimethylsilyl ether	$C_{27}H_{56}O_4Si_2$
67	38.552	992	73	Per-O-trimethylsilyl-(3-O-.beta.-d-mannopyranosyl-d-glucitol)	$C_{39}H_{96}O_{11}Si_9$
68	38.953	440	73	Tetracosanoic acid	$C_{27}H_{56}O_2Si$
69	39.448	650	73	Catechine	$C_{30}H_{54}O_6Si_5$
70	44.975	484	83	Stigmasterol	$C_{32}H_{56}OSi$
71	46.030	458	129	Cholesterol	$C_{30}H_{54}OSi$
72	46.335	481	91	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-,17-[O-(phenyl methyl)oxime]	$C_{29}H_{43}NO_3Si$
73	77.439	496	75	9,12,15-Octadecatrienoic acid	$C_{27}H_{52}O_4Si_2$
74	77.518	640	371	1,3-Dipalmitin trimethylsilyl ether	$C_{38}H_{76}O_5Si$

4. Conclusions

The GC-MS study reveals that *Theobroma cacao* husk contains a number of phytoconstituents which are to be used for various therapeutic purposes or in food industry. In this study, 74 compounds were identified from GCMS data. The compounds identified can be also used as biomarkers especially for *T. cacao* husk because little research has been published for this part of the plant. Chromatographic profiles reveal high predominance of fatty acids, aminoacids and reductants sugars, and two important alkaloids: caffeine and theobromine, which may be considered a source of important phytochemicals with bioactive properties to be explored for pharmaceutical applications. This is the first report with complete GC/MS data for these compounds found in the underutilized husk of *Theobroma cacao* that grows in Cuba.

5. ConflicT OF Interest StatemENT

We declare that we have no conflict of interest.

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