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A comparative study of phytoconstituents and antibacterial activity of *in vitro* derived materials of four *Passiflora* species

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ABSTRACT

Passiflora species are well known for their common use in popular medicine for the treatment of several diseases, such as insomnia, anxiety, and hysteria, in addition to their anti-inflammatory, antioxidant, analgesic and antibacterial potential. However, few data about the chemical composition and the medicinal potential of *in vitro* derived materials are available. Therefore, the goal of this work was to compare, for the first time, the phytoconstituents of *in vitro* derived materials of four *Passiflora* species, and evaluate the antibacterial potential of their extracts against 20 Gram-positive and negative strains. Chromatographic analysis indicated the presence of saponins in roots extracts from all studied species, whereas leaf extracts presented both saponins and flavonoids. Extracts from leaves and roots of *P. alata* and *P. foetida* exhibited a selective inhibitory activity against *B. thuringiensis* and *S. pyogenes,* which might be related to the presence of a high concentration of secondary metabolites, including flavonoids and saponins.

Key words: antibacterial, chromatography, flavonoids, *Passiflora*, saponins.

INTRODUCTION

Bacteria, fungi, viruses and nematodes have been recognized as the main causes of human and animal infections in tropical and subtropical countries. However, the indiscriminate use of synthetic antimicrobial drugs is leading to an increasing number of multiple drug resistance microorganisms, therefore directing studies towards the discovery of new antimicrobials (Bax et al. 2000). Since medicinal plants may constitute alternative sources of new, natural antimicrobial agents, considerable efforts have been made to investigate the potential of bioactive compounds for the treatment of several infectious diseases (Pan et al. 2009).

The genus *Passiflora* is the largest and more diverse of the family Passifloraceae, comprising more than 560 species of vines, lianas, trees, and shrubs, commonly used for their fruits and derivatives, and as ornamental and medicinal plants (Ulmer and MacDougal 2004). Leaves, fruits and roots of *Passiflora* species are traditionally used in several countries for the treatment of insomnia, anxiety and irritability (Dhawan et al. 2004).

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Pharmacological studies carried out in the last 20 years reported distinct biological activities in the genus, including diuretic, anxiolytic, anti-inflammatory, antioxidant, analgesic, antiviral, and antihyperglycemic (Montefusco-Pereira et al. 2013, Colomeu et al. 2014), as well as antibacterial activity (Bendini et al. 2006, Mohanasundari et al. 2007, Ripa et al. 2009, Baby et al. 2010, Ingale and Hivrale 2010, Ramaiya et al. 2014, Razia et al. 2014, Subramani et al. 2014). These pharmacological activities have been associated to the presence of alkaloids, flavonoids, saponins, cyanogenic compounds, essential oils, and carotenoids (Dhawan et al. 2004, Gosmann et al. 2011).

In this work, we compared the phytochemical composition and antibacterial potential of four *Passiflora* species. *Passiflora alata* and *P. foetida* belong to the subgenus *Passiflora*, and are widely used in folk medicine. *Passiflora alata* is one of the commercially exploited passionfruit, and is present as an official drug in the Brazilian Pharmacopoeia (2010), in addition to its use in phytopharmaceutical preparations due to its sedative and anxiolytic properties. *Passiflora foetida* is a wild species commonly used for the treatment of hysteria, asthma and skin diseases with inflammation (Dhawan et al. 2004). *Passiflora suberosa* and *P. pohlii* are wild species from the subgenus *Decaloba*, which have important agronomic potential due to their tolerance to virus, fungi, and soil-borne pathogens that cause damages to the passionfruit culture (Gardner 1989, Junqueira et al. 2005). These species are also used as ornamental plants, due to the small size of their fruits and flowers (Ulmer and MacDougal 2004). Leaves of *P. suberosa* are commonly used as a sedative and to treat hypertension, diabetes, and skin diseases (Miller 1998). Although there are no reports on the use of *P. pohlii* in folk medicine, Simão et al. (2016) recently described a high antioxidant potential of its roots.

The over-exploitation of natural resources, caused by the great demand for supplying high-value phytochemicals, as well as the problems associated to seasonal variations and other environmental factors, constitute important limitations to the use of medicinal plants. Considering this, biotechnological methods, including plant cell and tissue cultures, provide alternative tools for large-scale production of secondary metabolites (Wilson and Roberts 2012). However, in spite of the well known medicinal potential of *Passiflora* species, only a few data about the chemical composition and pharmacological activities of *in vitro* derived materials of these species are available (Antognoni et al. 2007, Fraccaroli et al. 2008, Lugato et al. 2014, Simão et al. 2016). Therefore, the aim of this work was to investigate the presence of flavonoids and saponins, and compare the antibacterial activity of extracts from leaves and roots excised from *in vitro*-grown plants of four *Passiflora* species (*P. alata, P. pohlii, P. suberosa* and *P. foetida*) against different bacterial strains.

MATERIALS AND METHODS

PLANT MATERIAL AND TISSUE CULTURE CONDITIONS

In vitro-grown plants of *P. alata, P. foetida, P. pohlii* and *P. suberosa,* derived from seed germination (Garcia et al. 2011, Pacheco et al. 2012, Merhy et al. 2014), and maintained by bimonthly subcultures of nodal segments on solidified ½ MSM medium (Monteiro et al. 2000), supplemented with 1.5 % sucrose, and solidified with 0.7 % agar (Merck) were used as sources of plant materials for extract preparation. Cultures were maintained in a growth chamber at 25 ± 2 °C, under a 16 h photoperiod, using a total irradiance of 46 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps.

EXTRACT PREPARATION

Extracts from dry leaves and roots excised from *in vitro*-grown plants were prepared separately, using 40 % ethanol under reflux (1:10, plant:solvent, w/v) for one hour (1 h), as described by Birk et al. (2005). For the evaluation of antibacterial activity, 50 mg of the extracts were solubilized in 200 mL of dimethyl sulfoxide (DMSO). The extracts (10 mg) were also resuspended in methanol for Thin Layer Chromatography (TLC) analysis.

TLC ANALYSIS

The TLC analysis was carried out as described by Simão et al. (2016). Each sample was directly applied on silica gel-coated TLC aluminum plates (Si gel 60 UV_{254nm}, Marcherey–Nagel, 20x20 cm plates).

For flavonoid analysis, the mobile phase was prepared according to Wagner and Bladt (2001), using AcOEt:formic acid:AcOH:H_2O (100:11:11:26, v/v). The plate was sprayed using 1 % methanolic solution of diphenylboryloxyethylamine (Sigma Aldrich®), followed by 5 % (w/v) PEG 4000 (Natural Product Reagent—polyethyleneglycol) as color reagents. Spots were observed under UV (365 nm).

The saponins analysis was carried out with $CHCl_3$:AcOH:MeOH:H_2O (60:32:12:8, v/v) as the mobile phase (Wagner and Bladt 2001). TLC plates were then visualized using anisaldehyde-H_2SO_4 before heating (100 °C) for 5–10 min.

ANTIBACTERIAL ASSAY

The antibacterial potential of *Passiflora* extracts was evaluated by the agar dilution method (macrodilution) described by Soberón et al. (2007) with modifications (Barboza et al. 2015). An aliquot of the extracts was solubilized in 20 mL of pre-warmed Mueller-Hinton Agar (MHA, Oxoid, Ltda.), obtaining the test concentrations of 500 and 1000 μg mL^{-1}. The final content was poured into sterile Petri dishes. After the solidification of the medium, 2 μL of each bacterial suspension previously grown in Mueller-Hinton Broth (MHB, Oxoid, Ltda.) at 37 °C for 18 h, were cultivated in duplicate. After 24 h of incubation at 37 ºC, the growth of the colonies was observed and compared to the growth of the control plates containing MHA without extracts (positive control) or with 0.5 % DMSO (negative control). The experiments were repeated three times for each plant material.

Twenty bacterial strains, encompassing Gram-positive and Gram-negative were evaluated (Table I). All the tested strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and from the collection of the Department of Microbiology, Immunology, and Parasitology, from the Rio de Janeiro State University.

The evaluation of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the inhibitory concentration of 50 % of the population (IC_{50}) were determined by the dilution method in 96-well plates. For these assays, only the strains that showed inhibition of growth by the macrodilution method were used.

The bacterial suspensions, adjusted to 0.5 on the McFarland scale, were cultured on MHB at 37 °C for 18 h, and then adjusted to 0.14 of optic density at 580 nm. The suspensions (100 μL) were cultured in 96-well plates in the presence of different concentrations (100 - 1500 μg mL^{-1}) of the extracts (100 μL) for 18 h, in quadruplicates. After the incubation period, an aliquot of 2 μL of each well was plated onto MHA medium and grown for 18 h at 37 °C for proof of the MBC. The plates were analyzed in the microplate reader at 492 nm and the absorbances were used to measure the MIC and calculate the IC_{50} by non-linear regression in the GraphPad Prism®.

To eliminate the possible influence of color of the extracts, three wells containing only the

TABLE I
Bacterial strains used for the evaluation of antibacterial activity of different *in vitro* derived materials of *Passiflora* species.

	Species	Strains
Gram-positive	*Bacillus thuringiensis*	(ATCC 33679)
	Enterococcus faecalis	(29212)
	Staphylococcus aureus	(ATCC 25923)
	Staphylococcus simulans	(ATCC 27851)
	Staphylococcus saprophyticus	(ATCC 15305)
	Streptococcus pyogenes	(ATCC 8668)
Gram-negative	*Aeromonas caviae*	(ATCC 15468)
	Aeromonas hydrophila	(ATCC 7966)
	Citrobacter freundii	(ATCC 12241)
	Escherichia coli	(17-2)
	Escherichia coli	(ATCC 25922)
	Escherichia coli	(ATCC 35218)
	Escherichia coli K-12	(HB 101)
	Escherichia coli K-12	(C600)
	Enteroaggregative *E. coli*	(EAEC 042)
	Klebsiella pneumoniae	(ATCC 700603)
	Pseudomonas aeruginosa	(ATCC 27853)
	Salmonella typhimurium	(C20)
	Serratia marcescens	(7145)
	Shigella sonnei	(ATCC 25931)

extracts at different concentrations, without bacterial suspensions, were analyzed in each assay. The mean value of each triplicate was discounted of the absorbance values for MIC and IC_{50}.

The experimental controls used were the Mueller-Hinton medium without extracts (positive control) and the same medium supplemented with 0.5 % DMSO (negative control). The experiments were repeated four times and all the materials and culture media were previously sterilized at 121 °C for 20 minutes.

RESULTS AND DISCUSSION

Biotechnological approaches are often employed for the stable or increased production of secondary metabolites, without the interference of external factors (Wilson and Roberts 2012). In this work, we investigated the presence of flavonoids and saponins and compared the antibacterial potential of roots and leaves from *in vitro*-grown plants of four *Passiflora* species.

The genus *Passiflora* is a unique source of several phytochemicals, including alkaloids, flavonoids, saponins, essential oils and carotenoids, as well as minerals, fibers and vitamins, which are already explored for human use. The TLC analysis presented here using both NP/ PEG 4000 and anisaldehyde-H_2SO_4 as color reagents revealed the presence of flavonoids and saponins in leaf and root extracts from *in vitro*-grown plants of the four *Passiflora* species studied.

Characteristic flavonoid fluorescent spots were only observed in leaf extracts. However, each extract presented a distinct chromatographic profile, with different flavonoid-related spots. Extracts from leaves of *P. pohlii* and *P. foetida* displayed fluorescent yellow spots (R_F = 0.35

and 0.43, respectively), whereas *P. alata* extracts showed major yellow ($R_F = 0.42$) and orange ($R_F = 0.38$) spots. Leaves of *P. suberosa* presented three pale orange ($R_F = 0.28$, 0.40 and 0.63), and a blue spot ($R_F = 0.86$) not detected elsewhere (Figure 1). The flavonoid profile observed here in leaf extracts of *in vitro* plants of *P. alata* and *P. suberosa* was similar to the observed by Birk et al. (2005) in extracts of aerial parts of *in vivo*-grown plants of these two species.

Although flavonoids are more frequently reported in the genus, saponins could also be associated with some *Passiflora* species. *Passiflora alata* presents saponins as its major phytoconstituent, and five saponins have already been isolated and identified from leaves of this species, one steroidal and four triterpenes (Reginatto et al. 2001). In addition, Simão et al. (2016) have recently described the presence of saponins in different plant materials of *P. pohlii*. In this work, saponins were detected in extracts from the four studied species, characterized by orange, purple and dark spots (Figure 2). Leaves of *P. suberosa, P. foetida*, as well as roots of *P. alata* presented similar purple spots ($R_F = 0.48$), which suggests that they might be the same substance. Four dark spots with the same color and R_F (0.34) were observed in root extracts of the four species. Roots of *P. alata, P.*

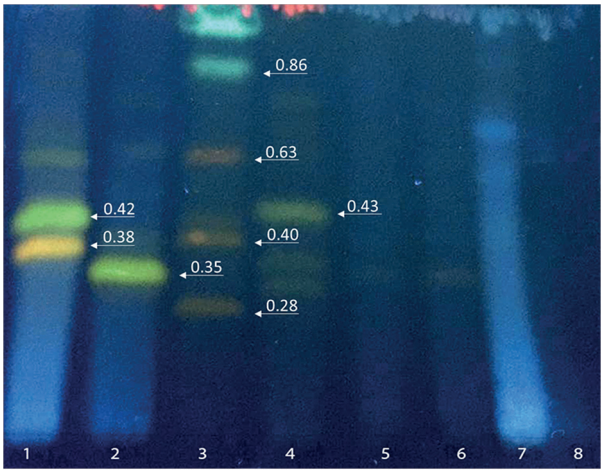

Figure 1 - TLC plate for the identification of flavonoids in the extracts from *in vitro* derived materials of four *Passiflora* species. 1-4 Leaves of *P. alata* (1), *P. pohlii* (2), *P. suberosa* (3), *P. foetida* (4). 5-8 Roots of *P. alata* (5), *P. pohlii* (6), *P. suberosa* (7), *P. foetida* (8). Mobile phase: AcOEt:formic acid:AcOH:H_2O (100:11:11:26, v/v). Visualization: NP/PEG 4000/UV_{365nm}.

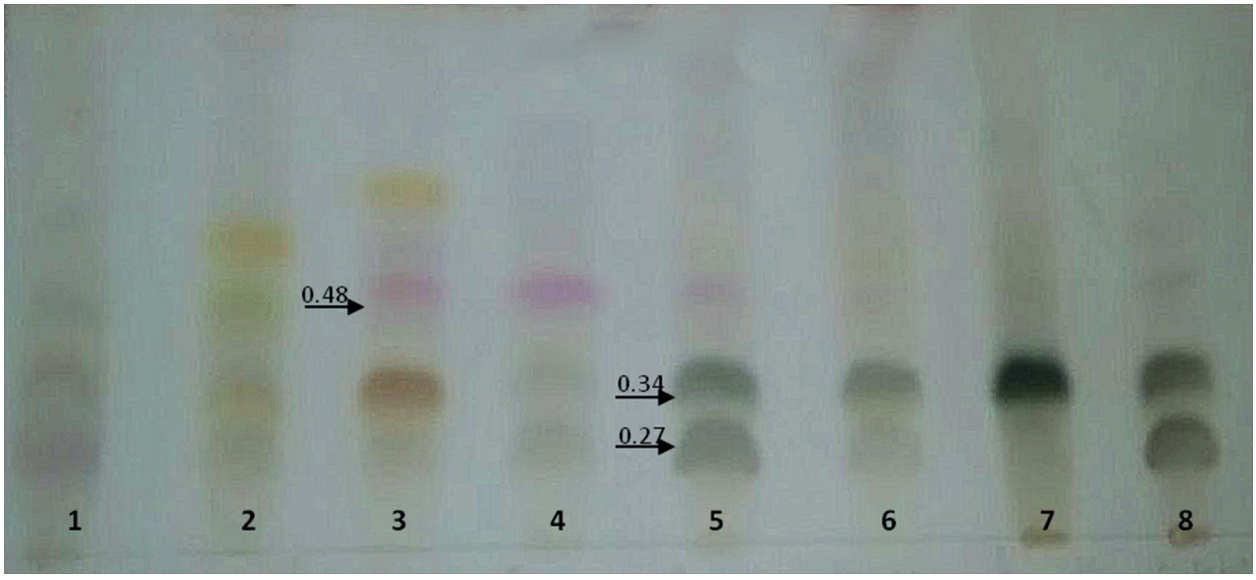

Figure 2 - TLC plate for the identification of saponins in the extracts from *in vitro* derived materials of four *Passiflora* species. 1-4 Leaves of *P. alata* (1), *P. pohlii* (2), *P. suberosa* (3), *P. foetida* (4). 5-8 Roots of *P. alata* (5), *P. pohlii* (6), *P. suberosa* (7), *P. foetida* (8). Mobile phase: $CHCl_3$:AcOH:MeOH:H_2O (60:32:12:8, v/v). Visualization: anisaldehyde-H_2SO_4/heating (100 ºC).

pohlii and *P. foetida* also presented grey spots, with the same R_F (0.27).

The antibacterial potential of crude extracts from *in vitro* derived leaves and roots was also evaluated in this work. Extracts from leaves and roots of *P. alata* and *P. foetida* showed a selective inhibitory activity on the growth of only two strains, namely *Bacillus thuringiensis* (ATCC 33679) and *Streptococcus pyogenes* (ATCC 8668). Root extracts of *P. alata* also reduced the growth of *Pseudomonas aeruginosa* (ATCC 27853). On the other hand, an increased growth was observed in almost all strains tested in response to extracts from *in vitro* derived leaves of *P. alata* and leaves and roots of *P. foetida,* when compared to the control (Table II).

Leaves and roots of *P. pohlii* showed an increase of growth of all tested strains, except for *Streptococcus pyogenes,* which presented similar growth as the control plate. In contrast, *P. suberosa* extracts did not alter the growth of the 20 strains tested (Table II).

Based on these results, extracts from *P. alata* and *P. foetida* were tested for IC_{50}, MIC and MBC for the inhibition of *B. thuringiensis* and *S. pyogenes* growth and the results are shown in Table III. Root extracts of *P. alata* showed the lowest IC_{50} for both strains when compared to the other three extracts tested, with 28.98 μg mL^{-1} for *S. pyogenes* and 9.08 μg mL^{-1} for *B. thuringiensis*. Root extracts of *P. alata* and *P. foetida* also presented the lowest MIC for both strains, which were less than 100 μg

TABLE II
Evaluation of the antibacterial activity of crude extracts (1000 μg.mL^{-1}) from *in vitro*-derived materials of four *Passiflora* species using the macrodilution method.

	Strains	*P. alata*		*P. foetida*		*P. pohlii*		*P. suberosa*	
		Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Gram-positive	*B. thuringiensis*	IN	IN	IN	IN	I	I	-	-
	E. faecalis	I	-	I	I	I	I	-	-
	S. aureus	I	-	I	I	I	I	-	-
	S. simulans	I	-	I	I	I	I	-	-
	S. saprophyticus	I	-	I	I	I	I	-	-
	S. pyogenes	IN	IN	IN	IN	-	-	-	-
Gram-negative	*A. caviae*	I	-	I	I	I	I	-	-
	A. hydrophila	I	-	-	I	I	I	-	-
	C. freundii	I	-	I	I	I	I	-	-
	E. coli (17-2)	I	-	I	I	I	I	-	-
	E. coli (ATCC 25922)	I	-	I	I	I	I	-	-
	E. coli (ATCC 35218)	I	-	I	I	I	I	-	-
	E. coli K-12 (HB 101)	I	-	I	I	I	I	-	-
	E. coli K-12 (C600)	I	-	I	I	I	I	-	-
	E. coli (EAEC 042)	I	-	-	I	I	I	-	-
	K. pneumoniae	I	-	-	I	I	I	-	-
	P. aeruginosa	-	RD	-	-	I	I	-	-
	S. typhimurium	I	-	I	I	I	I	-	-
	S. marcescens	I	-	I	I	I	I	-	-
	S. sonnei	I	-	I	I	I	I	-	-

I = increase; RD = reduction; IN = inhibition; Trace = similar growth in the control MHA.

TABLE III
Inhibitory concentration of 50 % of the population (IC_{50}), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (µg.mL^{-1}) of crude extracts of leaves and roots excised from *in vitro*-grown plants of *P. alata* and *P. foetida*.

Extracts		*Streptococcus pyogenes*			*Bacillus thuringiensis*		
IC_{50}		MIC	MBC	IC_{50}	MIC	MBC	
P. alata	Leaves	488	>750	1000	75.67	>250	>1000
	Roots	28.98	>100	500	9.08	>100	750
P. foetida	Leaves	104.74	>250	>500	75.3	>250	>500
	Roots	36.89	>100	>250	37.51	>100	500

mL^{-1}. The best MBC for *S. pyogenes* was displayed by roots extracts of *P. foetida,* with values under 250 µg mL^{-1}. For *B. thuringiensis,* the lowest MBC was displayed by leaf extracts of *P. foetida*, with values under 500 µg mL^{-1} (Table III).

Previous reports described antibacterial activity of extracts from different plant materials of *in vivo*-grown plants of *P. foetida* against both Gram-positive and Gram-negative strains. Bendini et al. (2006) reported high antimicrobial activity of leaf extracts of *P. foetida*. Mohanasundari et al. (2007) observed the effect of the extracts from leaves and fruits against *Pseudomonas putida*, *Vibrio cholerae*, *Shigella flexneri* and *Streptococcus pyogenes* using the well-in-agar method. Baby et al. (2010) used the Kirby-Bauer disc diffusion method to test the effect of root extracts against *Staphylococcus epidermidis*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*. On the contrary to the observed in this work, these studies reported better antibacterial activity against Gram-negative strains.

Although the role played by the secondary metabolites is not yet fully understood, their presence in plant extracts has already been associated with antibacterial potential (Bukke et al. 2015, Dzotam et al. 2016). In the genus *Passiflora*, the production of total phenols, flavonoids and tannins, as well as the evaluation of the antibacterial potential of leaf extracts of *P. alata* prepared with different solvents was reported by Vasic et al. (2012). They observed that the ethyl acetate extract showed stronger antibacterial activity when compared to the other extracts tested, especially against Gram-positive strains. However, they detected low levels of phenols and flavonoids in this extract, suggesting that the antibacterial activity was not related to these phytoconstituents. Johnson et al. (2008) also attributed the high inhibition rates of chloroform extracts of callus of *P. edulis* against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Aeromonas* spp., *Serratia* and *Escherichia coli* to the presence of a diversity of compounds, including saponins, tannins, triterpenoids, alkaloids and flavonoids.

To our knowledge, this is the first report on the antibacterial potential of *in vitro* derived materials of *P. alata*, *P. foetida*, *P. pohlii* and *P. suberosa.* Although further studies are required aiming at the isolation and identification of the flavonoids and saponins observed in the extracts of the four studied species, the solid inhibiting effect on the growth of two bacterial strains, as seen by the results of MIC and MBC analyses, may be associated with the presence of these compounds in the extracts of *P. alata* and *P. foetida*.

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