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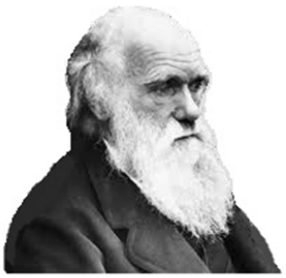

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Characterization of Catechins from *Smilax domingensis* Willd. in Cuba

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ABSTRACT

Gas Chromatography coupled with mass spectrometry was applied to study the fragmentation of selected flavonoids from the ethanolic extract of *Smilax domingensis* Willd., Smilacaceae, known as zarzaparrilla, after Soxhlet extraction during 20 hours. Compounds belonging to one of the major subgroups found in common plants, i.e. flavanols were studied. Following solvent extraction and derivatization using BSTFA, possibly two different metabolites from the same chemical group were characterized in one analytical run: (+)-catechin and (-)-epicatechin. For the very first time, qualitative data on these analytes in the drug were determined after detailed validation of a sensitive, cheap and reliable GC-MS method.

***Keywords*:** *Smilax domingensis*, GC/MS, flavanols, catechins, ethanolic extract

1. INTRODUCTION

S. domingensis is native from Mexico to Panama and the Antilles, it has been collected from 0-2000 masl; it grows on most soils over nearly all types of tutor tree in areas with yearly precipitation from 1400-3500 mm (Stevens et al., 2001). The most common name in Cuba is raíz de china, but it is also known as zarzaparrilla, cuculmeca, bejuco de canasta (Costa Rica), chiquihuite (México), palo de la vida, corona de Cristo, tietie, China-root (Guatemala,

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Honduras, Belize), and bejuco de membrillo (Puerto Rico). The rhizome is popularly used in medicine as anti-inflammatory, antiseptic, depurative, sudorific, antasthmatic, antiherpetic, antirheumatic and for venereal diseases (Roig, 2014).

It is an evergreen dioic woody vine, 2-4 m high with lignified rhizomes (Fig. 1). Alternate leaves, ovate-acuminated short petiole with two lignified tendrils. Flowers are arranged in umbels. Staminated flowers usually have six stamens. Pistillated flowers with ovary superior. Fruit is a berry, red, purple, or black. Rhizome is partially lignified, voluminous, with tuberous swelling, reddish brown in color. Roots are adventitious, growing from the rhizomes (Cáceres et al., 2012).

The chemistry of Smilax has been described primarily for the long roots and small rhizome type of species, which include steroidal saponins, flavonoids, polyphenols and stigmasterol (Bérdy et al., 1982). There is no published information on the chemical composition of *S. domingensis* rhizome. Phytochemical screening revealed the possible presence of alkaloids, oils and/or fats, coumarins, saponins, flavonoids, pyrogallol-type tannins, quinones, catechins, reductants sugars, triterpens and steroids and absence of resins, aminoacids, cardiotonic glycosides, anthocyanidins and astringent and/or bitter principles, realized under WHO parameters (J. G. Yaque *et al*., 2017). Data presented here refer to evaluations with wild material. The aim of this article was to characterize the chemical components present in ethanolic extracts of *S. domingensis* that grows in Cuba for the standardization as drug.

Fig. 1. Macroscopic views of rhizomes from *S. domingensis* in Cuba.

2. MATERIAL AND METHODS

2. 1. Plant Material and Reagents

The *S. domingensis* Willd. rhizome was collected from Sierra Cristal, Sagua de Tánamo, Holguín Province, Cuba, 850-1000 masl, by Elio M. García Fargie in March, 2016. Voucher specimen (**HAJB 089193**) is deposited at the herbarium of National Botany Garden in Havana, Cuba. The plant material was authenticated by Dr. Jorge E. Gutiérrez Amaro. The harvested

rhizomes were dried in the shade at room temperature (temperature 30 - 40 ºC) on the Research Lab Table in the Faculty of Pharmacy and Foods (Havana University), ground into powdered form (1 mm) and stored in airtight containers. All reagents used were of analytical grade (Merck). All solvents were degassed prior to use in an ultrasonic bath without filtration.

2. 2. Extracts Preparation

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

2. 3. 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 ºC with a heating ramp of 10 ºC/min to 300 ºC 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 J.G. Yaque et al., 2017. Silylation agent was *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) CAS 25561-30-2 (Lot: 0901-1) Macherey-Nagel GmbH & C. KG.

3. RESULTS AND DISCUSSION

The highly diverse group of plant flavonoids comprise flavonols (e.g., kaempferol and quercetin), flavanones (e.g., naringenin and hesperidin), flavones (e.g., luteolin and apigenin), flavan-3-ols (e.g., catechin and gallocatechin), and flavanonols (e.g., taxifolin). All these structures, either as aglycon or glycoside, are characterized by a certain number of hydroxy groups which can be silylated. However, due to relatively higher molecular weight of glycosylated polyphenols, the detection and structure elucidation of intact glycosides is preferably achieved on LC platforms, which is also true for hydolyzable tannins. (Rohloff, 2015).

According with the GC/MS database report of the Shimadzu QP2010 equipment utilized in the chemical characterization of ethanolic extracts from rhizomes of *S. domingensis* Willd. that grows in Cuba, 125 different kinds of chemical components were identified in the sample (Fig. 2).

Among them, the authors consider appropriate that according to the obtained results, the extracts contain two closely related compounds: catechin (5TMS) **CT** and epicatechin (5TMS) **ECT**. Both components belongs to flavan-3-ol subgroup (catechin derivatives), having as unique structural difference the insertion at the main backbone the OH in 3 position and the same molecular weight of 290 amu (Tsimogiannis et al., 2007) (Fig. 3).

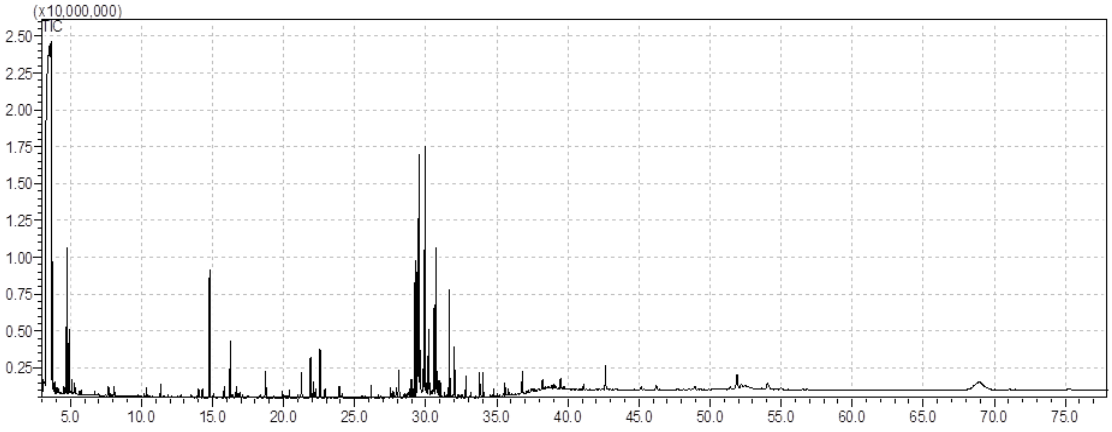

Fig. 2. Current chromatogram of ethanolic extract from *S. domingensis* in Cuba.

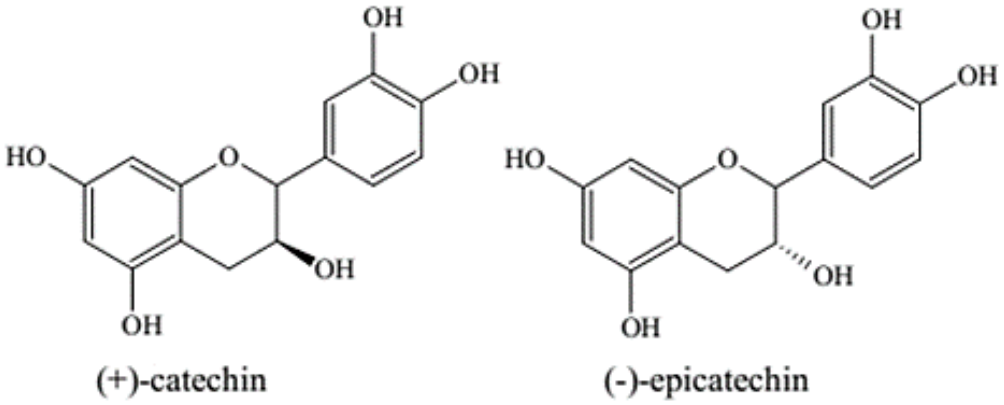

Fig. 3. The structures of the standard flavonoids (flavanol subgroup).

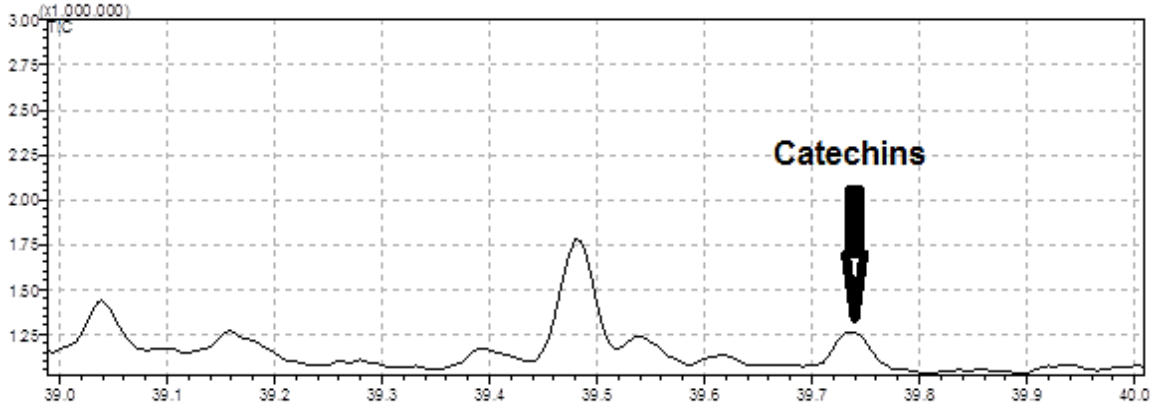

Fig. 4. Chromatogram of ethanolic extract from *S. domingensis* in Cuba (R. Time 39.735).

Magnifiying the area around 39.735 minutes was observed a little peak attributed to catechins (Fig. 4). As we can see, the peak of reference is not prominent, but show a good resolution. Comparing with NIST107 Library the selected peak corresponds to a chemical compound with MF $C_{30}H_{54}O_6Si_5$. The resulting search allow to inferred that this compound have a MW of m/z 290 after the rest of 5 TMS (360 amu) from the derivatization process. The Base Peak is 73, CAS# 89267-68-5, Serial# 106536 and Compound Name: Catechine, penta-TMS-ether, (2R-cis)-, and as Synonyms: 2H-1-benzopyran-3,5,7-triyl]tris(oxy)]tris[trimethyl-, (2R-cis)- or (+)-(2R:3S)-5,7,3',4'-Tetrahydroxyflavan-3-ol, etc.

According to the aforementioned data the fragmentations of the compounds of each flavonoid subgroup are specific and therefore these fragmentations, combined with the molecular weight of the parent ion, the retention times and the UV spectrum can allow the tentative identification of a compound, which can be further verified by the use of suitable standards, when available (Sarria-Villa et al., 2017) (Figures 5 and 6).

The MS spectra of commonly found TMS derivatives of polyphenolic structures such as catechin (5TMS) (CT) and epicatechin (5TMS) (ECT) are depicted in the figures below. MS spectra of CT and ECT can be found in the Golm Metabolome Database [G], Human Metabolome Database [H], MassBank [M], and/or NIST Chemistry WebBook [N] (Fig. 7).

Using HPLC-MS/MS flavanols presented defining C-ring fragments identical to those of the studied flavanones. However the high relative abundance of the three main fragments of C-ring fission i.e. $^{1,2}B^+$, $^{1,3}A^+$ and $^{1,4}B^+$ is a characteristic quality of flavanols, presented even by the methoxylated ones, according to the spectra of Cren-Olivé et al., 2000, and they can distinguish these flavonoids from any other subgroup (Tsimogiannis et al., 2007). Flavanols (3-hydroxy-flavanones) show fragmentation patterns similar to those of flavanones; the main difference is that positive ESI spectra of flavanols are dominated by loss of water and homolytic H loss from the 3-hydroxy group to generate the $[M-H]^{\bullet+}$ ion (Zhang et al., 2008).

Despite limitations in GC-MS with respect to the mass range and polarity of metabolites, the utilization of derivatization techniques and automation technology have extended the range of separable and detectable compounds in high-throughput profiling experiments. Beside the qualitative and quantitative analysis of trimethylsilyl derivatives of highly abundant compounds found in plant samples such as sugars, amino acids and polyols, instrument sensitivity and resolution also allows for the successful detection of minor constituents such as plant secondary metabolites (Kumari et al., 2011; Menikarachchi et al., 2013).

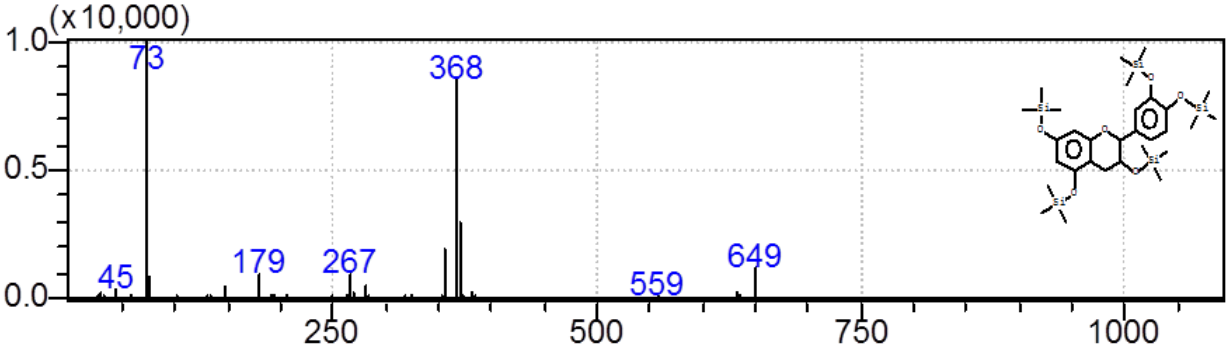

Fig. 5. Mass spectrum of Catechine, penta-TMS-ether, (2R-cis) – at 39.735 min (NIST 107 LIB)

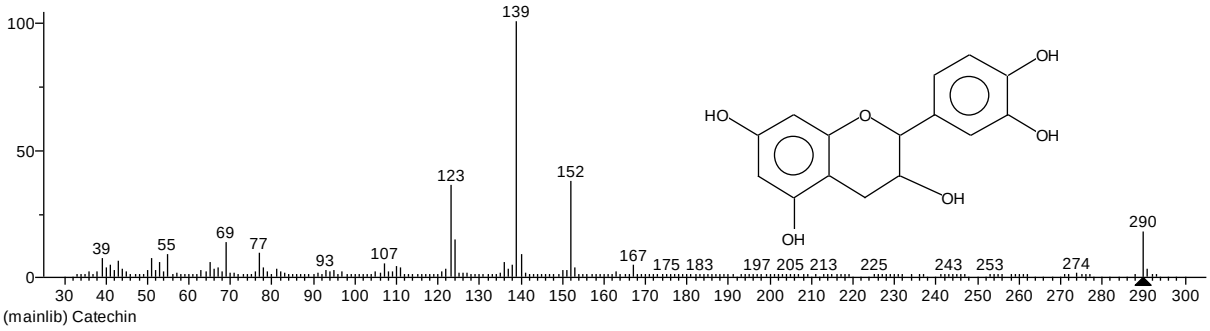

Fig. 6. Mass spectrum of Catechine (NIST MS Search 2.0 LIB).

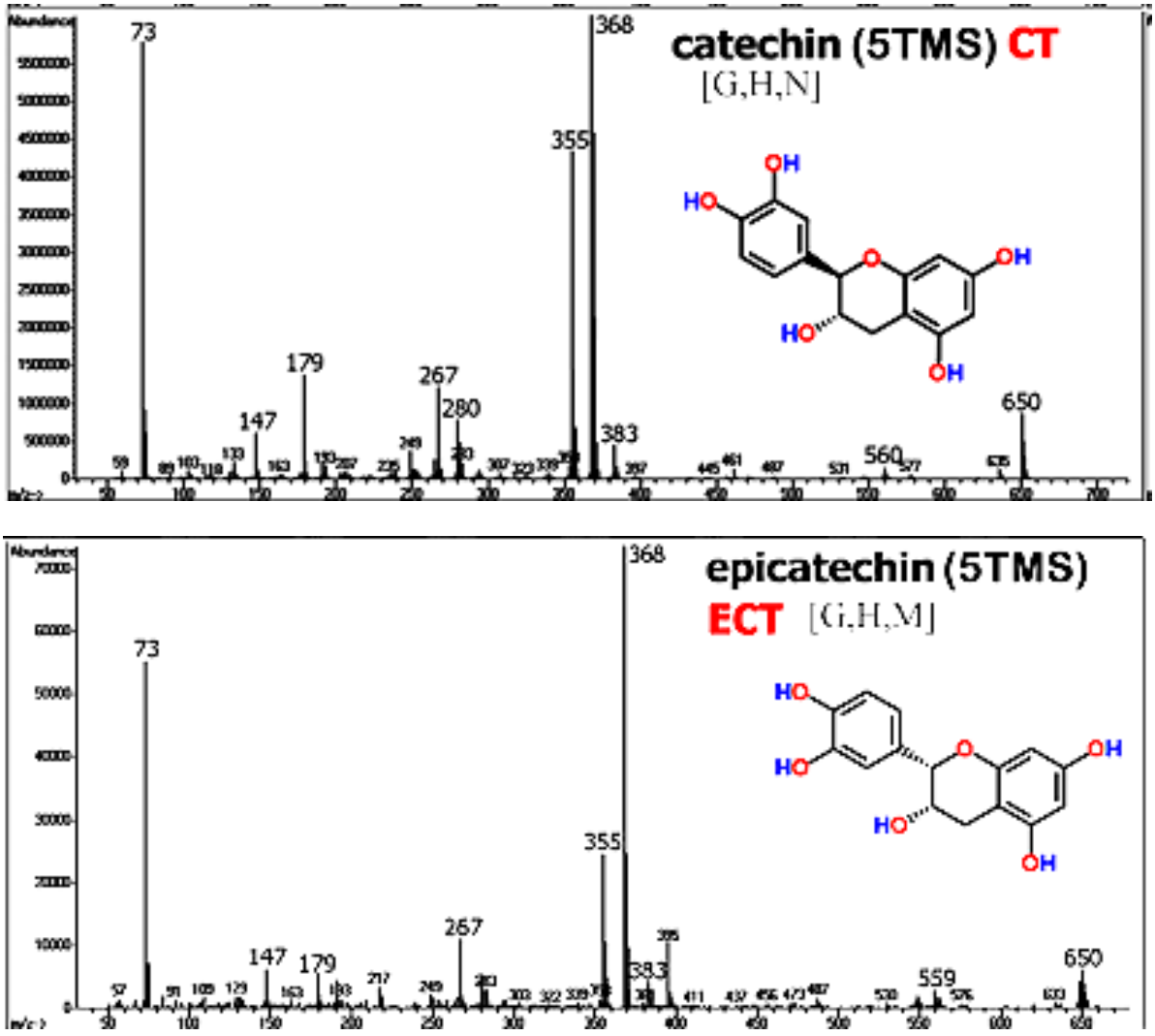

Fig. 7. MS spectra of commonly found TMS derivatives of polyphenolic structures such as catechin (5TMS) (**CT**) and epicatechin (5TMS) (**ECT**) (NIST Chemistry WebBook [N]).

Taking into account the probably fragmentation pathway of this type of flavonoid subgroup by GC/MS is totally justified that the peak of 649 m/z corresponding to loss of H, while peak at 634 was attributed to loss of 15 units corresponding to CH_3, etc.

These results are in concorcance with Zhang and Zuo in 2004, when they publicated their results after the use as derivatization reagent *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS), Retention Times and Characteristic Ions Present in the MassSpectra of Silylated Derivatives in Standard Solution, Cranberry Juice, and Plasma Samples (m/z 368, 355, 283,179, 73).

Table 1 summarized the proposal fragmentation patterns of those kinds of flavonoid subgroup that are present in the extracts of the plant and according to the phytochemical screening done by the authors in 2017. The same result was observed by Cáceres et al., 2012, when in cooperation with the National Institute of Engineering, Technology and Innovation (INETI) from Lisbon, Portugal, several compounds were identified by HPLC in tinctures and extracts in order to select markers for extracts standardization, among them, catechin, epicatechin, epigallocatechin, epicatechin gallate, and gallocatechin.

Several artefacts caused by the derivatization reagent were observed in the GC-MS chromatograms. This observation has earlier been noted by Little (1999). BSTFA used by itself does not generate artefacts with carboxylic acids, but it does with phenol. It cannot derivatize totally the phenol functional group hence producing artefacts. Incomplet silylation of compounds lead to multiple peaks hence affecting the determination of the number of components present in a sample. Little (1999) suggested that BSTFA should be used with DMF to have complete derivatization for phenol (Kiprop et al., 2013).

Table 1. Assigment of proposal structure fragments produced by GC/MS.

Flavanol/fragments	**m/z**
M+5TMS	650
M+5TMS-H	649
M+5TMS-CH_3	634
M+5TMS-C_6H_3	559
^{1,3}A+2TMS	383
^{1,3}A+2TMS-CH_3	368
^{1,3}A+2TMS-(CH or C_2H_5)	355
-2H_2O+H	318
^{1,2}B+2TMS	267
-C_3H_7OH	207
-CO	179

-CH_3CH_2OH	133
-CH_3CH_2OCO- or $(CH_3)_3Si$	73
-$CH_3CH_2O^{\bullet}$	45
C_2H_5	29

4. CONCLUSIONS

GC-MS is frequently applied to characterize the chemical complexity of analytical samples based on its separation and identification capacity. For the first time, the presence of catechins in the ethanolic extracts of rhizomes from *S. domingensis* Willd. in Cuba was proposed. Based on experimental data from own research, the present study has emphasized the capabilities of GC-MS to deduce chemical information on phenolics and cyclic compounds found in complex mixtures of plant metabolites. More deeply investigations will be necessary in the future to complete the chemical fingerprint of this promising medicinal plant.

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