EXTRACTION ASSISTED BY ULTRASOUND BATH AND CHEMICAL CHARACTERIZATION BY HPLC-ESI-UV-MS/MS

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Abstract

A sample of petals from the flowers of *Talipariti elatum* (Sw.) dried at shadow at room temperature was extracted with ethanol 95 % using for the first time an Ultrasound Bath after 3 hours. Liquid chromatography (LC) coupled with UV detection and electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was used for the generation of chemical fingerprints and the identification of phenolic compounds in Blue Mahoe from Martinica Island. Fifty seventh compounds were detected by HPLC and among them, 21 of these detected by MS after base peak filtration. The structures of eleven compounds found in this hydroethanolic extract were suggested mainly by MS data conjugated with the UV spectra, reference compounds and available mass spectra data in literature. To the best of our knowledge, with exception of gossypetin-3'-*O*-glucoside found in the petals of the flowers, nothing has been published about the chemical composition of *T. elatum* in Martinica.

Keywords: Taliparitti, HPLC-ESI-UV-MS/MS, petals, ethanolic extract, ultrasound.

Resumen

Una muestra de pétalos de las flores *de Talipariti elatum* (Sw.) secada a la sombra a temperatura ambiente fue extraída con etanol 95 % usando por primera vez un Baño de Ultrasonido durante 3 horas. Se usó cromatografía líquida (CL) acoplada a un detector de UV y espectrometría de masas en tándem (EM/EM) con ionización por aspersion (IA) para la generación de huellas digitales químicas y la identificación de compuestos fenólicos en la majagua de la Isla Martinica. Cincuenta y siete compuestos fueron descubiertos por HPLC y entre ellos, 21 de éstos descubiertos por EM después

de la filtración de pico base. Las estructuras de once compuestos encontrados en este extracto hidroetanólico fueron sugeridas principalmente por datos de EM conjugados con los de espectros UV, compuestos de referencia y datos de espectros de masas disponibles en la literatura. Según nuestro conocimiento, con la excepción de gossypetina-3'-*O*-glucósido, encontrado en los pétalos de las flores, nada ha sido publicado sobre la composición química *de T. elatum* en Martinica.

Palabras clave: *Talipariti*, HPLC-ESI-UV-MS/MS, pétalos, extracto etanólico, ultrasonido.

Introduction

Over years, American *Hibiscus species* (*Hibiscus rosa-sinensis* L., *Hibiscus elatus* Sw. and *Hibiscus tiliaceus* L) have been used as an effective therapeutic option in persistent spasmodic coughs, flu and asthma. Since 2007, Areces and Fryxell renamed the Blue Mahoe (from *Hibiscus elatus* Sw. to *Talipariti elatum* Sw.) due to their arborescent behavior, prominent stipules that close the terminal yams, foliar lameness coriaceous, entire margin and a relative higher chromosomal number (2n = ca. 80, 90, ca. 92, ca. 96 and 120) (Fryxell. 2001). The same procedure was done with *Hibiscus tiliaceus* L (now *Talipariti tiliaceum* L.). Both species belongs to *Malvaceae* family.

T. elatum Sw. is a big tree that grows naturally in wet mountain forests of Cuba and Jamaica. It produces all year beautiful flowers orange to red, and was domesticated and planted in almost all the islands and countries of the Caribbean basin (Fig. 1). In Martinica, under the name Blue Mahoe or Mountain Mahoe, the uses are less known and therefore less common, and a complete exploration of the biochemical and biological properties remains to be done. In 2015, a Martinican research team isolated and characterized from the petals of the flowers the isomeric form of gossypitrin: gossypetin-3'-*O*-glucoside, utilizing 1,2-dimetoxiethane by Soxhlet extraction (Françoise-Haugrin *et al*., 2016).

The aim of this work is to extract and characterize the main chemical components from the petals of the flower using an Ultrasound Bath with ethanol at 95 % for the generation of chemical fingerprints and the identification of chemical compounds in this part of the plant.

Fig. 1. Flowers of *Talipariti elatum* Sw.

2. Material and Methods

2.1 Plant material

Flowers were collected in January 2015 in Martinica along Route de la Trace. Flowers were washed before drying in the laboratory at room temperature during five days. A voucher specimen is deposited and registered in French Pharmacopeia as Fournet 1752 (4232 Guad). Martinican specimens are registered as *Hibiscus elatus* S.w.

2.2 Solvents

All solvents were purchase from Merck (Darmstadt, Germany). LCMS grade water; analytical grade ethanol and LCMS grade methanol were used in the analysis work. All solvents were degassing previously before used in an ultrasonic bath without filtration.

2.3 Extract and Samples Preparation

Dark red flowering types were collected daily. The isolated petals used were dried at shadow at room temperature during 5 days. The extract was prepared with 75.1 mL of ethanol 95 % and the ground material (38.49 g) using a WARING (Blender 8010EB), Model HGBTWT without screen in an Ultrasound Bath (Elmasonic S 70 H) during 3 hours (Fig. 2). Grounded material was previously humectant with 80 mL of ethanol to get a final amount of 155.1 mL of extract. The ethanolic extract was concentrated and evaporated under vacuum at 120 rpm, a temperature of 70˚C and 500 mbar to obtain finally 80 mL.

Fig. 2. Ultrasound Bath Elmasonic S 70 H.

2.4. HPLC-UV-ESI-MS/MS Procedures, Instrumentation and Parameters

The LC system consisted of a Varian 500 (USA) equipped with UV detector at 280 nm, scan mode running: ion positive coupled to a Mass Spectrometer Ion Trap 500 MS Varian (USA) fitted with an Electrospray source with the scan range from m/z 100 to 2000, using manual injection with a flow of 1 mL/min. The collision energy was -5 to -35 V. Scan program used was Varian MS Workstation Version 6.9, Column Pinnade DB C-18, 5 µm (i.d), 250 x 4,6 mm of diameter (Made in USA). Capillary voltage 60 V and needle 5000 V. Chromatographic analyses by HPLC/MS was done using as eluent A methanol and B water. A gradient of 15-85 % of B during 30 min at 1 mL/min follow by holding of gradient, increasing up to 50 % A during 10 min sustained, reversing up to 0 % B during 5 min and equilibrating during five minutes. Full scan LC was running in 60 min. Total extract was introduced directly into the HPLC system.

3. Results and Discussion

Figure 3 shows the total chromatographic profile of the investigated extract by LC-MS. The LC conditions permitted a good separation of these compounds and were optimized for further separations of crude plant extracts containing aglycones or glycosylated flavonoids derivatives and other chemical constituents in 60 min.

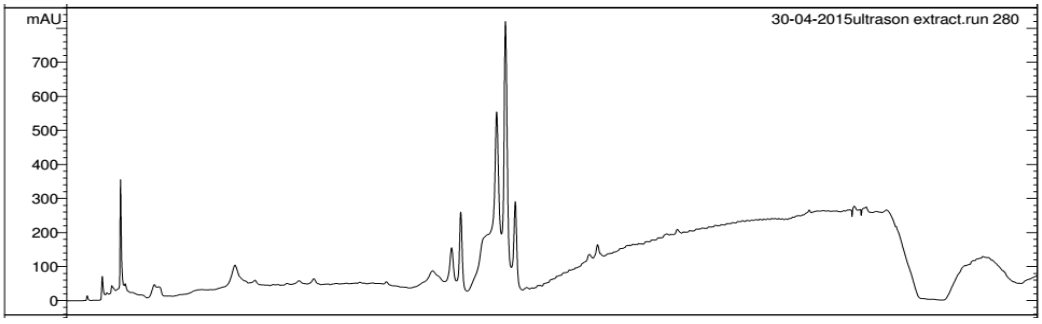


Fig. 3. Chromatogram of ethanolic extract from the petals of *T. elatum* at 280 nm.

After the running, 57 different chemical compounds were found in the ethanolic extract using the gradient with A (MeOH) and B (H_2O). Fig. 4 shows the current chromatogram of ethanolic extract from

the plant searching the phenolic profile. In the LC, at least 21 different kinds of chemical constituents are found, giving an idea that the utilized gradient gives a good resolution to get the information about how many phenolic compounds where in the ethanolic extract. All compounds were registered between 1.255 and 58.547 min, respectively. Three most prominent peaks were at 26.578, 27.122 and 27.727 joined together with a little shoulder, while another two at 3.320 and 24.356 are very significant too.

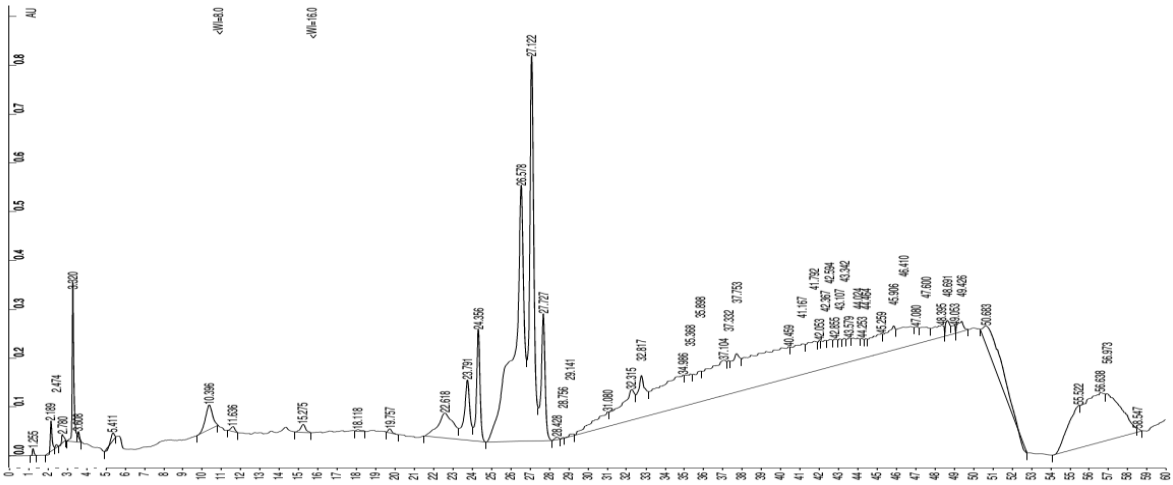


Fig. 4. Total ion current chromatogram obtained through the analysis of hydroethanolic extract from petals of *T. elatum* in positive ion mode.

The interpretation of the MS/MS and UV spectra compared to reported data and reference compounds were the main tool for the proposal of the structure of the compounds found in this extract. Through MS analyses no information can be obtained about the stereochemistry of the glycan part of the flavonoids glycosides. However, the sugar types (hexoses, deoxyhexoses and pentoses) can be indirectly deduced from the difference of the mass of pseudo-molecular ions and the masses of corresponding fragment (Ma *et al*., 1997; Vukics and Guttman, 2010; Ferreres *et al*., 2011).

Little structural information regarding the aglycone fragment could be obtained from spectra data (Table 1). HPLC-UV-MS analyses were performed for the characterization of the aglycone of flavonols-*O*-glycosides. Fig. 5 show the full scan MS of total ethanolic extract from petals of *T. elatum* done between m/z 100 and 2000. After that, the MS/MS scan was analyzed after base peak filtration including three chemical compounds in each separated zones. Seven MS/MS spectrums were obtained which including 21 different chemical components. For a best resolution of the results, all scan was filtered taking into account the wavelength at 280 nm and only one at m/z 481.

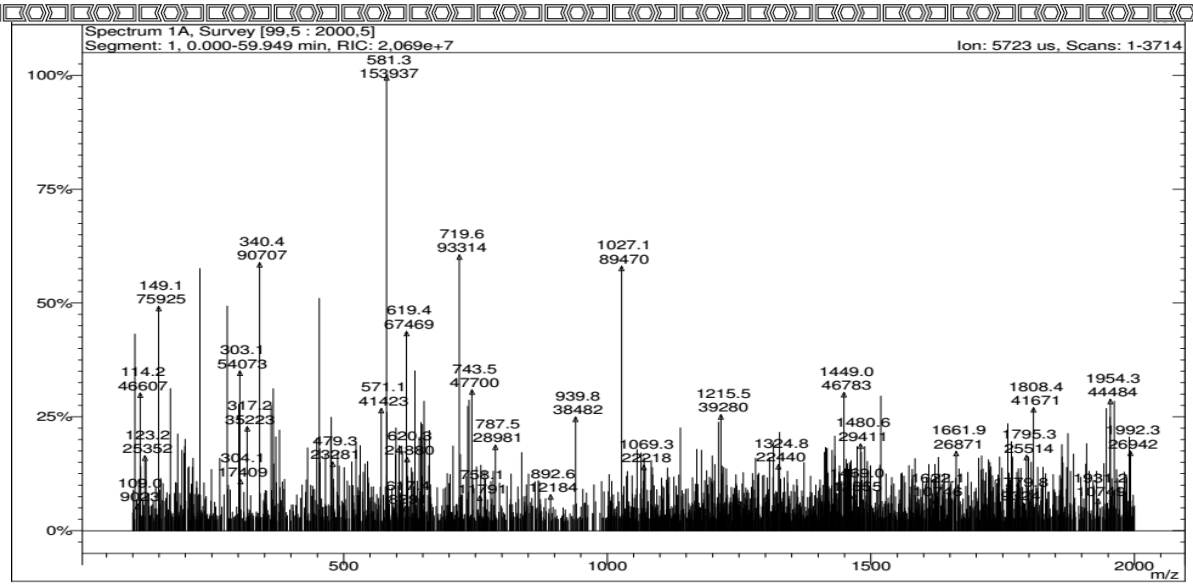


Fig. 5. ESI-MS spectrum of protonated molecules obtained through the analysis of hydroethanolic extract from the petals of *T. elatum* in positive ion mode.

All selected chemical compounds were analyzed according to its intensities (100 %) in all cases and depending of the particular case, taking into account the molecular ion (M, M+1, M-1 or M-2). As mentioned, in flavonoids glycosides the sugar types (hexoses, deoxyhexoses and pentoses) can be indirectly deduced from the difference of the mass of pseudo-molecular ions and the masses of corresponding fragment (Ma *et al*., 1997; Vukics and Guttman, 2010; Ferreres *et al*., 2011). Fig. 6 shows the selected MS scan after filter base peak selection.

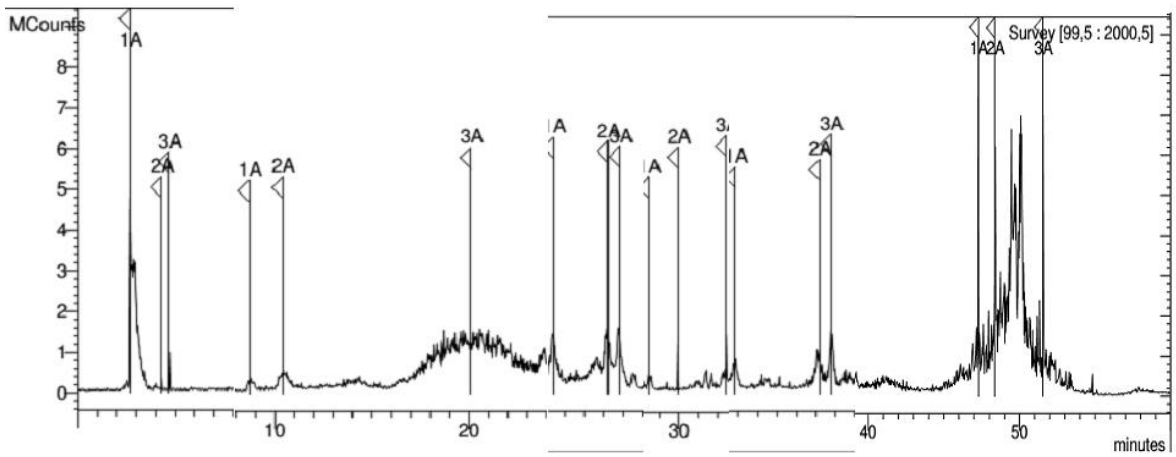


Fig. 6. Selected MS/MS scan after base peak filtration at 280 nm.

After base peak filtered at m/z 481 three different peaks were found, with the particularity that they showed the same molecular weight at different retention times. Their individual retention times are between peak **7** (24.407 min) and **8** (27.214 min). As shown in Fig. 7, the resulting protonated peaks

are in correspondence with flavonoid aglycones, glycosylated flavonoids or their derivatives. The MS spectrum are suggesting the presence of three flavonoid closely relationated.

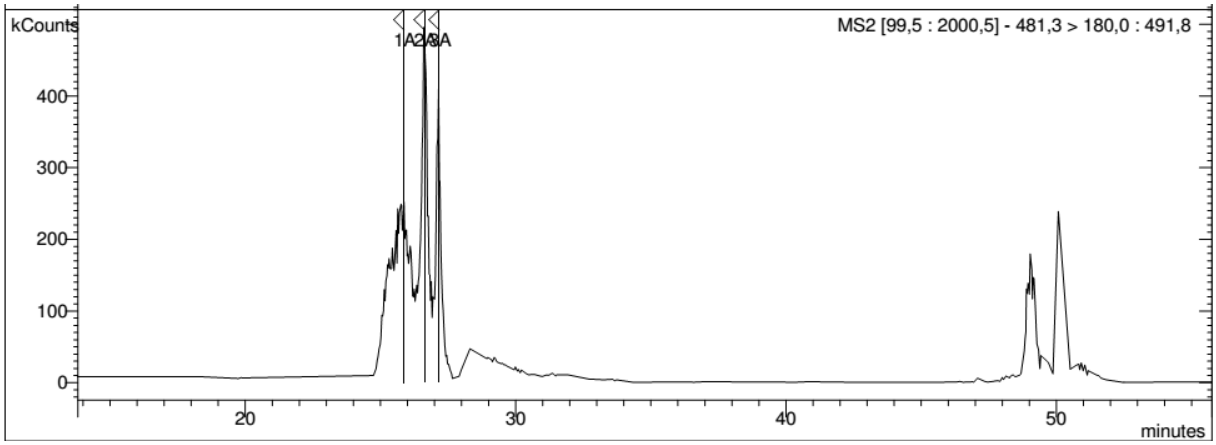


Fig. 7. Chemical compounds found after base peak filtered at 280 nm with m/z 481 only.

Our experimental experiences indicate that this result agrees with the presence of gossypetin aglycone (m/z 318), gossypitrin (m/z 480) and gossypetin-3'-*O*-glucoside (m/z 480) (J. G. Yaque *et a*l., 2016 a, b; González *et al*., 2017). For us, the first peak (25.870 min) corresponding to gossypetin aglycone, the second one to gossypitrin (26.626 min) and the third one to gossypetin-3'-*O*-glucoside. This suggestion must be experimental confirmed properly by LC-NMR, because of the same molecular mass of last both chemical compounds and due to in general they cannot be identified by their respective UV and IR spectral data (González *et al*., 2016).

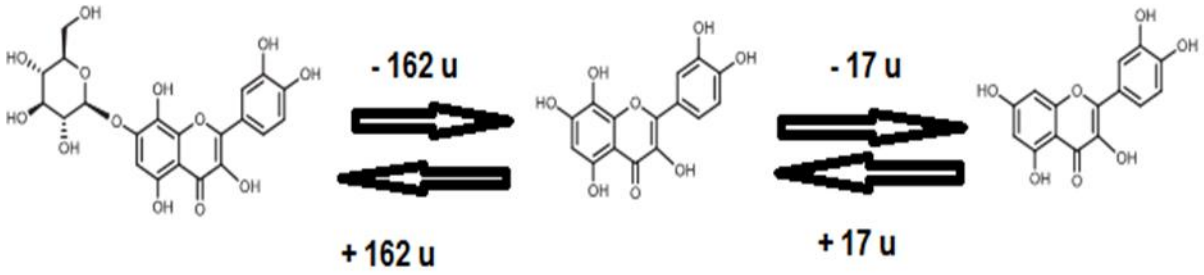


Fig. 8. Neutral losses of gossypitrin/gossypetin-3'-*O*-glucoside after base peak filtered at 280 nm with m/z 481 only.

As is known, both flavonoid glycosides are isomers differentiated by the sugar position. The ESI-MS spectrum exhibited protonated molecule at m/z 481 and a base peak at m/z 319 which probably corresponds to the loss of 162 u (glucose) produced from the homolytic cleavage of the *O*-glycosidic bond, resulting in the formation of a radical aglycone cation (Pinheiro and Justino, 2012). The neutral loss of 162 u (glucose moiety) yielded the radical aglycone ion at m/z 319, which according to Vukics & Guttman, 2010 is one of the most common neutral losses of sugar attached to flavonoid aglycones. The presence of quercetin as aglycone was also suggested by a fragment ion at m/z 302 for the

subsequent neutral loss of 17 u (OH) yield to the formation of quercetin (m/z 302) in both cases (Simirgiotis, 2013; González, 2016).

Compounds **8** and **9** showed MS spectra characteristic of flavonols. Little structural information regarding the aglycone fragment could be obtained from spectra data (Table 1). Herbacetin, quercetin could be considered to suggest their presence into the ethanolic extract suggested by a fragment ion at m/z 303 with their respective retention times at 27.214 and 27.827 min. Compound **7** was tentatively identified as ellagic acid, a gallotannin, at 24.407 min of retention time (Fig. 9) (Fracassetti et al., 2013).

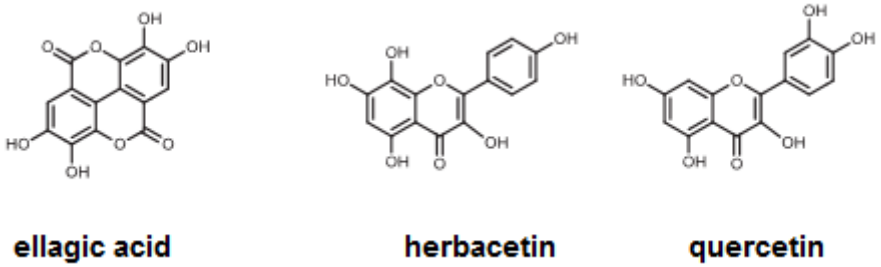
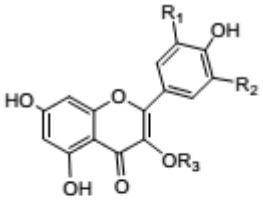


Fig. 9. Structures of compounds 7, 8 and 9 identified in *T. elatum*.

Compounds **10** (28.439 min), **14** (37.182 min) and **15** (37.764 min) were suggested to be myricitrin (Myricetin-3-*O*-galactose) (m/z 480.3) and two isomers suggested being myricetin-3-*O*-(6″galloyl)-galactose (m/z 633.3) by comparison against published data (Fracassetti *et al*., 2013, Rodriguez Perez *et al*., 2013, Simirgiotis *et al*., 2013, Negri *et al*., 2013) (Fig. 10). None evidence of the presence of myricetin as aglycone was found in this research.



R_1	R_2	R_3
OH	OH	(6” galloyl) Galactose
H	OH	Galactose
OH	H	(6” galloyl) Galactose

Fig. 10. Structures of compounds 10, 14 and 15 identified in *T. elatum*.

The molecular masses of compounds **1** and **4** (m/z 118.2; m/z 104.2) are suggesting the presence of molecules with impair number of mass, due to the at least one N atom in their molecular structures.

We are suggesting the presence of two closely related amino carboxylic acids: 2-aminopentanoic acid (m/z 118) and 2-aminobutanoic acid (m/z 104), respectively.

Eight chemical constituents remain unidentified. Compounds **6** (20.099 min) and **13** (32.865 min) with ions at 581.5 and 487.3 u.m.a are chemical closely related with flavonoid glycosides. Compounds **2**, **3**, **11**, **12**, **16** and **18** showed the highest molecular masses among 1405.0 and 1851.5 u.m.a., some of them with impair mass number, probably alkaloids, as we suggest previously in 2016 when our research team found out a chemical constituent in a sample of petals from Martinica dried at shadow during a week with m/z 480 in positive ion mode (J. G. Yaque *et al*., 2016 a).

Table 1 lists the retention times (Rt), MS data spectra and maximal ultraviolet wavelength (λ max) for the chemical constituents found in the extract.

Table 1. Identified compounds in *T. elatum* by HPLC-UV-ESI-MS/MS.

Peak #	Rt (min)	λ max (nm)	$[M+H]^+$ (m/z)	Fragment ions (m/z)	Polyphenols Identity
1	2.667	280	118.2	-	2-aminopentanoic acid
2	4.292	280	1545.7	-	Unknown
3	4.703	280	1851.5	-	Unknown
4	8.682	280	104.2	-	2-aminobutanoic acid
5	10.400	280	479.3	480.3, 501.2	Myricitrin derivative
6	20.099	280	581.5	582.4	Unknown flavonoid glycoside
7	24.407	280	303.2	-	Ellagic acid
8	27.214	280	303.1	-	Herbacetin
9	27.827	280	303.2	304.2	Quercetin
10	28.439	280	480.3	481.3, 503.1	Myricetin -3-*O*-galactose (**myricitrin**)
11	29.968	280	1482.0	1482.8	Unknown
12	32.443	280	1405.0	-	Unknown
13	32.865	280	487.3	488.4	Unknown flavonoid glycoside
14	37.182	280	633.3	634.4, 635.2	Myricetin-3-*O*-(6″galloyl)-galactose

15	37.764	280	633.3	634.4	Myricetin-3-*O*-(6″galloyl)-galactose
16	47.273	280	1430.4	-	Unknown
17	48.380	280	467.4	468.5	Quercetin-*O*-glucoside derivative
18	51.557	280	1569.2	-	Unknown
19*	25.870	280	319.3	317.3	**Gossypetin (aglycone)**
20*	26.626	280	319.3	320.3	**Gossypitrin**
21*	27.176	280	319.3	318.2	**Gossypetin-3'-*O*-glucoside**

***Base Peak Filtered at m/z 481 only.**

4. Conclusions

The HPLC fingerprints showed in this work can be used to authenticate and differentiate the flowers of *T. elatum* that grows in Martinica and Cuba, which are similar in appearance and are grown in the different location and used for similar medical purposes. Based on our LC/UV and LC/MS experiments, the distribution of different phenolics in the specie has been analyzed and a total of 57 compounds were detected and among them 11 characterized, or tentatively identified for the first time for the spice in Martinica Island many of which have not been described hitherto in these plant materials which might be related with the number of phenolic compounds and total phenolic content found in these extracts. The compounds identified can be also used as biomarkers especially for *T. elatum* since little research has been published for this species that belong to Malvaceae family. The phenolic profiles of the different plant parts revealed high predominance of flavonoids, which are antioxidant compounds that modulate a variety of beneficial biological events. For the first time, in this part of the flowers of *T. elatum,* was utilized the extraction assisted by UB suggesting that it is an appropriated method to get good results in the elaboration of medicinal extracts with this part of the plant.

Acknowledgements

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Conflict of Interest

The authors have declared no conflict of interest.

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