

Chemical constituents and antioxidant activity of extracts obtained from branch bark of *Bursera simaruba*

[Constituyentes químicos y actividad antioxidante de extractos obtenidos de corteza de ramas de *Bursera simaruba*]

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Abstract: The chemical constituents of the hexane and methanol extracts obtained from the branch bark of *Bursera simaruba* (Burseraceae) grown in Querétaro, Mexico, were investigated by GC-MS, HPLC coupled to DAD, and NMR techniques. Seventeen compounds, including terpenoids, flavonoids, phenolic acids, long-chain fatty acids (FA), methyl esters of FA and sucrose, were identified. In addition, an assessment of the antiradical activity of the methanol extract (ME) was also carried out using DPPH, ABTS, FRAP and DPV assays. The DPPH, ABTS and FRAP assays showed a low antioxidant capacity for the ME. This was in accordance with the relatively low quantities of phenols found in the extract. However, according to the differential pulse voltammetry assay (DVP), this extract exhibited an oxidation potential close to those of quercetin and (+)-catechin, two of the flavonoids with recognized good antioxidant power. This indicated that the ME does contain compounds with good antioxidant capacity and suggested that sometimes the most popular methods commonly used might be underestimating the true antioxidant capacities of plant samples and how the DPV is a valuable complementary tool to be taken into consideration when conducting these in vitro assays.

Keywords: *Bursera simaruba*, phenols, terpenes, sucrose, antioxidant activity, DPV.

Resumen: Los constituyentes químicos de los extractos hexánicos y metanólicos de la corteza de ramas de *Bursera simaruba* (Burseraceae) recolectada en Querétaro, México, fueron investigados mediante las Cromatografías de Gases acoplada a Espectrometría de masas (GC-MS) y de líquidos de alta resolución (HPLC) acoplada a un detector de arreglo de diodos (DAD) (HPLC-DAD) y mediante RMN. Diecisiete compuestos, incluyendo terpenos, flavonoides, ácidos fenólicos, ácidos grasos de cadena larga (AG), ésteres metílicos de AG y la sacarosa fueron identificados. De manera adicional, se determinó la actividad antioxidante del extracto metanólico utilizando los ensayos de DPPH, ABTS, FRAP y DPV (Voltametría de Pulso Diferencial). Los métodos de DPPH, ABTS y FRAP indicaron una baja capacidad antioxidante para este extracto. Esta observación estuvo de acuerdo con las bajas cantidades de fenólicos encontrados en este extracto. Sin embargo, mediante el método DPV, el extracto tuvo un potencial de oxidación cercano a los de la quercetina y la (+)-catequina, dos de los flavonoides con reconocida buena capacidad antioxidante. Estos resultados indican que el extracto sí contiene metabolitos secundarios con buena capacidad antioxidante y sugieren además que los métodos más comunes pueden subestimar la verdadera capacidad antioxidante de extractos de plantas y resalta la importancia del método DPV como complementario a tener en cuenta en estos tipos de estudios in vitro.

Palabras clave: *Bursera simaruba*, fenólicos, terpenos, sacarosa, actividad antioxidante, DPV

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INTRODUCTION

Mexico is one of the mega diverse countries in the world and both its rural and urban inhabitants make extensive use of medicinal plants. *Bursera simaruba* (L.) Sarg. is one of the plants widely distributed in our territory and several other Central American countries. In Mexico, it is disseminated throughout the whole country, growing on lands up to 1800 m above sea level and its different parts are employed for diverse medicinal purposes according to each particular locality. In the state of Querétaro (Mexico), where it is commonly known as “chaka”, its bark is traditionally used as an antipyretic and a nasal anti hemorrhagic agent, and to alleviate muscle pain. It is also used for the treatment of skin sores and ovary inflammation (Vázquez-Yanes *et al.*, 1999). There is evidence suggesting that free radicals and excited-state chemical species play a key role in inflammatory processes (Closa and Folch-Puy, 2004; Kao *et al.*, 2005; Kielland *et al.*, 2009; Reuter *et al.*, 2010). So, it is plausible to assert that antioxidants may prevent or suppress inflammation. Phenolics, the most widespread antioxidants contained in medicinal plants, have proven their antioxidant and/or anti-inflammatory activities (Scalbert *et al.*, 2005; Kao *et al.*, 2005; Yoshino *et al.*, 2006; Jensen *et al.*, 2008). These compounds, including lignans and proanthocyanidins, have been isolated (Maldini *et al.*, 2009a) or just identified in extracts obtained from the branch bark of *Bursera* (Maldini *et al.*, 2009b).

There are many other studies supporting the relationship between antioxidant and anti-inflammatory activities (Scalbert *et al.*, 2005; Jensen *et al.*, 2008). At least, these effects have been proven *in vivo* in a rat arthritis model (Yoshino *et al.*, 2006).

The anti-inflammatory activities of extracts obtained from the bark (Sosa *et al.*, 2002) and from the leaves (Noguera *et al.*, 2004; Carretero *et al.*, 2008) of *B. simaruba* collected elsewhere have been reported. These activities have been attributed both to triterpenes (Carretero *et al.*, 2008) and to the lignan methyl β -peltatin A (Noguera *et al.*, 2004).

As people commonly use medicinal plants as crude drugs, it is important to determine the chemical constituents of these preparations in order to assess their therapeutic potential and safety. Great attention has been paid in the last decade to the antioxidant properties of plant extracts, owing to the fact that many of the chronic illnesses (cancer, inflammation and arthritis, cardiovascular, Alzheimer, diabetes) are

related to the imbalance between endogenous antioxidant factors and oxidative chemical species produced inherently or ingested from food or from our environment (Willcox *et al.*, 2004; Soobrattee *et al.*, 2005). On the understanding that plant extracts having good *in vitro* antioxidant activity might also be helpful in the treatment or prevention of these illnesses, the present study was undertaken to investigate the chemical constituents and the *in vitro* antioxidant effects of the methanol and hexane extracts prepared from the branch bark of *B. simaruba* grown in Querétaro, Mexico.

MATERIALS AND METHODS

General

GC-MS analyses were performed on an Agilent chromatograph 6850 GC System Series coupled with Agilent 5973 Network Mass Selective Detector. Analytes were separated on a 30 m \times 0.25 mm nonpolar capillary column (HP-5MS) with a phase thickness of 0.25 μ m (5% phenylmethylsiloxane) and interfaced with a quadrupole mass spectrometer. The injector and interface temperatures were kept at 250 $^{\circ}$ C, and the column temperature was maintained at 70 $^{\circ}$ C from 0 to 5 min and then programmed from 70 $^{\circ}$ C to 300 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min. This highest temperature was maintained for the next 35 min. Helium was used as the carrier gas at the flow of 1 mL/min. Injection volume was 0.1 μ L of the sample dissolved either in CHCl_3 or in MeOH and using split mode (split ratio: 1:20). The MS operating parameters were: ionization voltage: 70 eV, scan range: 40-500 amu, run time: 60 min, solvent delay: 4 min. Identification of compounds was achieved by comparison of their mass spectra and those reported by the NIST98 MS SEARCH 2.0 library.

HPLC separations were conducted using a Waters apparatus (Millipore Corp., Waters Chromatography Division, Milford, MA, USA), composed of a 600E multisolvent delivery system and a 2998 PDA detector. Control of this equipment, data acquisition, processing, and management of the chromatographic information were performed by the Empower v2 software (Waters). NMR spectra were run on a Varian Inova NMR spectrometer equipped with 5 mm ^1H and ^{13}C probes and operating respectively at 500 and 125.7 MHz, with TMS as the internal standard.

Plant material

Branches of *B. simaruba* were collected at the locality of Jalpan de Serra, Querétaro, Mexico, on 13 November 2009. A voucher specimen (A. Cabrera 2183) has been deposited in the Ethnobotanical Collection of the Herbarium of Querétaro "Dr. Jerzy Rzedowski" (QMEX) located at the Faculty of Natural Sciences, Autonomous University of Querétaro, Mexico. The sample was dried in an oven set at 45 °C. Complete drying process needed three weeks. The bark was then detached and milled before extraction.

Extraction and fractionation

The milled dry plant material (2.092 kg) was successively extracted by maceration with hexane, methanol and 30% aqueous methanol (v/v) (8 L each solvent system). The extracts were evaporated until dry under reduced pressure. A portion of the methanol extract (42 g) was fractionated in an open silica gel column chromatography (CC) employing a gradient elution with hexane, ethyl acetate and MeOH. A total of 418 fractions (250 mL each) were collected and pooled according to their similarities into 21 fractions (I–XXI) that were then dried under reduced pressure. Fraction XIII eluted with EtOAc-MeOH (95:5) was mixed with H₂O and successively extracted with hexane, CHCl₃, EtOAc and *n*-BuOH. The alcoholic fraction (230 mg) was analysed by HPLC and some of its compounds purified by semi-preparative RP-HPLC [column YMC ODS AQTM, S5 µm, 12 nm, 250 × 20 mm id; MeOH-H₂O (25:75) as the mobile phase at the flow rate of 4 mL/min; injection volumes 500 µL; detection at 254 nm]. Five compounds were collected and their ¹H NMR spectra recorded. Fraction XVIII eluted with MeOH was subjected to preparative TLC (MeOH-CH₂Cl₂ 8:2), affording 10.6 mg of a white amorphous powder whose ¹H and ¹³C NMR data were recorded in CD₃OD. Finally, fractions III and V were analyzed by GC-MS and many of their components identified.

Acid and base hydrolyses of Methanol and aqueous extracts

Five mL of 2M HCl or 2N NaOH were added to a solution of one hundred mg of extract dissolved in 10 mL of MeOH or water (HPLC grade). The dissolution was subjected to reflux for two hours. Twenty µL of the hydrolyzed mixture or diluted preparations were then analyzed by reversed phase HPLC for identification and quantification of the phenolic aglycones.

HPLC determination of phenolics

Five phenolic acids (gallic, protocatechuic, caffeic, *p*-coumaric, and rosmarinic) and ten flavonoids (hesperidin, rutin, myricetin, luteolin, (-)-epicatechin, quercetin, apigenin, naringenin, hesperetin, and kaempferol) available in our files were used as standards. The following analytical conditions were employed: column: Symmetry C-18 (5 µm, 250 x 4.6 mm i.d.); mobile phase: linear gradient of CH₃CN (eluent A) and 0.0125 N aqueous-acetic acid (eluent B), starting from 95% A and reaching 50% in 20 min and then returning to 95% from 20 min to 25 min, composition which was then maintained until 35 min; the flow rate was 1 mL/min; peaks were detected at 280 nm and the injection volume was 20 µL. The detection was made at 280 nm.

Antioxidant activity of the original extracts

DPPH method

Antiradical activity (ARA) was determined using the stable radical DPPH, according to the method reported by Fukumoto & Mazza (2000). All reactions were conducted in 96 well micro plates. A twenty µL aliquot of a methanol solution of the extracts at various concentrations (2.2, 2.5, 2.8, 3.1, 3.4, 3.7 mg/mL) was mixed with 200 µL of 150 µM of DPPH in 80 % methanol. The absorbance was recorded at 520 nm in a Spectra Max Tunable Micro plate Reader (Molecular Devices Co., Sunnyvale, CA, U.S.A.). The percentage of absorbance inhibition was calculated according to Fukumoto and Mazza (2000).

ABTS method

The estimation of the antiradical activity (ARA) was performed using the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay described by Nenadis *et al.* (2004). The method was modified to be conducted in 96-well microplates (Nalge Nunc International, NY, USA). Briefly, a 20 µL aliquot of the extracts (2.2, 2.5, 2.8, 3.1, 3.4, 3.7 mg/mL) were mixed with 230 µL of previously prepared ABTS^{•+} solution. The absorbance was recorded at 730 nm at 0 and 6 minutes in a Spectra Max Tunable Microplate Reader (Molecular Devices Co., Sunnyvale, CA, USA). The radical scavenging activities for the DPPH and ABTS results were expressed as the median inhibitory concentration (IC₅₀) values. The IC₅₀ was calculated from the log-dose inhibition curve obtained by a nonlinear regression algorithm (Prism, 4.0, GraphPad).

Ferric reducing antioxidant power (FRAP)

FRAP values were obtained according to the method reported by Firuzi *et al.* (2005). Briefly, 75 μ L of sample dissolved in methanol (2.5 mg/mL) were placed in a 96-well microplate (Nalge Nunc International, NY, USA). Then 175 μ L of freshly prepared and warm (37 °C) FRAP solution were added. The absorbances at 595 nm were monitored by a Spectra Max tunable micro plate reader (Molecular Devices Co., Sunnyvale, CA, USA). The results were expressed as mM of FeSO₄ equivalents (mM eq FeSO₄) at 30 min. All data were reported as means \pm standard error.

Electrochemical experiments

The electrochemical experiments were performed using a BAS-Epsilon Potentiostat/Galvanostat coupled to a C3-BAS Cell Stand. The differential pulse voltammetry (DPV) experiments were performed in deoxygenated solutions by previously bubbling pure nitrogen gas for 20 min and using an electrochemical 10 mL cell in which a Pt counter electrode, a Ag/AgCl 3 M NaCl reference electrode and a glassy carbon working electrode (0.08 cm²) were properly fitted. Before each experiment was carried out, the working electrode was polished using slurry of 0.05 μ m alumina and a Milli-Q system purified water on a felt surface. In a typical DPV experiment, 30 μ L of methanol solution (10 mg extract dissolved in 500 μ L MeOH) were added to 2970 μ L of Sørensen buffer (pH = 7.0). Parameters for DPV were 70 mV pulse amplitude and 5 mV/s.

RESULTS AND DISCUSSION

Chemical study

Chemical study of the most abundant fraction XIII (eluted with EtOAc-MeOH 95:5) obtained from successive CC of the ME, followed by reversed phase semi preparative HPLC resulted in the isolation of five compounds, those ¹H NMR spectra showed almost all

their signals in the region between δ 3.2 and 4.2, which is characteristic of carbohydrates (Bah and Pereda-Miranda, 1996). No more spectral studies were conducted on these components, since carbohydrates were not our main targeted compounds. Analytical and preparative TLC of the quite distant fraction XVIII led to the purification of another carbohydrate whose chemical structure was established as sucrose on the basis of its NMR spectral data analysis (¹H and ¹³C, and ¹H-¹H and ¹H-¹³C NMR correlations). Although spectral data of this disaccharide are already reported in C₅D₅N, DMSO-d₆ and D₂O (Mehta *et al.*, 2008; Zhao *et al.*, 2008), we report here those now recorded in CD₃OD and slightly different.

Sucrose: ¹H NMR (500 MHz, CD₃OD, ppm; Glu = α -D-glucopyranosyl and Fru = Fructofuranosyl): 5.38 (d, *J* = 3.5 Hz, Glu-1), 4.09 (d, *J* = 8.0 Hz, Fru-3), 4.02 (dd, *J* = 12.5 Hz and 8.0 Hz, Fru-4), 3.82 (overlapped m, Glu-5), 3.80 (dd, *J* = 12.0 HZ and 2.0 Hz, Glu-6a), 3.78 (m, Fru-6a), 3.77 (m, Fru-5), 3.75 (m, Fru-6b), 3.72 (dd, *J* = 2.5 Hz and 2.5 Hz, Glu-6b), 3.71 (dd, *J* = 9.5 Hz and 9.5 Hz, Glu-3), 3.63 (d, *J* 12.5 Hz, Fru-1a), 3.60 (d, *J* = 12.5 Hz, Fru-1b), 3.42 (dd, *J* = 3.5 Hz and 9.5 Hz, Glu-2). ¹³C NMR (125 MHz, CD₃OD, ppm): 105.3 (Fru-2), 93.6 (Glu-1), 83.8 (Fru-5), 79.4 (Fru-3), 75.8 (Fru-4), 74.7 (Glu-3), 74.4 (Glu-5), 73.2 (Glu-2), 71.4 (Glu-4), 64.1 (Fru-1), 63.4 (Fru-6), 62.2 (Glu-6).

HPLC and GC-MS analysis of the extracts

On account of the poor results obtained in the purification processes, two hyphenated analytical techniques involving HPLC-DAD and GC-MS were used to identify at least more chemical constituents of the extracts. By the first method, aqueous-ME and ME gave on superimposition nearly the same chromatogram (Figure N° 1).

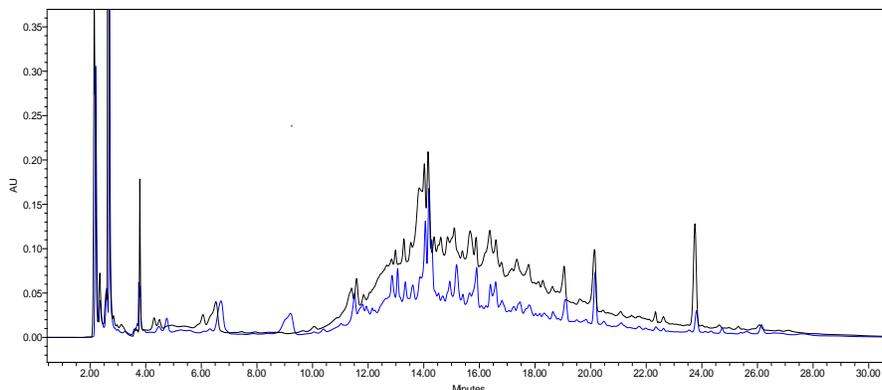
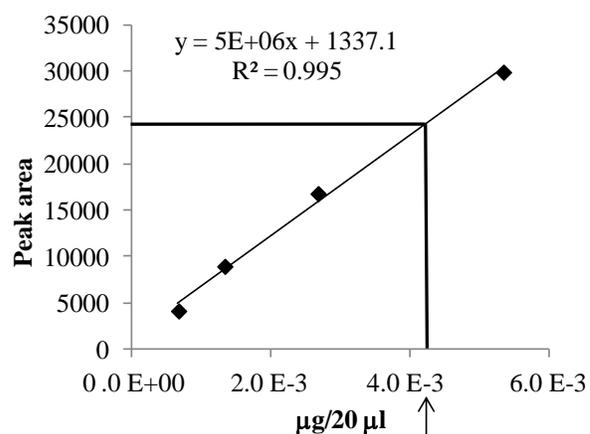


Figure 1
Comparative chromatograms of the methanol (black) and aqueous-methanol (blue) extracts (for HPLC analytical conditions, see text).

So, the presence of the above mentioned phenolic acids and flavonoids was investigated in the non-hydrolyzed and in the acid and base-hydrolyzed MEs. Retention times (Rt), co-chromatography and UV spectra were used to assess the correct identification of the phenolic compounds.

Of all the phenolic standards investigated, only six were detected. Densitograms were used to quantify these phenolics. As an example, Figure 2 shows the densitogram used for quantification of luteolin. Protocatechuic acid (Rt 11.53 min) and gallic acid (Rt 5.94 min) were found in their free forms in the non-hydrolyzed methanol extract, with concentrations of 32 mg/g and 0.58 mg/g dry plant material (DPM), respectively. In the acid-hydrolyzed ME, only (–)-epicatechin was detected, though in such a low quantity that it could not be quantified. However, this low yield is not so surprising, as this compound is the building block of a considerable amount of condensed tannins (proanthocyanidins) recently determined by LC-ESI-MS in the bark of this species (Maldini *et al.*, 2009b). Also, low quantity of apigenin (0.42 mg/g DPM) and even very low amounts of luteolin and kaempferol were present in the acid-hydrolyzed ME, indicating that these three flavonoids were present only in their glycoside forms.



Experimental concentration found (0.0973 µg/20 µl)

| Luteolin | |
|--------------------------|-----------|
| Concentration (µg/ 20µL) | Peak area |
| 6.67E-04 | 4134 |
| 1.33E-03 | 8953 |
| 2.67E-03 | 16816 |
| 5.33E-03 | 29947 |
| 4.3E-03 (ME) | 23882 |

Figure 2
Main fragments and structures of some of the compounds identified.

GC-MS analysis of the less polar fractions (III and V) led to the identification of long-chain fatty acids (FAs), methyl esters of FAs and three triterpenes. Identification of these compounds was assessed by comparison of their fragmentation pattern and data reported in the NIST library. The structures of some of the compounds identified and the main experimental fragments observed in their mass spectra are indicated in Figure 3.

A glucopyranoside of β -sitosterol has been previously described in the leaves of *B. graveolens* (Nakanishi *et al.*, 2003), while the corresponding

aglycone has been identified in the leaves of *Bursera simaruba* (Robles-Camargo, 2000). According to a literature review, this is the first report of occurrence of phytol, ursa-9(11)-12-dien-3- β -ol, and 24(28)-methylenecycloartanol (also known as 24-methylen-9,19-cyclolanostan-3- β -ol) in the genus *Bursera*. Other compounds identified were *n*-hexadecanoic acid, 9*Z*,12*Z*-octadecadienoic acid, 2*E*,4*E*-decadienal, methyl esters of hexacosanoic, octacosanoic and triacontanoic acids.

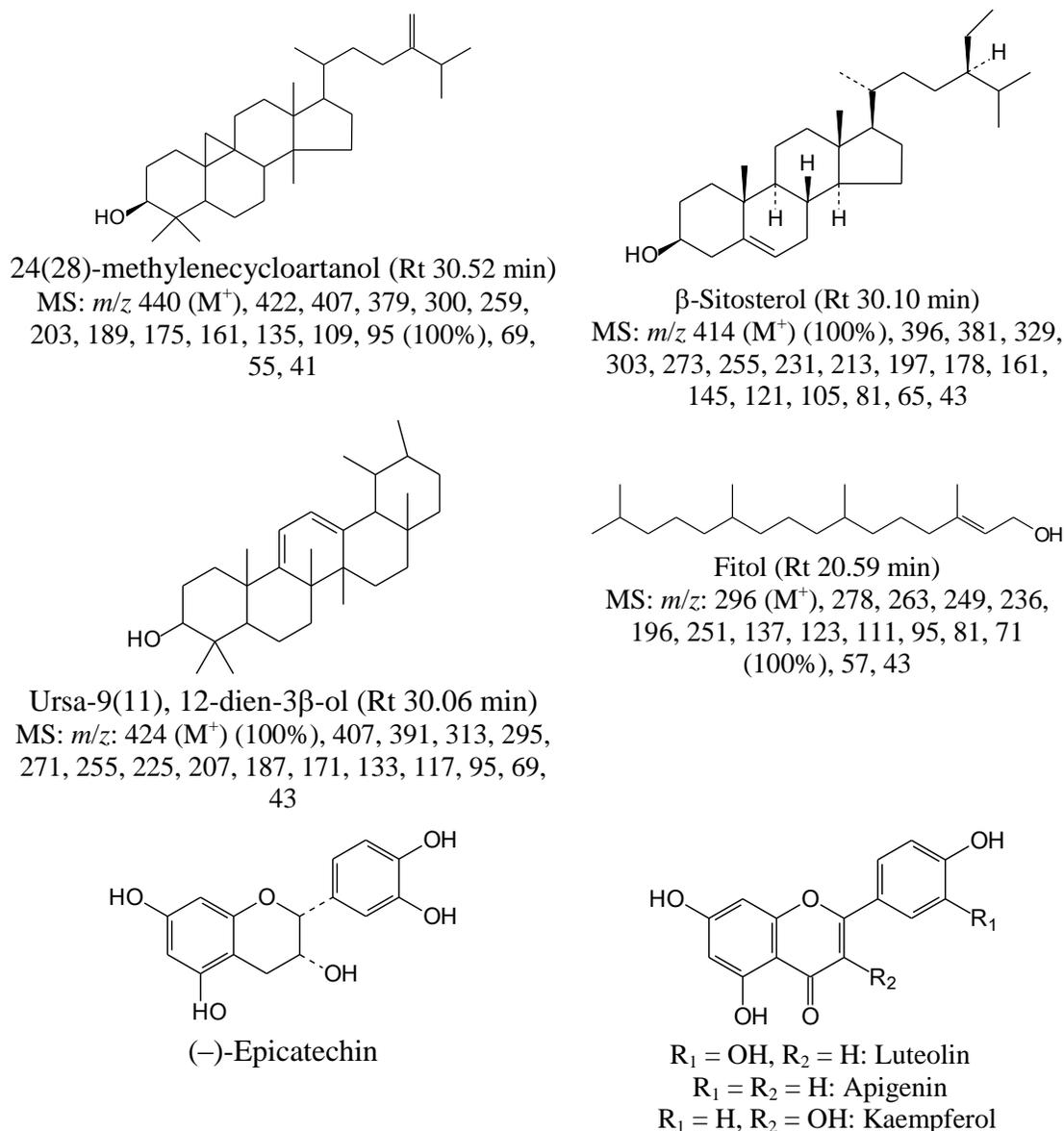


Figure 3

Densitogram obtained during quantification of luteolin and showing concentration found on extrapolation.

Some of the compounds identified in this work are frequently found in fruits, vegetables and cereals of the human diet and in medicinal plants, and have important biological activities. Literature is full of pharmacological studies which associate polyphenols with many beneficial effects on health. For example, anti-invasive activity (Kaur *et al.*, 2009) and antiinflammototy effect in mice (Kroes *et al.*, 1992; Pal *et al.*, 2010) have been recognized for gallic acid, while protocatechuic acid inhibits cancer cell metastasis (Hui-Hsuan *et al.*, 2011). Also, many terpenoids show anti-inflammatory activity (Singh *et al.*, 2002; de las Heras *et al.*, 2009; Shuang *et al.*, 2010; Zhang *et al.*, 2012).

Antioxidant activity

Several *in vitro* methodologies have been proposed to determine the antioxidant activity of food and plant extracts, among these DPPH, FRAP, ABTS, CUPRAC and TBARS assays (Roginsky and Lissi, 2005). The free radical scavenging properties of the hexane and methanol extracts of *B. simaruba* were determined by the DPPH and ABTS assays, where both radicals are reduced by the antioxidant compounds to their stable derivatives. The hexane extract produced negligible reducing power in any of the assays. ME was capable of scavenging DPPH and ABTS radicals in a concentration dependent fashion, with an IC_{50} of 2.117 mg/mL and 2.574 mg/mL, respectively. These values indicated poor antioxidant capacity if compared with that of Trolox (DPPH, $IC_{50} = 0.506 \pm 0.008$ mg/mL, ABTS, $IC_{50} = 0.525 \pm 0.01$ mg/mL). The FRAP assay was performed to evaluate the total antioxidant activity through the capacity of the extract to reduce Fe^{3+} to Fe^{2+} ions. At a concentration of 2.5 mg/mL, the extract exhibited a FRAP value of 44.93 mM eq of $FeSO_4$, demonstrating again a poor antioxidant capacity compared with a 39 μ g/mL solution of Trolox that exhibited 94.85 mM eq of $FeSO_4$ (Moreno-Escobar *et al.*, 2011). The low quantities of phenolic compounds in the ME might correlate with the poor antioxidant capacities observed in these assays. However, solubility restrictions, suitability of the positive controls and the response of the radicals may account for underestimation of the antioxidant content. Due to the complexity of the composition of food,

plants and biological samples, and more importantly the synergic and antagonism effects derived from the components involved in the target extract, analytical strategies based on development of screening methods for fast and reliable estimation of antioxidant activity have been proposed. In this way, Blasco *et al.*, (2004) have introduced the "Electrochemical Index" (EI) concept, defined as the total amount of antioxidants obtained by selective oxidation detecting antioxidant substances that can be selectively quantified through tunable working potentials. For electrochemical studies, two sets of parameters may be obtained: a) the biological oxidation potentials, which reflect the specific reducing power of a component or components with similar potential, and b) the intensity of the current which is correlated to the concentration of the components (Chevion *et al.*, 2000). One of the most used electrochemical techniques to evaluate the antioxidant activity of natural extracts or complex biological samples is the differential pulse voltammetry (DPV). Its inherent analytical potential is based on the fact that the oxidation potential is conceptually correlated with the antioxidant capacity. It would be expected that the lower the oxidation potential of a target sample extract, the stronger the antioxidant capacity. In this study, the DPV response of the extracts of *B. simaruba* on glassy carbon was obtained at pH 7. The ME revealed two oxidation signals at + 0.159 V and + 0.579 V vs Ag/AgCl (Figure N° 4). This result indicated that despite the poor radical scavenging properties exhibited by the ME through the DPPH, ABTS and FRAP assays, it does contain electro active compounds that exhibit oxidation potentials close to those of quercetin (0.090V) and (+)-catechin (0.148V), flavonoids with good antioxidant capacity (Blasco *et al.*, 2004). So, the integrated antioxidant capacity derived from the voltammograms recorded at a glassy carbon electrode of the ME which includes the contribution of low molecular weight antioxidants revealed that at physiological pH, there are compounds that exhibited low oxidation potential, therefore high antioxidant properties. This may account, at least in part, for the sustained use of the bark of this plant species in the treatment of ovary inflammation.

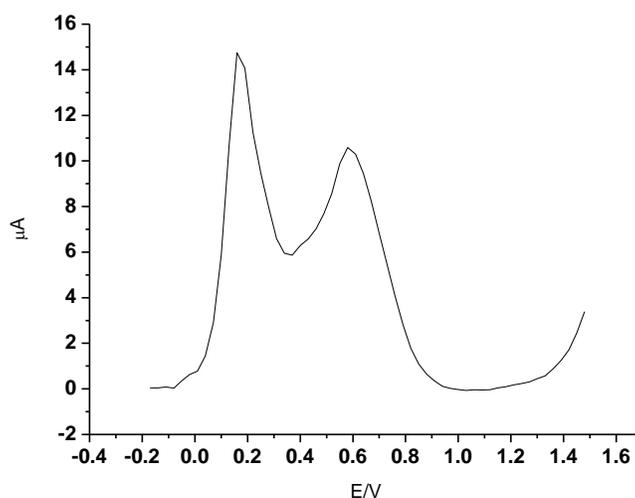


Figure 4

Differential pulse voltammogram of *B. simaruba* methanol extract in Sørensen buffer at pH = 7 at a glassy carbon electrode vs Ag/AgCl, scan rate: 5 mV/s.

CONCLUSIONS

The chemical constituents of the methanol and hexane extracts of the branch bark of *B. simaruba* consisted mainly of carbohydrates and triterpenes. Besides the phenols identified in this study, others non-identified and the terpenoids might contribute to the anti-inflammatory properties of this plant species and in that way, support its ethno medical use. The electrochemical results suggest the use of this simple, cheap and reliable tool to determine the true antioxidant capacities of extracts, and in connection with well-established separation techniques to isolate the antioxidant components.

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