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Determination of phenolic profile by HPLC-ESI-MS/MS and antibacterial activity of *Eugenia platysema* against mollicutes strains

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ABSTRACT

This study evaluated the phenolic composition of the extract and fractions from *E. platysema* leaves, in addition to antibacterial activity against different types of bacteria, like mollicutes and cel wall bacteria. The crude extract (CHE) was partitioned with solvents to yield fractions of different polarities (dichloromethane, ethyl acetate, butanol and water). The chemical composition of CHE and its fractions were evaluated by HPLC-LC-ESI-MS/MS together with 76 standard phenolic compounds. Nine known compounds were identified for the first time from this species: hydroxybenzoic-*O*-hexoside acid **[1]**, gallocatechin **[2]**, syringic acid **[3]**, 4-hydroxycinammic acid **[4]**, salycilic acid **[5]**, isoquercetin **[6]**, isorhamnetin **[7]**, luteolin-3'-*O*-glucuronide **[8]** and luteolin **[9]**. The evaluation of antibacterial activity was performed using the broth dilution method. The CHE and fractions of *E. platysema* were active against mollicutes strains, being the best result presented by the dichloromethane fraction against *U. urealyticum*, *M. mycoides* subsp. *Capri* and *M. hominis* (125 μg mL^{-1}).

INTRODUCTION

Microorganisms are one of the most frequent causes of diseases, representing as a serious public health problem for a large portion of the population. The mollicutes are organisms responsible for several diseases such as pneumonia and pyelonephritis, both in humans and in animals. Some strains like *Mycoplasma hominis* and *Ureaplasma urealyticum* are a common commensal of the female genital tract and have been associated with pyelonephritis, bacterial vaginosis, cervicitis, endometritis, and postpartum septicemia. *U. urealyticum* is considered as the main cause of nonchlamydial, non-gonococcal urethritis, chorioamnionitis, preterm delivery, abortion, preterm birth, bacterial vaginosis and cervicitis (Amirmozafari *et al.*, 2009; Falk *et al.*, 2005; Naessens *et al.*, 1989; Taylor *et al.*, 2013). The mollicutes have the peculiarity of the fact they do not have cell walls, and contain cholesterol in the composition of their cell membrane (Cordova *et al.*, 2010). The lack of a rigid cell wall renders genital *Mycoplasmas* innately resistant to antimicrobial agents, such as β-lactam antibiotics and vancomycin, which act in this target.

Tetracyclines, macrolides, and quinolones are the major antibiotics used in the treatment of infections caused by mycoplasmas, however, their therapeutic efficacy may be unpredictable due to increasing resistance (Bayraktar *et al.*, 2010). Therefore, there is an importance in researching new active compounds against these microorganisms from various sources, including natural products (Benfatti *et al.*, 2010).

It is known that the *Eugenia* genus has innumerable therapeutic properties, among them are antimicrobial, antifungal, larvicide, besides to have hypotensive, diuretic, hipoglicemic, anti-inflamatory, cytotoxic, antioxidant, hypoglycemic and cardio protective properties (Auricchio and Bacchi, 2003; Bag *et al.*, 2012; Famuyiwa and Adebajo, 2012; Garmus *et al.*, 2014; Saha *et al.*, 2013; Victoria *et al.*, 2012;). Many compounds have been described for the *Eugenia* genus, especially phenolic compounds, including the flavonoids (Baliga *et al.*, 2011; Oliveira *et al.*, 2014).

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These compounds are a large class within polyphenols, where there are more than 4000 structures with have important medicinal and biological activity, especially antimicrobial, antioxidant and to prevent cancer effects (Shafaghat *et al.*, 2014).

Eugenia platysema, popularly known as "Guamirim", is a perennial tree of the *Eugenia* genus (Myrtaceae) native to South America (Reitz *et al.*, 1969). There are few studies on this species related in the scientific literature. Apel *et al.*, (2002), evaluated the chemical composition of the essential oil from leaves of *E. platysema*, which showed β-selinene (17.9%) and allo-aromadendrene (12.6%) as major compounds. Tenfen *et al.*, (2016) also analyzed the essential oil of *E. platysema* leaves and presented it as major compound the diterpene (6-*E*, 10-*Z*) pseudo phytol (65.55% ± 0.81).

Nevertheless, this is the first study that evaluates chemically and biologically the extract and fractions of *E. platysema*. Thus, the aim of this study was to identificate the phenolic compounds presents in the crude extract and fractions of *E. platysema* leaves by HPLC-ESI- MS/MS and evaluate their antibacterial activities against cell wall and mollicutes strains.

MATERIAL AND METHODS

Plant material

Leaves of *Eugenia platysema* were collected in June 2013, in Blumenau, Santa Catarina State, Southern Brazil (26°54'26.8"S; 49°04'52.4" W). The sample was identified by Prof. Dr. Marcos Eduardo Guerra Sobral, (Departamento de Ciências Naturais, Universidade Federal de São João Del-Rey/MG) and André Luis de Gasper (Departamento de Ciências Naturais, FURB). A voucher specimen was deposited in the Herbarium Roberto Miguel Klein (FURB) under registration number 42336.

The dried and ground plant material was macerated in hydroalcoholic solution (70 %) at room temperature for 7 days. The crude extract resulting (CHE) from maceration was concentrated under reduced pressure to completely dry. After concentration, the crude extract was resuspended in water and partitioned by liquid-liquid fractionation using solvents of different polarities yielding dichloromethane (FDCM), ethyl acetate (FAE) n-butanol (FBu) and aqueous fractions (FAq).

Identification of phenolic compounds by HPLC-LC-ESI-MS/MS

The profile of crude extract, FDCM, FAE, FBu and FAq fractions were analysed by HPLC-ESI-MS/MS in LABEC/INCT-Catálise at Federal University of Santa Catarina (UFSC). Analysis were conducted in an Agilent® 1200 chromatograph, with TurbolonSpray® as ionization source, coupled to a Qtrap® 3200 mass spectrometer, with a Phenomenex® Synergi 4μ Polar-RP 80A (150 mm x 2 mm ID, particle size of 4 μm) at the temperature of 30 ºC. The eluents were formed by mixing solvents A ($MeOH/H_2O$ in ratio of 95:5, v v^{-1}) and B (H_2O ultrapure/formic acid (0,1 %) as follows: 1st stage – 10 % solvent A and 90 % B (isocratic mode) for 5 minutes; 2nd stage – linear gradient of solvents A and B (from 10 to 90 % of A) for 2 minutes; 3rd stage – 90 % solvent A and 10 % B (isocratic mode) for 3 minutes; 4th stage – linear gradient of solvents A and B (from 90 to 10 % of A) for 7 minutes with a flow rate of 250 μL $min^{-1}$of mobile phase. In all analyses, the injected volume was 5 uL at a concentration of 300 mg L^{-1}.

For the compounds identification, 76 standard phenolic compounds (3,4-dihydroxybenzoic acid, 4-aminobenzoic acid, 4-hydroxybenzoic acid, 4-methyl-umbelliferone, acacetin, 4-hydroxymethylbenzoic acid, *p*-anisic acid, caffeic acid, carnosic acid, cinnamic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, mandelic acid, methoxyphenylacetic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, rosmarinic acid, salicylic acid, sinapic acid, syringic acid, vanillic acid, apigenin-7-*O*-glucoside, apigenin, apigenin-7-*O*-rutinoside, apiine, aromadendrin, caffeic-*O*-hexoside acid, carnosol, catechin, chrysin, cirsimaritin, coniferaldehyde, coumarin, dicaffeoylquinic acid, epicatechin, epigallocatechin-gallate, epirosmanol, eriodictyol, scopoletin, fustin, galangin, gallocatechin, hydroxybenzoic-*O*-hexoside acid, hispidulin, isoquercetrin, isorhamnetin, isorhamnetin-3-O-hexoside, kaempferol, luteolin, luteolin-3'-*O*-glucuronide, luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside, medioresinol, metoxy carnosol, methyl carnosate, myricetrin, naringenin, naringin, poridzin, pinocembrin, protocatechuic acid, quercetin-3-*O*-hexoside, quercetin, resveratrol, rosmadial, rutin, sinapaldehyde, syringaldehyde, taxifolin, thymol, umbelliferone, vanillin and vitexin) diluted in methanol (1 mg L^{-1}) were analysed in the same conditions as described above.

The liquid chromatograph was coupled to a mass spectrometer with electrospray ionization source using negative ionization mode with the following source parameters: ion spray interface at 400 °C; ion spray voltage of 4500 V; curtain gas, 10 psi; nebulizer gas, 45 psi; auxiliary gas, 45 psi; collision gas, medium. The Analyst® (version 1.5.1) software was used for recording and processing the data. Pairs of ions were monitored in MRM (Multiple Reaction Monitoring) mode.

Evaluation of antibacterial activity

Microorganisms and medium

The Laboratory of Clinical Microbiology from FURB provided the bacterial strains. Tests were evaluated against Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and against Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Mollicutes strains (no-cell-wall bacteria) *Mycoplasma mycoides* subsp. *capri* (NCTC 10137); *Mycoplasma hominis* (ATCC 33530), and *Ureaplasma urealyticum* (ATCC 27618) were also assessed. For the growth of bacterial strain, Müller-Hinton broth was used for *S. aureus*, *S. E. coli* and *P. aeruginosa*, U10 broth was used for *U. urealyticum*, SP4 broth to *M. mycoides* subsp. *capri* and *M. hominis* (Velleca *et al.*, 1979).

Determination of minimum inhibitory concentration (MIC)

The antibacterial activity of the crude extract and fractions from *E. platysema* was evaluated by determination of the minimum inhibitory concentration (MIC). The microdilution broth assay was performed in sterile 96-well microplates, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012) for cell-wall bacteria and Bebear and Roberteson (1996) for mollicutes.

The samples were properly prepared and transferred to each microplate well with the appropriate culture medium, in order to obtain a twofold serial dilution of the original extract in a 10 % H_2O/dimethyl sulfoxide (DMSO) solution, obtaining sample concentrations ranging between 1000 μg mL^{-1} to 7.81 μg mL^{-1}. The inoculum containing 10^4 to 10^5 microorganisms per mL were then added to each well.

A number of wells were reserved in each plate to test for sterility control (no inoculum added), positive control (gentamycin or ciprofloxacin), inoculum viability (no extract added), and the DMSO inhibitory effect.

The microplates were incubated at 37 °C ± 1 °C for 24 hours to cell-wall bacteria and 48 hours for mollicutes. Thereafter, growth of mollicutes strains was detected by observing the colour change in the medium. For cell-wall bacteria, a methanolic solution of triphenyl tetrazolium chloride (5 mg mL^{-1}) was added into each well, and the presence of a reddish bacterial "button" observed at the bottom of each well. The MIC was defined as the lowest concentration of the sample able to inhibit bacterial growth.

RESULTS AND DISCUSSION

Identification of phenolic compounds by HPLC-ESI-MS/MS

Chemical profiling of 76 phenolic compounds from 5 samples using the HPLC-ESI-MS/MS method were carried out. The results are shown on Table 1. These results indicated that in the crude extract four phenolic compounds were identified: gallocatechin **[2]**, syringic acid **[3]**, salycilic acid **[5]** and isorhamnetin **[7]**. In the FDCM, syringic acid **[3]**, 4-hidroxycinammic acid **[4]**, and luteolin **[9]** were identified. In the FAE salycilic acid **[5]**, syringic acid **[3]**, 4-hidroxycinammic acid **[4]** besides luteolin-3´-*O*-glucuronide **[8]** were also identified. In the FBu, isoquercetin **[6]**, salycilic acid **[5]**, syringic acid **[3]** and 4-hidroxycinammic acid **[4]** were identified. The compounds hydroxybenzoic-*O*-hexoside acid **[1],** gallocatechin **[2]**, salycilic acid **[5]**, isoquercetin **[6]** and isorhamnetin **[7]** were identified in the aqueous fraction.

The skeletons of identified compounds are characteristics from the *Eugenia* genus (Baliga *et al.*, 2010; Einbond *et al.*, 2004; Oliveira *et al.*, 2014). However, this is the first report of identification of salycilic acid and hydroxybenzoic-*O*-hexoside acid on leaves of *Eugenia* genus. Futhermore, this is the first report about the presence of these compounds in *Eugenia platysema.* The flavonoids isoquercetin and gallocatechin have been previously described in another plants of *Eugenia* genus, as *E. brasiliensis* (Pietrovski *et al.*, 2008), *E. jambolana* (Baliga *et al.*, 2011) and *E. uniflora* (Lee *et al.*, 2000). Syringic acid were described in *E. pyriformis* and *E. jambolana* (Sharma *et al.*, 2016); luteolin were identified in *E. cariophyllata* (Gülçin *et al.*, 2004).

Antibacterial Activity

For the evaluation of the antibacterial activity, a criterion established by Machado *et al.*, (2005), was used. Samples with MIC values lower than 10 μg mL^{-1} were considered to have excellent antibacterial activity; between 10 and 100 μg mL^{-1} were considered good; values between 100 and 500 μg mL^{-1} were considered of moderate activity; values between 500 and 1,000 μg mL^{-1} of low activity, and for MIC values above 1,000 μg mL^{-1}, samples were considered inactive. The results for MIC of all samples are shown in Table 2.

Table 1: Phenolic compounds identified in *E. platysema* by HPLC-ESI-MS/MS.

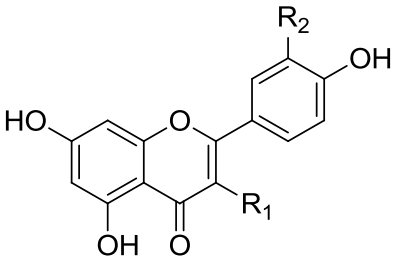
	Compound	Rt* (min)	Calculated Mass	Experimental mass [M – H]	MS/MS (*m/z*)	Identified compounds				
						CHE	FDCM	FAE	FBu	FAq
1	Hydroxybenzoic-*O*-hexoside acid	7.85	299.80	299.07	137.00					x
2	Gallocatechin	9.28	306.26	305.06	225.00	x				x
3	Syringic acid	9.69	198.17	196.86	119.60	x	x	x	x	x
4	4-hydroxycinammic acid	10.22	164.16	162.86	116.40		x	x	x	
5	Salycilic acid	10.58	138.12	136.85	90.11	x		x		
6	Isoquercetin	10.73	464.38	462.90	297.30				x	x
7	Isorhamnetin	12.28	316.26	315.04	300.00	x			x	x
8	Luteolin-3'-*O*-glucuronide	12.62	462.36	461.07	285.00			x		
9	Luteolin	13.24	286.24	285.03	267.00		x			

* Rt = retention time (minutes); CHE = crude hydroalcoholic extract; FDCM = Dichloromethane fraction; FAE = Ethyl acetate fraction; FBu = Butanolic fraction; FAq = Aqueous fraction.

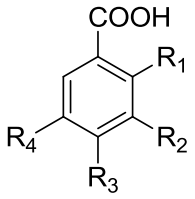
Table 2. Antibacterial activity of crude extract and fractions from *E. platysema*.

	MIC[a] (μg mL^{-1})					
	CHE	FDCM	FAE	FBu	FAq	Positive Control[b]
U. urealyticum	125	125	500	>1000	>1000	40
M. mycoides subsp. *capri*	250	125	250	500	>1000	40
M. hominis	250	125	250	500	>1000	20
S. aureus	>1000	>1000	>1000	>1000	>1000	15.6
P. aeruginosa	>1000	1000	500	>1000	>1000	7.3
E. coli	>1000	1000	500	>1000	>1000	15.6

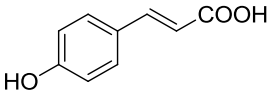
[a]MIC =Minimal Inibitory Concentration. [b]Positive Control= ciprofloxacin, for mollicutes; gentamicin, for cel wall bacterials.



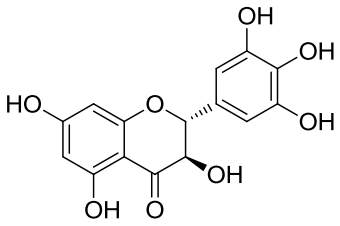
	R_1	R_2
[6]	*O*-glu	OH
[7]	OH	OCH_3
[8]	H	*O*-glucuronide
[9]	H	OH



	R_1	R_2	R_3	R_4
[1]	H	H	*O*-glucose	H
[3]	H	OCH_3	OH	OCH_3
[5]	OH	H	H	H



[4]



[2]

Fig. 1: Phenolic compounds identified in the leaves of *Eugenia platysema.*

Among the tested samples, the fractions which had the highest activity was FDCM against *U. urealyticum*, *M. hominis* and *M. mycoides* (125 μg mL^{-1}) and FAE against *U. urealyticum* and *M. mycoides* subsp. capri (125 μg mL^{-1}). The best activity of crude extract was against *U. urealyticum* (125 μg mL^{-1}).

These results are important because the mollicutes are responsible for various diseases such as pneumonia, vaginitis, urethritis, and pyelonephritis in humans (Cordova *et al.*, 2010). Reports of resistance to these microorganisms in conventional treatments also increased (Yechouron *et al.*, 1992), in addition these microorganisms do not have cell wall, which makes them completely resistant to β-lactam antibiotics and other drugs that act on it (Silva, 2006). For the cell-wall bacteria none of samples tested were active, showing with MIC values above 1,000 μg mL^{-1}. This indicates that the mechanism of action of the extract and the fractions of *E. platysema* to inhibit bacterial growth is not involved with the bacterial cell wall.

Some of the identified phenolic compounds were report on literature to have antimicrobial properties. Luteolin, a well-known polyphenolic compound identified in the dichloromethane fraction, has diverse biological benefits that include antimicrobial effects. Joung *et al.*, (2016) reported that luteolin showed synergistic activity by increasing cytoplasmic membrane permeability and inhibiting ATPase. This is important on the effects of the membrane permeabilizing agent and ATP-binding cassette (ABC) transporter inhibiting agent, since most bacteria produce ABC transporter that is an essential uptake system for amino acids in the bacterial membrane, and this mechanism can be a determinant of bacterial antibiotic resistance.

Other identified phenolic compounds like syringic acid has been reported to exhibit antibacterial ability against various microorganisms (Shi *et al.*, 2016). The syringic acid retarded bacterial growth, and caused cell membrane dysfunction, with decrease of intracellular ATP concentration, cell membrane hyperpolarization and changes in cellular morphology (Shi *et al.*, 2016).

However, this is the first study that evaluated the antibacterial activity of samples containing the syringic acid in its composition against mollicute strains. The presence of syringic acid and luteolin in the FDCM fraction probably contributes to the antibacterial potential of this fraction. These mechanisms may help to explain some of the good antibacterial activity shown by the dichloromethane fraction. However, further studies with the isolated compounds should be conducted, together with the identification of other classes of compounds, to searching for the responsible compounds for the antibacterial activity in these fractions and to better answer these questions.

CONCLUSIONS

Present results showed that nine phenolic compounds were identified in the extract and fractions from *E. platysema*. All of these compounds were reported the first time in this species. Interesting antibacterial activity for the extract and fractions of this species against mollicutes strains were observed, especially in the FDCM fraction. Further phytochemical studies should be performed to isolate and identify the active constituents for this activity.

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