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Residues from the Brazilian pepper tree (*Schinus terebinthifolia* Raddi) processing industry: Chemical profile and antimicrobial activity of extracts against hospital bacteria

Rodrigo Borges de Araujo Gomes[a,b,*], Erica Santana de Souza[c], Nataly Senna Gerhardt Barraqui[d], Cristina Luz Tosta[a], Ana Paula Ferreira Nunes[c], Ricardo Pinto Schuenck[c], Fabiana Gomes Ruas[e], José Aires Ventura[e], Paulo Roberto Filgueiras[a], Ricardo Machado Kuster[a,*]

[a] Laboratório de Cromatografia, Departamento de Química, Universidade Federal do Espírito Santo, Vitória, ES, Brazil
[b] Instituto Federal do Espírito Santo – IFES, Aracruz, ES, Brazil
[c] Programa de Pós-Graduação em Doenças Infecciosas, Universidade Federal do Espírito Santo, Vitória, ES, Brazil
[d] Programa de Pós-Graduação em Biologia Vegetal, Universidade Federal do Espírito Santo, Vitória, ES, Brazil
[e] Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural – Incaper, Vitória, ES, Brazil

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ABSTRACT

Schinus terebinthifolia Raddi is a plant used in folk medicine in the treatment of various diseases and has several biological potentials. Its fruit is used as condiment and has high demand in the spice market. In the present study extracts of different polarities prepared from residues from the Brazilian pepper tree processing industry were characterized chemically by gas chromatography/mass spectrometry (GC–MS) and negative-ion mode electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI(-)FT-ICR MS). The antibacterial activity of the extracts was evaluated against multidrug-resistant strains of hospital origin (*Staphylococcus aureus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and standard strains (ATCC). The apolar fractions (dichloromethane and hexane) presented triterpenes as main components and the polar extracts (methanol and hydroethanolic extracts) were characterized by high contents of phenolic compounds, especially gallotannins, gallic acid and flavonoids. The methanolic fraction and the hydroethanolic extract of the residues were the most active mainly against *S. aureus* (MIC 0.60–0.90 mg/mL), *E. faecium* and *E. faecalis* (MIC 1.20–2.10 mg/mL). These results demonstrate the richness of bioactive compounds present in the residues and indicate a possible application of this material for the development of biotechnological products with potential against multidrug-resistant bacteria.

1. Introduction

Schinus terebinthifolia Raddi (Syn.: *Schinus terebinthifolius* Raddi) (Anacardiaceae), is known as Brazilian pepper tree or aroeira. It is a species widely distributed along the Brazilian coast (Morton, 1978; Carvalho et al., 2013). Its fruits are used as condiment and have high demand in the international market. In Brazil a large part of the fruit production originates from the extractive exploitation, mainly in Espírito Santo and Rio de Janeiro states. Currently, some producers in the state of Espírito Santo have been growing aroeira (rose pepper) for the international market due to high prices reached in this agribusiness (Neves et al., 2016). Therefore, Espírito Santo has become one of the most important Brazilian state for that agricultural production. In addition to being used in food industry, *S. terebinthifolia* is also widely used in folk medicine due to its different biological activities, such as anti-allergic (Cavalher-Machado et al., 2008), anti-inflammatory (Rosas et al., 2015; da Silva et al., 2017b) and antimicrobial (Gomes et al., 2013; Muhs et al., 2017; da Silva et al., 2017a). Phytochemical studies have described the presence of gallic acid, methyl gallate, ethyl gallate, flavonoids myricitrin, myricetin and quercitrin (Ceruks et al., 2007; Santana et al., 2012), anthocyanins, bioflavonoids, hydrolyzable tannins such as galloylglucose and galloyl shikimic acids (Feuereisen et al., 2014, 2017). This species is present in the Phytotherapeutic Form of the Brazilian Pharmacopoeia (2011) (Anvisa, 2011) and in the list of phytotherapics of the National Relation of Essential Medicines (Brazilian Ministry of Health, 2017).

* Corresponding authors at: Laboratório de Cromatografia, Departamento de Química, Universidade Federal do Espírito Santo, Vitória, ES, Brazil.
E-mail addresses: rodrigo.gomes@ifes.edu.br, rodrigobagomes@hotmail.com (R.B.d.A. Gomes), kusterrm@gmail.com (R.M. Kuster).

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Bacterial resistance is considered a global public health problem. Infections caused by bacteria resistant to antibiotics are associated with excess mortality, prolonged hospitalization and increased hospital costs (Cosgrove, 2006; de Kraker et al., 2011). Therefore, control initiatives have been proposed in the attempt to combat infections caused by resistant microorganisms. In 2017 the World Health Organization released a list of priority pathogens for research and development of new antibiotics. Among them are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* resistant to carbapenem (WHO, 2017). This information demonstrates the importance and the concern with the development of research on active agents against the strains of these microorganisms. Considering the potential of *S. terebinthifolia* Raddi as an antimicrobial plant of popular use, the high availability of raw material (residues) and the need for research involving these pathogens, this work aimed to characterize the residues from the Brazilian pepper tree processing industry and to evaluate its activity against multidrug-resistant bacteria.

2. Material and methods

2.1. Plant material

The residues were collected in an industry located in the municipality of Boa Esperança - ES, which receives fruits produced in the south and in the north of Espírito Santo under cultivation guidance by the Capixaba Institute of Research, Technical Assistance and Rural Extension (Incaper). This material is composed mostly of leaves and minority of unusable fruits. Another material composed exclusively of the fruit peels discarded in the process was also collected for the preparation of extracts.

2.2. Preparation of the extracts

2.2.1. Extraction of Brazilian pepper tree residues (leaves and fruits)

The extracts were prepared by subjecting the sample (50 g) to the organic solvents hexane (HF) (3 × 130 mL), dichloromethane (DF) (3 × 130 mL) and methanol (MF) (3 × 130 mL) successively. At each extraction, in the maceration mode, the solvent remained in contact with the sample for 24 h at room temperature and then it was percolated through the plant. Similarly, a new amount of sample (50 g) was subjected to extraction but using a 54% (v/v) hydroethanolic solution (HEE) as the extracting solvent. After being prepared, all extracts were dried in the rotary evaporator.

2.2.2. Extraction of Brazilian pepper tree fruit peels

The fruit peels that are discarded in the beneficiation process were weighed (50 g) and subjected to percolation with a 54% (v/v) hydroethanolic solution (FPE) (3 × 130 mL) at room temperature. After preparation, the extract was also dried in rotary evaporator.

2.3. Esterification reaction - dichloromethane fraction

The dichloromethane extract (0.8 g) was refluxed with 10 mL of 0.5 mol L^{-1} KOH solution for 10 min. Then the reactional medium was cooled and 5 mL of a methanolic solution of BF_3 were added to it and a new refluxing procedure was carried out for another 10 min. After cooling, the contents were transferred to a separatory funnel and partitioned between hexane and a saturated NaCl solution. The organic phase was collected, filtered and the solvent evaporated to give the esterified dichloromethane fraction.

2.4. Gas chromatography/mass spectrometry (GC/MS) analysis

The hexane, dichloromethane and esterified dichloromethane fractions were analyzed by gas chromatography coupled to mass spectrometry (GC–MS) on an Agilent 7890B (Agilent, California, USA) equipped with an HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm) and an Agilent 5977A MSD mass detector. The initial column temperature was set at 40 °C, remaining for 1 min. Thereafter, a heating rate of 5 °C/min was used up to 240 °C, increased to 310 °C at 30 °C/min and maintained for 20 min. The total analysis time was 63 min. The carrier gas was helium at a flow rate of 1 mL/min. Injector and detector temperatures were set at 290 °C and 310 °C, respectively. The MS was operated in electron impact ionization mode (70 eV, 50–500 Da). The samples were diluted in hexane (5.8 mg/mL) and dichloromethane (10 mg/mL) and the injected volume was 1.0 µL with the injector in split mode 1:10. A series of *n*-alkanes was analyzed under the same chromatographic conditions for the calculation of the retention index of the substances. The main chemical constituents of the samples were proposed by comparing the mass spectra with the NIST library data base, afterwards by comparing the retention indices to the literature (Adams, 2009; The Pherobase-Database, 2018; Chemistry WebBook, 2018).

2.5. ESI(-)FT-ICR MS and ESI(-)FT-ICR MS/MS analyses

The methanolic, dichloromethane and hydroethanolic extracts were analyzed by Fourier Transform Ion Cyclotron Mass Spectrometry (FT-ICR MS) to determine the chemical profile. The samples were solubilized (1 mg/mL) in a solution of acetonitrile:water (1:1) which was infused at a rate of 5 µL/min in the negative mode operated electrospray (ESI) source. The mass spectrometer (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany) was set to operate over a range of *m/z* 150–1500. Among the ESI source conditions used in the analyzes are the nebulizer gas pressure of 1.0 bar, the capillary voltage of 3.8 kV and the capillary transfer temperature of 200 °C. In addition, the accumulation time of the ions was 0.010 s and each spectrum was acquired by accumulating 32 scans of time-domain transient signals in 16 mega-point time-domain data sets. The tandem mass spectrometry (MS^2) experiments were performed on a quadrupole analyzer coupled to the FT-ICR mass spectrometer. The MS^2 spectra were acquired using the ion accumulation time of 1 s, isolation window of 1.0 (*m/z* units) and 25–45% of the collision energy. The mass spectra were acquired and processed using Data Analysis software (Bruker Daltonics, Bremen, Germany).

2.6. Antimicrobial activity

2.6.1. Microorganism strains

The antimicrobial activity was evaluated against 15 clinical bacterial strains isolated from different hospitals of Espírito Santo, Brazil: 5 strains of MRSA, 5 strains of VRE (2 strains of *E. faecium* and 3 strains of *E. faecalis*), 5 strains of carbapenemase-producers non-fermenting Gram-negative bacilli (2 strains of *A. baumannii* and 3 strains of *P. aeruginosa*). The following reference strains from American Type Culture Collection (ATCC) were also evaluated: *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *A. baumannii* (ATCC 19606) and *P. aeruginosa* (ATCC 27853). The Tables 1 and 2 of the Supporting Information showed the antimicrobial susceptibility profile and genes of antimicrobial resistance of clinical strains.

2.6.2. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were determined by the microdilution method according to the methodology proposed by Clinical and Laboratory Standards Institute (CLSI, 2017) using Mueller-Hinton broth (MHb) as culture medium and dimethylsulfoxide (DMSO) as emulsifier. The bacterial strains from cultures grown in non-selective solid medium for 20–24 h of incubation at 37 °C were suspended in 0.85% saline and adjusted to the standard of 0.5 of the McFarland scale. In the microplate with 96 wells, each well was filled with 180 µL of solution of each

Table 1
Chemical composition of the hexane fraction of residues.

No.	Retention time (min)	Compound	Area (%)	RI[a]
		Monoterpenes		
1	9.359	α-pinene	0.96	928
2	11.621	3-carene	1.65	1004
3	12.047	*p*-cymene	3.06	1018
4	12.182	limonene	1.92	1023
5	16.976	α-terpineol	0.19	1185
6	20.022	thymol	0.20	1295
		Sesquiterpenes		
7	21.039	δ-elemene	0.22	1333
8	22.076	α-copaene	0.34	1373
9	22.466	β-elemene	0.95	1388
10	23.218	β-caryophyllene	2.08	1417
11	23.503	γ-elemene	0.24	1429
12	23.706	aromadendrene	0.32	1437
13	24.064	humulene	0.14	1452
14	24.593	γ-muurolene	0.64	1473
15	24.743	germacrene D	0.23	1479
16	24.878	β-selinene	0.85	1484
17	25.086	α-selinene	1.07	1493
18	25.164	α-muurolene	0.23	1496
19	25.517	γ-cadinene	0.39	1511
20	25.709	δ-cadinene	0.88	1519
21	26.310	elemol	0.23	1545
22	27.032	spathulenol	2.22	1576
23	27.187	caryophyllene oxide	1.66	1582
24	27.743	rosifoliol	0.50	1606
25	28.256	γ-eudesmol	0.32	1629
26	28.438	α-muurolol	0.42	1638
27	28.687	β-eudesmol	0.75	1649
28	28.754	α-eudesmol	1.36	1652
		Triterpenes/Steroids/Others		
29	37.736	phytol	0.54	2102
30	44.803	squalene	9.69	2816
31	45.145	octacosane	8.20	2874
32	46.889	α-tocoferol	4.93	3136
33	48.814	β-sitosterol	3.63	3346
34	49.078	olean-12-en-3-one	1.82	3373
35	49.416	28-norolean-17-en-3-one	10.69	3405
36	49.592	lupenone	12.75	3417
37	49.867	lupeol	16.32	3437
38	51.097	lupeol acetate	4.10	3524
39	52.041	ursonic aldehyde 3-acetate	2.19	3592

[a] Retention index obtained with reference to a standard of *n*-alkanes using an HP-5MS column.

Table 2
Main compounds of the esterified dichloromethane fraction.

No.	Retention time (min)	Area (%)	Compound	RI[a]
1	30.352	8.74	methyl tetradecanoate	1725
2	34.472	26.51	methyl hexadecanoate	1928
3	37.658	7.69	methyl linoleate	2098
4	37.783	31.21	methyl linolenate	2104
5	38.218	4.60	methyl stearate	2129

[a] Retention index obtained with reference to a standard of *n*-alkanes using an HP-5MS column.

extract at concentrations raging from between 0.30 and 2.70 mg/mL and 20 µL of bacterial suspension. The results were based on the visual growth of the bacterial after 24 h of incubation at 37 °C, and confirmed with the addition of 20 µL of resazurin solution (0.015% w/v) after more 3 h of incubation at 37 °C. MIC was defined as the minimum extract concentration capable of inhibiting the visible growth of cells. Five microliters of solution from each well where no growth was observed were applied to plates containing nutrient agar. The plates were then incubated in a bacteriological oven for 24 h. MBC was considered the lowest concentration where bacterial growth was not observed. The experiments were performed in triplicate.

3. Results and discussion

3.1. Extraction yields

Yields for HEE, MF, DF and HF were 23.62% 22.94%, 2.55% and 4.39%, respectively. A higher extraction efficiency was observed when polar solvents were used. These results agree with the findings of Scheid et al. (2018) that reported higher yield of the polar extract (methanol) of the leaves when compared with hexane and dichloromethane fractions. Braga et al. (2007) and Barbieri et al. (2014) obtained yields of 26.67% and 24.31%, respectively, for the methanol extracts of the leaves of *S. terebinthifolia*. The FPE was the one that presented the highest yield (32.4%) and is close to the results obtained by Muhs et al. (2017) who obtained 28.46% for the methanol extract of fruits.

3.2. Gas chromatography/mass spectrometry (GC/MS) analysis

3.2.1. Hexane fraction

The chromatographic profile of the hexane fraction is shown in Fig. 1.

It was possible to identify 39 substances (Table 1). The pentacyclic triterpenes lupeol (16.32%), lupenone (12.75%) and 28-norolean-17-en-3-one (10.69%) as well as their precursor squalene (9.69%) are among the major compounds accounting for 49.45% of the total area of the chromatogram. Lupeol is a triterpene found in various fruits and vegetables such as olives, mango and fig, and in many medicinal herbs (Saleem et al., 2004). This substance was also identified in a hexane extract of *Schinus molle* L. together with lupenone, which was the main component (Batista et al., 2016). The literature describes the presence of triterpene 28-norolean-17-en-3-one in plants of the genus *Pistacia*, which belongs to the same family as *S. terebinthifolia* (Anacardiaceae) (Stern et al., 2003; Vuorinen et al. (2015)), being one of the main triterpene found in samples of *Pistacia lentiscus* (Assimopoulou and Papageorgiou, 2005).

As the residues are fragmented and exposed to the environment, smaller peaks related to monoterpenes (7.98%) and sesquiterpenes (16.06%) were expected due to the volatilization of these compounds. However, it was possible to identify some terpenes produced by this species such as the monoterpenes *p*-cymene (3.06%), limonene (1.92%), 3-carene (1.65%), α-pinene (0.96%) and the sesquiterpenes spatulenol (2.22%), β-caryophyllene (2.08%), caryophyllene oxide (1.66%) and α-eudesmol (1.36%). These substances were described as components of the essential oil of *S. terebinthifolia* (Lloyd et al., 1977; Bendaoud et al., 2010; Carvalho et al., 2013; Ennigrou et al., 2017). It has been reported that essential oils obtained from leaves of *S. terebinthifolia*, collected at different sites, have monoterpenes in their composition, mainly α-pinene, limonene and *p*-cymene (Barbosa et al., 2007). Regarding the volatile compounds of the green and mature fruits of this plant Schimitberger et al. (2018) identified α-pinene, δ-3-carene and limonene as the predominant substances and proposed them as chemical markers of the species.

3.2.2. Dichloromethane and dichloromethane esterified fractions

The GC–MS chromatogram of the dichloromethane fraction shows the predominance of peaks in the triterpene region between 45 and 54 min (Fig. 2A). The presence of the steroid β-sitosterol (11.29%) together with 28-norolean-17-en-3-one (29.18%), lupenone (8.04%) and lupeol (9.08%) were observed in the dichloromethane fraction. Regarding the esterified dichloromethane fraction (Fig. 2B), a significant difference between the both chromatographic profiles could be verified. In the esterified sample, intense peaks related to fatty acid methyl esters appear in the region between 30 and 38 min, with a decrease of the peaks of the triterpenes. The presence of these signals demonstrates the high concentration of fatty acids in comparison with triterpenes and indicates a satisfactory esterification process was, allowing the identification of these acids in the form of their methyl esters (Table 2).

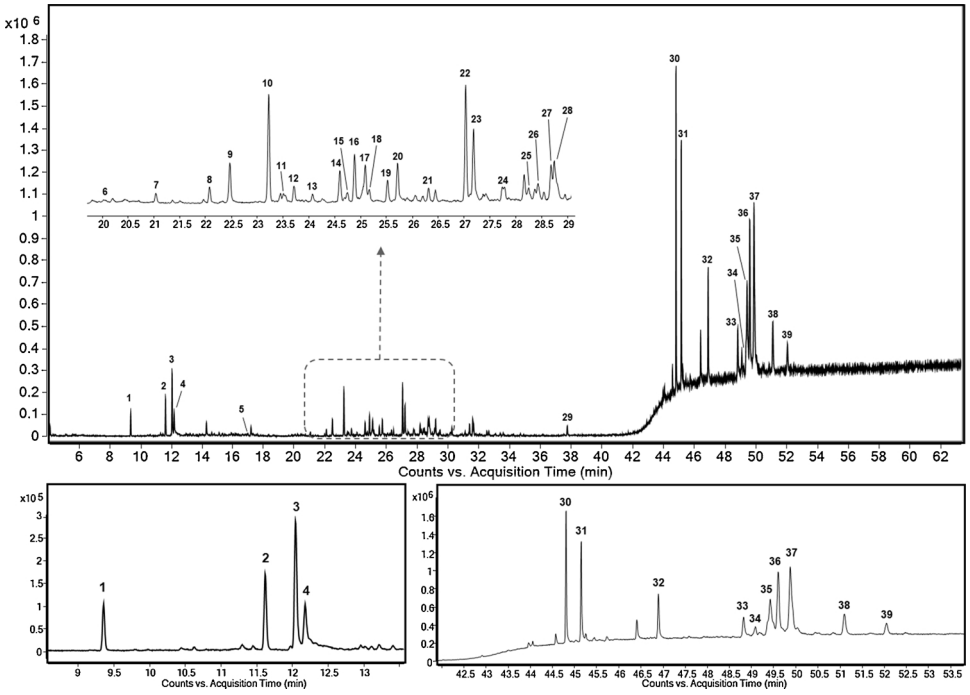

Fig. 1. Chromatogram of the hexane fraction of residues.

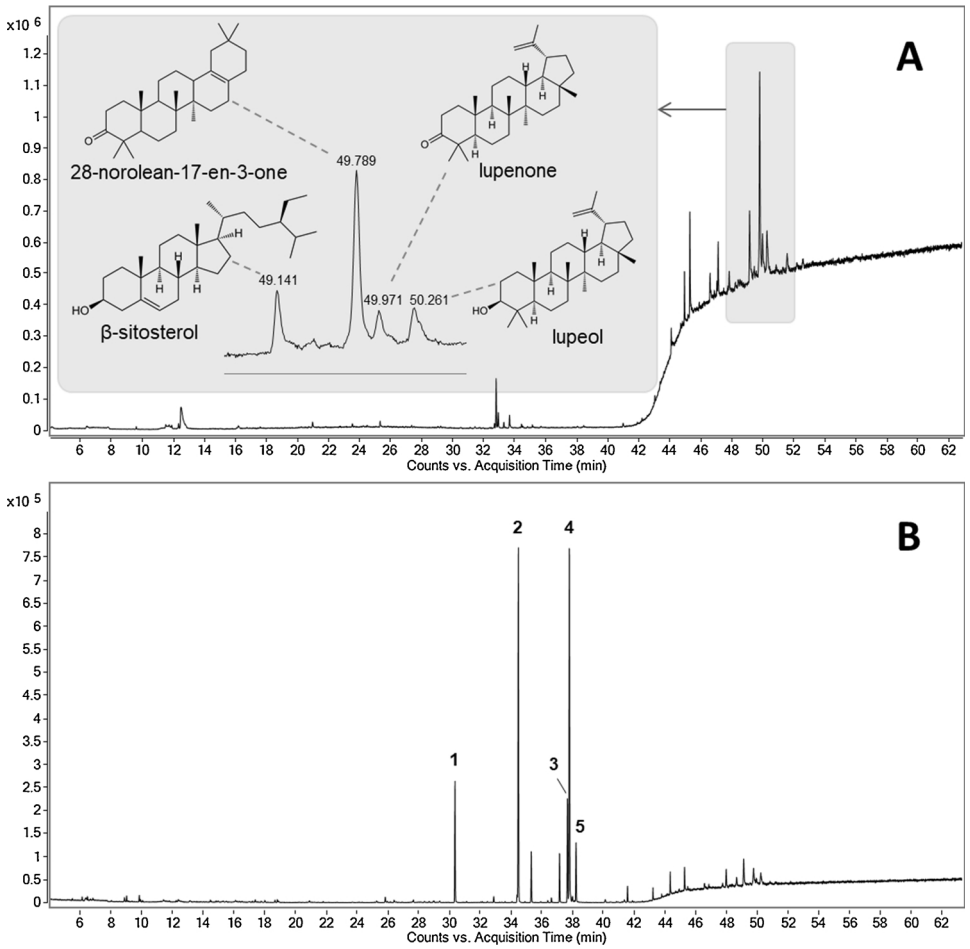

Fig. 2. (A) Chromatogram of the dichloromethane fraction of the residues. (B) Chromatogram of the esterified dichloromethane fraction.

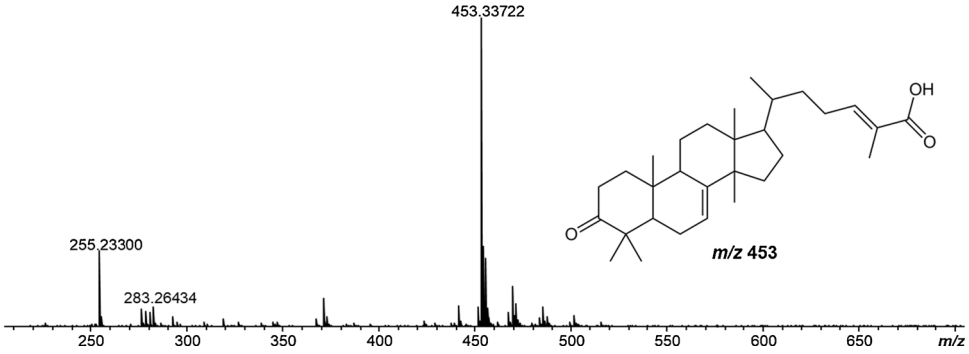

Fig. 3. ESI(-)FT-ICR mass spectra of dichloromethane fraction of the residues.

It was possible to identify esters of myristic, palmitic, linoleic, linolenic and stearic acids, with esters of linolenic and palmitic acids accounting for 57.72% of the total area. Recently, the composition of fatty acids in *S. terebinthifolia* Raddi leaves and twigs was determined by gas chromatography. Thirteen fatty acids were identified, and α-linolenic, palmitic and linoleic acids were the main components (Ennigrou et al., 2018).

3.3. ESI(-)FT-ICR MS and ESI(-)FT-ICR MS/MS analyses

3.3.1. Dichloromethane fraction of residues

In the mass spectra obtained (Fig. 3), the regions with the most intense signals are related to the main classes of compounds identified in the dichloromethane fraction, acid triterpenes (*m/z* 453 to 503) and fatty acids (*m/z* 227 to 339). Among the acid triterpenes, two of the main ones found in *S. terebinthifolia* were identified: masticadienoic acid (*m/z* 453) and schinol (*m/z* 455) (Jain et al., 1995; Morais et al., 2014). In addition, nine other ions had molecular formulas associated with acid triterpenes being one of them in glycoside form. These results indicate the presence of substances not yet isolated from this plant (Table 3). The analysis allowed the identification of other fatty acids beyond the five identified by GC–MS.

Table 3
Substances proposed from ESI(-)FT-ICR MS data for the dichloromethane fraction of the residues.

$[M-H]^-$ (*m/z*)	MS/MS fragments	Molecular formula $[M-H]^-$	Error (ppm)	DBE[a]	Proposed substance
227.20176	–	$C_{14}H_{27}O_2$	−0.46	1	myristic acid
241.21746	–	$C_{15}H_{29}O_2$	−0.63	1	pentadecanoic acid
255.23300	–	$C_{16}H_{31}O_2$	−0.18	1	palmitic acid
269.24865	–	$C_{17}H_{33}O_2$	−0.18	1	heptadecanoic acid
277.21739	–	$C_{18}H_{29}O_2$	−0.32	4	linolenic acid
279.23302	–	$C_{18}H_{31}O_2$	−0.22	3	linoleic acid
281.24870	–	$C_{18}H_{33}O_2$	−0.34	2	oleic acid
283.26434	–	$C_{18}H_{35}O_2$	−0.31	1	stearic acid
311.29568	–	$C_{20}H_{39}O_2$	−0.41	1	eicosanoic acid
339.32691	–	$C_{22}H_{43}O_2$	−0.15	1	behenic acid
453.33722	407	$C_{30}H_{45}O_3$	0.45	8	masticadienoic acid
455.35305	173	$C_{30}H_{47}O_3$	0.04	7	schinol
467.31677	423, 407	$C_{30}H_{43}O_4$	−0.18	9	triterpene acid
469.33235	439, 425, 409, 369	$C_{30}H_{45}O_4$	−0.03	8	triterpene acid
471.34808	–	$C_{30}H_{47}O_4$	−0.20	7	triterpene acid
483.31170	–	$C_{30}H_{43}O_5$	−0.20	9	triterpene acid
485.32732	455, 423, 387	$C_{30}H_{45}O_5$	−0.14	8	triterpene acid
487.34299	–	$C_{30}H_{47}O_5$	−0.19	7	triterpene acid
501.32231	–	$C_{30}H_{45}O_6$	−0.30	8	triterpene acid
503.33799	–	$C_{30}H_{47}O_6$	−0.35	7	triterpene acid
633.40121	453, 179	$C_{36}H_{57}O_9$	−0.63	8	triterpene glycoside

[a] Double bound equivalente (DBE).

These results are in agreement with da Silva et al. (2017a) who identified by ESI(-)-TOF MS the fatty acids (myristic, palmitic, stearic, among others) and acid triterpenes when different fractions of ethanolic extracts of fruits and leaves of *S. terebinthifolia* were analyzed. When studying antifungal compounds of the hydroethanolic extract of the leaves of this plant, Johann et al. (2010) isolated the schinol from the hexane fraction and identified it by ESI-MS and NMR. Years later Vieira et al. (2015) isolated the 3β-masticadienolic (schinol) and masticadienonic acids when performing the fractionation of a dichloromethane extract of the fruits.

3.3.2. Polar extracts

After the FT-ICR MS analysis, mass spectra of the MF, HEE and FPE were obtained (Fig. 4). The most intense signals were *m/z* 183 (methyl gallate), *m/z* 353 (quinic acid hexoside) and *m/z* 325 (galloylshikimic acid), for MF, HEE and FPE, respectively. The chemical composition of polar extracts analyzed by ESI(-)FT-ICR MS is presented in Table 4, where the substances or classes were proposed based on the generated ions, molecular formula, double bound equivalent (DBE) and literature data regarding the identification of the substances in the species, gender or family.

The literature describes the identification of gallic acid and its derivatives in the leaves and fruits of *S. terebinthifolia* (Ceruks et al., 2007; Santana et al., 2012; Feuereisen et al., 2014; Camaroti et al., 2018). The ion of the deprotonated molecule at *m/z* 169, its chloride adduct (*m/z* 205) and its dimer $[2M-H]^-$ at *m/z* 339 confirm the presence of this substance. In relation to their derivatives, the ions at *m/z* 183 and 197 suggest the presence of the methyl and ethyl gallates, respectively, in addition to their adducts with chloride (*m/z* 219 and 233). Beyond gallic acid, shikimic acid (*m/z* 173) was also identified in the deprotonated form. It is one of the precursors of the phenolic compound biosynthesis (Dewick, 2009).

Some sugars were identified in the samples as disaccharides (*m/z* 341), monosaccharide (*m/z* 179) and their adducts with chloride (*m/z* 215 and 377), mainly in the fruit peels. The disaccharide was also detected clustering with water (*m/z* 359).

The presence of flavonoids was detected at *m/z* 301, 317, 433, 447, 449, 463, 477, 479, and 493. Some of them have been found in *S. terebinthifolia*, such as quercetin (*m/z* 301), quercitrin (*m/z* 447), myricetin (*m/z* 317) and myricitrin (*m/z* 463) (Ceruks et al., 2007; Carvalho et al., 2013). Myricitrin was found to form a cluster with the chloride anion (*m/z* 499) and in a more complex structure at *m/z* 615. Likewise, quercitrin was also detected as part of another structure, at *m/z* 599. ESI(-) MS/MS experiments were performed to aid in the identification of these compounds formed by these flavonoids (Fig. 5).

The ions of the deprotonated molecules at *m/z* 599 and 615 were identified as quercitrin *O*-gallate and myricitrin *O*-gallate. The two structures lose the galloyl fraction (−152 Da) to give the fragments *m/z* 447 (quercitrin) and 463 (myricitrin), which, with subsequent loss of the sugar portion (−146 Da), do originate *m/z* 301 and 317 signals

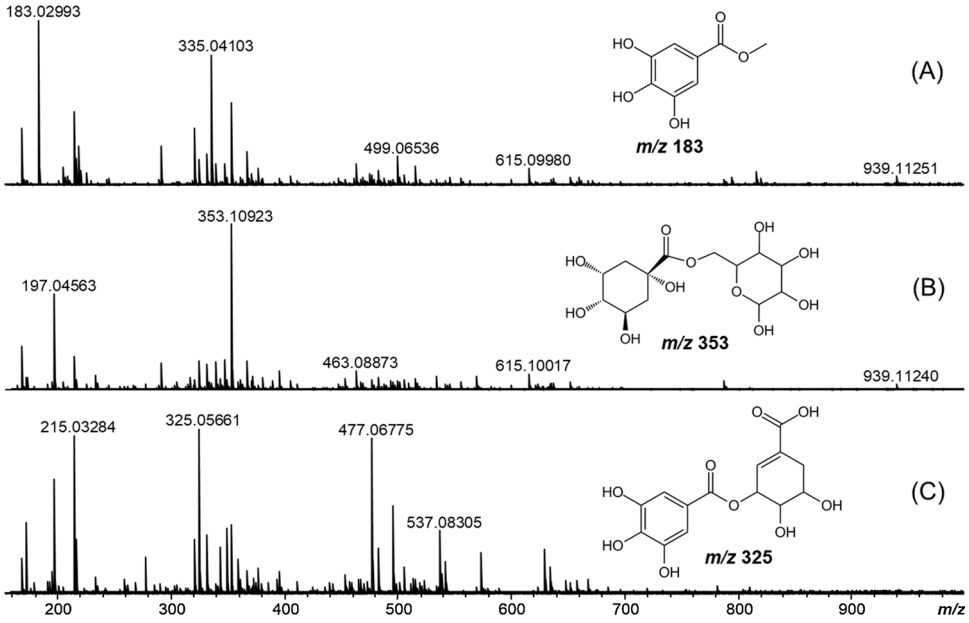

Fig. 4. ESI(-)FT-ICR mass spectra: (A) MF, (B) HEE, (C) FPE.

characteristic of the aglycones quercetin and myricetin, respectively. Similar compounds were identified in fruits of *Rhus coriaria* L. (Anacardiaceae) (Abu-Reidah et al., 2015). As far as we know, the quercitrin derivative is reported here for the first time in *S. terebinthifolia*.

Signals at *m/z* 537 and 541 related to biflavonoids have been identified. They are agathisflavone or its amentoflavone isomer for *m/z* 537 and tetrahydroamentoflavone or its isomer (tetrahydrorobustaflavone) at *m/z* 541. The literature describes the presence of these substances in the fruits of *S. terebinthifolia* (Feuereisen et al., 2017; Muhs et al., 2017).

The polar extracts were abundant in gallotannins and these substances were responsible for some of the most intense signals of the mass spectra. The ion at *m/z* 331 was identified as galloylglucose. The major fragments formed from MS/MS (Table 4) were related to two losses of two formaldehyde moieties $[M\text{-}H\text{-}60]^-$ and $[M\text{-}H\text{-}60\text{-}60]^-$ from glucose to give the ions *m/z* 271 and 211, respectively (Tan et al., 2011). Loss of glucose from *m/z* 331 $[M\text{-}H\text{-}162]^-$ to originate the ion at *m/z* 169. The ion *m/z* 483 (digalloylglucose) suffered decarboxylation $[M\text{-}H\text{-}44]^-$ (*m/z* 439) and showed loss of a galloyl fraction (−152 Da) and of gallic acid (−170 Da) to give the signals *m/z* 331 and 313 respectively. The ion at *m/z* 635 lost galloyl $[M\text{-}H\text{-}152]^-$ (*m/z* 483) and gallic acid $[M\text{-}H\text{-}170]^-$ (*m/z* 465) and has been identified as trigalloylglucose. The ion at *m/z* 939 also loses galloyl (−152 Da) and gallic acid (−170 Da) to give the ions *m/z* 787 and 769, respectively. A further loss of these structures can also be verified with the signals *m/z* 635 and 617. The fragments shown correspond to the pentagalloylglucose molecule (Fig. 1, Supporting Information).

Collision induced dissociation (CID) of the ion *m/z* 325 showed the presence of a fragment at *m/z* 169 for the deprotonated gallic acid as a result of the loss of a shikimate residue (−156 Da). Based on this information, galloylshikimic acid was proposed for the compound. For the ion *m/z* 477, digalloylshikimic acid was attributed to it as it provided the fragment at *m/z* 325 originated from the loss of galloyl (−152 Da) and the *m/z* 169 fragment. The *m/z* 629 ion showed the signals 477 and 325 generated by the consecutive loss of galloyl units. This ion was attributed to trigalloylshikimic acid (Fig. 2, Supporting Information). Gallotannins, galloylglucose and galloylshikimic acids compounds are described as metabolites identified in *S. terebinthifolia* (Cavalher-Machado et al., 2008; Feuereisen et al., 2014).

The deprotonated molecules in *m/z* 335 and 349 showed loss of a galloyl fraction (−152 Da) to give the *m/z* 183 and 197 fragments, respectively. The compounds were characterized as methyl digallate and ethyl digallate. The ions of *m/z* 343 and 495 were identified as mono- and digalloylquinic acids due to sequential loss of galloyl moieties (−152 Da) and the formation of ions *m/z* 191 (deprotonated quinic acid) and *m/z* 169 (deprotonated gallic acid). In the fragmentation of the *m/z* 353 ion signals were formed in *m/z* 179 for the loss of a fraction of the quinic acid (−174 Da) and in *m/z* 173 for the formation of that acid with its subsequent dehydration. These fragments suggest that this substance is the quinic acid hexoside.

The presence of methyl gallate (*m/z* 183) in the methanolic fraction and of ethyl gallate (*m/z* 197) in the hydroethanolic extracts was observed. It was suspected that the extractive solvent had reacted with some substance present in the sample to furnish the gallates in question. Thus, extracts of the residues were prepared with different solvents (methanol, ethanol, water and acetone) and analyzed by the same technique (ESI(-)FT-ICR MS) to verify the appearance of the corresponding gallate signals (Fig. 6).

ESI(-)FT-ICR MS showed the presence of methyl galate (*m/z* 183) in the methanolic extract, whereas the ethyl gallate (*m/z* 197) was found only in the ethanolic extract. It was observed the absence of these ions in the other extracts indicating the interference of the extracting solvent in the production of these gallates. Studies in which methanol was used as the extracting solvent described the identification of methyl gallate (Cavalher-Machado et al., 2008; Rosas et al., 2015; da Silva et al., 2017b). In contrast, those using ethanol as the extracting solvent described ethyl gallate (Bulla et al., 2015; da Silva et al., 2017a). There are also papers that report the two substances (Ceruks et al., 2007; Santana et al., 2012), but the plant extract had contact with both the solvents, one in the extraction process and the other in the purification step. The literature has described the methanolysis reaction, when gallotannins with a higher degree of galloylation are treated with methanol to give products such as methyl gallate, methyl digallate and pentagalloylglucose. This reaction also assists in the quantification by HPLC of hydrolysable tannins present in foods (Chen and Hagerman, 2004; Newsome et al., 2016). A further indication of the solvent effect was the presence of methyl digallate (*m/z* 335) only in the methanolic fraction and ethyl digallate (*m/z* 349) in the hydroethanolic extract (Table 4). Therefore, it is possible to suppose that the formation of these substances occurs during the preparation of extracts since hydrolysable tannins under heating (for example, during extract concentration) can

Table 4
Substances proposed from ESI(-)FT-ICR MS data for polar extracts.

$[M-H]^-$ (*m/z*)	MS/MS fragments	Molecular formula $[M-H]^-$	Error (ppm)	DBE[a]	Proposed substance	MF[b]	HEE[c]	FPE[d]
169.01428	–	$C_7H_5O_5$	−0.20	5	gallic acid	+	+	+
173.04559	–	$C_7H_9O_5$	−0.26	3	shikimic acid	+	+	+
179.05609	–	$C_6H_{11}O_6$	0.09	1	monosaccharide	–	–	+
183.02993	168	$C_8H_7O_5$	−0.19	5	methyl gallate	+	–	–
197.04559	169, 168	$C_9H_9O_5$	−0.21	5	ethyl gallate	–	+	+
204.99097	169	$C_7H_6ClO_5$	−0.20	5	gallic acid chloride cluster	+	–	+
215.03284	179	$C_6H_{12}ClO_6$	−0.23	1	monosaccharide chloride cluster	+	+	+
219.00666	183	$C_8H_8ClO_5$	−0.08	5	methyl gallate chloride cluster	+	–	–
233.02229	197	$C_9H_{10}ClO_5$	−0.28	5	ethyl gallate chloride cluster	–	+	+
301.03530	–	$C_{15}H_9O_7$	0.25	11	quercetin	+	+	–
317.03055	–	$C_{15}H_9O_8$	−0.81	11	myricetin	+	–	–
321.02537	169	$C_{14}H_9O_9$	−0.51	10	digallic acid	+	+	+
325.05661	169	$C_{14}H_{13}O_9$	−0.33	8	galloylshikimic acid	+	+	+
331.06723	271, 241, 211, 169	$C_{13}H_{15}O_{10}$	−0.47	6	galloylglucose	+	+	+
335.04098	183	$C_{15}H_{11}O_9$	−0.38	10	methyl digallate	+	–	–
339.03596	169	$C_{14}H_{11}O_{10}$	−0.57	9	gallic acid cluster	+	+	+
341.10920	–	$C_{12}H_{21}O_{11}$	−0.77	2	disaccharide	–	+	–
343.06722	191, 173, 169	$C_{14}H_{15}O_{10}$	−0.44	7	galloylquinic acid	–	+	+
349.05666	197	$C_{16}H_{13}O_9$	−0.43	10	ethyl digallate	–	+	+
353.10913	179, 173	$C_{13}H_{21}O_{11}$	−0.55	3	quinic acid hexoside	+	+	+
359.11972	179, 161	$C_{12}H_{23}O_{12}$	−0.61	1	disaccharide / H_2O	–	+	+
367.04392	331	$C_{13}H_{16}ClO_{10}$	−0.47	5	galloylglucose chloride cluster	+	–	+
377.08584	341, 215, 179	$C_{12}H_{22}ClO_{11}$	−0.60	1	disaccharide chloride cluster	+	+	+
433.07793	–	$C_{20}H_{17}O_{11}$	−0.67	12	flavonoid	+	–	–
447.09364	301, 273, 271, 179	$C_{21}H_{19}O_{11}$	−0.79	12	quercitrin	+	–	–
449.07280	–	$C_{20}H_{17}O_{12}$	−0.56	12	flavonoid	+	–	–
453.33762	407	$C_{30}H_{45}O_3$	−0.45	8	masticadienoic acid	–	+	+
455.35321	173	$C_{30}H_{47}O_3$	−0.30	7	schinol	–	+	+
463.08855	317, 179	$C_{21}H_{19}O_{12}$	−0.76	12	myricitrin	+	+	+
469.33231	439, 425, 409, 369	$C_{30}H_{45}O_4$	0.05	8	triterpene acid	–	+	+
471.34861	–	$C_{30}H_{47}O_4$	−1.33	7	triterpene acid	–	+	–
477.06775	325, 313, 289, 263, 169	$C_{21}H_{17}O_{13}$	−0.59	13	digalloylshikimic acid	–	+	+
477.10413	–	$C_{22}H_{21}O_{12}$	−0.59	12	flavonoid	+	+	+
479.08363	–	$C_{21}H_{19}O_{13}$	−1.07	12	flavonoid	+	+	–
483.07835	439, 331, 313, 271, 169	$C_{20}H_{19}O_{14}$	−0.67	11	digalloylglucose	+	+	+
485.32768	455, 423, 387	$C_{30}H_{45}O_5$	−0.89	8	triterpene acid	–	+	–
493.06275	–	$C_{21}H_{17}O_{14}$	−0.76	13	flavonoid	+	+	–
495.07832	343, 191, 169	$C_{21}H_{19}O_{14}$	−0.59	12	digalloylquinic acid	+	+	+
499.06536	463	$C_{21}H_{20}ClO_{12}$	−0.96	11	myricitrin chloride cluster	+	+	–
501.32251	–	$C_{30}H_{45}O_6$	−0.69	8	triterpene acid	–	+	–
537.08305	443, 375	$C_{30}H_{17}O_{10}$	−0.61	22	agathisflavone/ amentoflavone	–	+	+
541.11446	415, 389	$C_{30}H_{21}O_{10}$	−0.81	20	tetrahydroamentoflavone/ tetrahydrorobustaflavone	–	+	+
599.10484	447, 301	$C_{28}H_{23}O_{15}$	−1.00	17	quercitrin *O*-gallate	+	+	–
615.09980	463, 317	$C_{28}H_{23}O_{16}$	−1.04	17	myricitrin *O*-gallate	+	+	–
629,07902	477, 325	$C_{28}H_{21}O_{17}$	−0,96	18	trigalloylshikimic acid	–	–	+
633.40135	453, 179	$C_{36}H_{57}O_9$	−0.86	8	triterpene glycoside	–	+	+
635.08929	483, 465	$C_{27}H_{23}O_{18}$	−0.48	16	trigalloylglucose	+	+	+
635.41745	455, 179	$C_{36}H_{59}O_9$	−1.57	7	triterpene glycoside	–	–	+
649.39632	469, 179	$C_{36}H_{57}O_{10}$	−0.92	8	triterpene glycoside	–	–	+
939.11240	787, 769, 635, 617	$C_{41}H_{31}O_{26}$	−1.59	26	pentagalloylglucose	+	+	–

(+) - Substance present.
(-) - Substance absent.
[a] Double bound equivalent (DBE).
[b] Methanolic fraction (MF).
[c] Hydroethanolic extract (HEE).
[d] Fruit peels extract (FPE).

suffer solvolysis. The Fig. 7 shows the reaction between the solvent and the hydrolysable tannins present in the extract. When performing the nucleophilic attack (1) the solvent will induce the production of methyl or ethyl digallate. Another possibility would be the reaction (2) producing methyl or ethyl gallate.

3.4. Antimicrobial activity

The results of the antimicrobial activity are shown in Table 5. The extracts MF and HEE were the most active, mainly against Gram-positive bacteria. The higher sensitivity of these strains may be related to the difference in the constitution and arrangement of the cell membrane of these microorganisms in relation to the Gram-negative (Kołodziejczyk et al., 2013). The most susceptible strains were those of *S. aureus* with the two extracts presenting MIC 0.6–0.9 mg/mL and MBC from 1.8 mg/mL onwards. For strains 10A and ATCC 29213 the MBC was not reached at the concentrations tested. These extracts showed activity against strains of *E. faecium* and *E. faecalis* with MIC values 1.20–1.50 mg/mL and MBC 1.80–2.70 mg/mL for MF and MIC 1.20–2.10 mg/mL for HEE, in addition to inhibiting the growth of one *A. baumannii* strain (30B) and three multidrug-resistant *P. aeruginosa* strains. Although FPE, DF and HF had no bactericidal effect at the concentrations tested, FPE showed inhibitory activity against *S. aureus* (MIC 1.20–1.80 mg/mL), *E. faecium* (101E), *E. faecalis* (ATCC 29212)

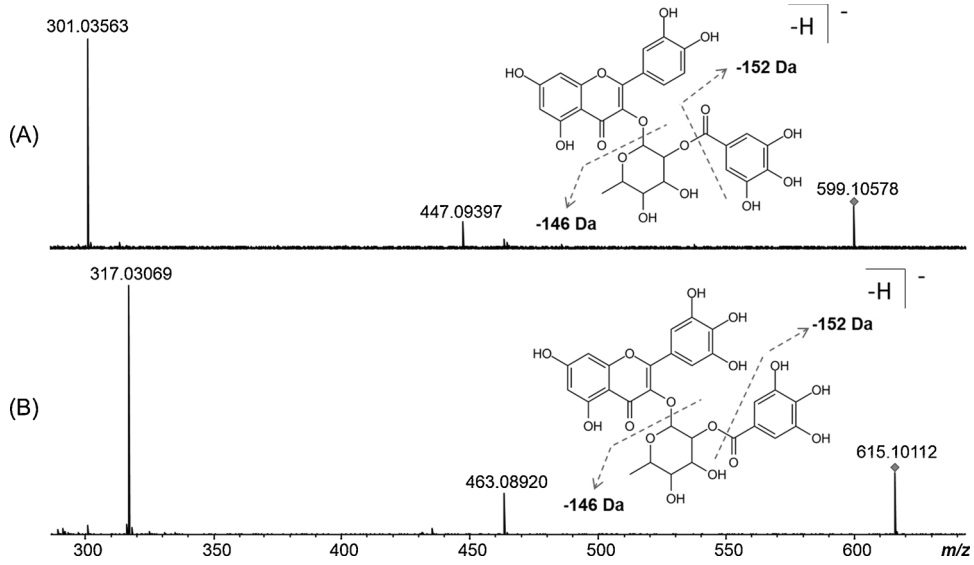

Fig. 5. ESI(-) MS/MS - Proposal for fragmentation of *m/z* 599 (A) and 615 (B) ions.

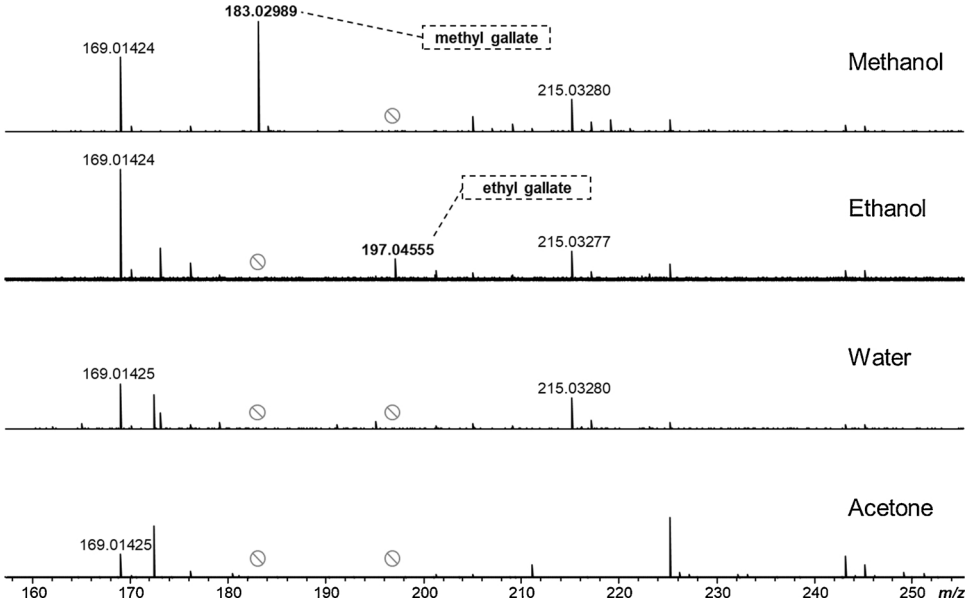

Fig. 6. Mass spectra of *S. terebinthifolia* extracts prepared with methanol, ethanol, water and acetone.

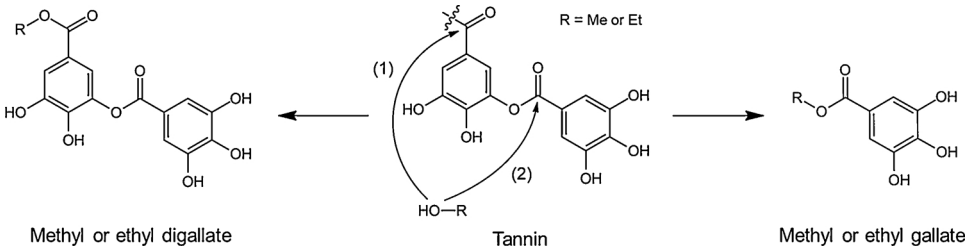

Fig. 7. Reaction between the solvent (methanol or ethanol) and a hydrolysable tannin with a higher degree of galloylation.

and *A. baumannii* (101B). The activity against *S. aureus* is in agreement with Muhs et al. (2017) who verified that one of the fractions of the methanolic extract of the fruits presented inhibitory activity against MRSA and demonstrated a significant reduction in the dermonecrosis caused by this microorganism. The DF was active against two strains of *S. aureus* and was the only extract that inhibited all strains of *A. baumannii* (MIC 2.40 mg/mL). This activity may be related to the acid triterpenes identified in the sample. When studying natural Mediterranean plants, Karygianni et al. (2014) verified that extracts containing acid triterpenes were active against oral bacteria, especially Gram-negative bacteria. The HF also showed no bactericidal activity but inhibited the growth of all *S. aureus* strains (MIC 2.40–2.70 mg/mL). In general, the polar extracts were more active, and this higher activity may be related to the presence of phenolic compounds, because

Table 5
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of residues extracts from the Brazilian pepper tree processing industry.

		MF[b]		HEE[c]		FPE[d]		DF[e]		HF[f]	
Species	Strains	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]
S. aureus	10A	0.90	> 2.70	0.90	> 2.70	1.50	> 2.70	2.40	> 2.70	2.40	> 2.70
	23A	0.90	1.80	0.60	1.80	1.20	> 2.70	> 2.70	> 2.70	2.70	> 2.70
	35A	0.60	1.80	0.60	1.80	1.50	> 2.70	> 2.70	> 2.70	2.40	> 2.70
	67A	0.60	2.10	0.90	1.80	1.50	> 2.70	> 2.70	> 2.70	2.40	> 2.70
	114A	0.60	2.70	0.60	1.80	1.20	> 2.70	> 2.70	> 2.70	2.40	> 2.70
	ATCC 29213	0.60	> 2.70	0.90	> 2.70	1.80	> 2.70	1.80	> 2.70	2.40	> 2.70
E. faecium	70E	1.50	2.10	2.10	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	101E	1.50	2.70	1.80	> 2.70	2.10	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
E. faecalis	1277	1.50	2.70	1.80	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	6885	1.20	2.70	2.10	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	168557	1.20	2.70	1.80	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	ATCC 29212	1.20	1.80	1.20	2.70	1.50	> 2.70	2.40	> 2.70	> 2.70	> 2.70
A. baumannii	30B	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	2.40	> 2.70	> 2.70	> 2.70
	101B	1.20	> 2.70	1.80	> 2.70	2.70	> 2.70	2.40	> 2.70	> 2.70	> 2.70
	ATCC 19606	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	2.40	> 2.70	> 2.70	> 2.70
P. aeruginosa	34B	2.40	> 2.70	2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	39B	2.40	> 2.70	2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	121B	2.40	> 2.70	2.40	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70
	ATCC 27853	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70	> 2.70

[a] Values expressed in mg/mL.
[b] Methanolic fraction (MF).
[c] Hydroethanolic extract (HEE).
[d] Fruit peels extract (FPE).
[e] Dichloromethane fraction (DF).
[f] Hexane fraction (HF).

substances belonging to this class such as gallic acid, methyl gallate, galloylglucose compounds and flavonoids have shown antimicrobial activity (Kang et al., 2008; Cushnie and Lamb, 2011; Engels et al., 2012; Farhadi et al., 2018). Phenolic compounds are also known to have strong antioxidant activity (Cai et al., 2004). Phenolic substances identified in extracts of *S. terebinthifolia* were considered responsible for the antioxidant activity observed (El-Massry et al., 2009; Bernardes et al., 2014; Uliana et al., 2016; da Silva et al., 2017b; Tlili et al., 2018).

Our results agree with the literature that describes the antimicrobial potential of *S. terebinthifolia*. Assays performed with alcoholic extracts of leaves against *S. aureus* obtained MIC values close to those found in the present study, 0.75 mg/mL (El-Massry et al., 2009) and 0.50 mg/mL (Uliana et al., 2016). The differences in the results presented in these works may be related to the variation of the chemical composition of each of the extracts. In addition to activity against *S. aureus*, the polar extracts were also active against *E. coli*, *P. aeruginosa*, *C. albicans* (Guerra et al., 2000; El-Massry et al., 2009; Uliana et al., 2016) and *E. faecalis* (de Costa et al., 2012). In another study the antimicrobial potential of fruit extract against *S. aureus* and *Bacillus cereus* was related to the phenolic compounds present in the sample (Degáspari et al., 2005). In addition to the extracts, the essential oils are also described with antimicrobial activity (El-Massry et al., 2009; Cole et al., 2014; Ennigrou et al., 2018). Even a study was carried out aiming at the application of the essential oil as a biopreservative food (da Silva Dannenberg et al., 2016).

4. Conclusion

It was possible to identify different classes of substances present in the residues from the Brazilian pepper tree processing industry, mainly phenolic compounds such as gallic acid, gallotannins and flavonoids in the polar extracts and triterpenes in the apolar extracts. This work describes for the first time the occurrence of quercitrin *O*-gallate in the species. Extracts rich in phenolic substances showed significant activity against multidrug-resistant bacteria, mainly against MRSA and VRE. Due to the richness of these compounds, residues from the Brazilian pepper tree processing industry can be used as source of substances with antimicrobial potential, antioxidant activity, besides the application as food preservative, being necessary more studies on the use of this material. These findings provide opportunities to explore the use of industrial by-products to reduce residues streams and recover bioactive compounds.

Conflicts of interest

The authors declare no conflict of interest related to this work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.05.079.

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