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Inhibitory activity of *Varronia curassavica* and *Mikania laevigata* fractions against pathogens associated with persistent dental infections

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Abstract

Herbal medicines have been studied as potential antimicrobial agents, emerging as treatments against oral diseases. The current study evaluated the antimicrobial activity of the crude extract and fractions of *Mikania laevigata Schultz* Bip. ex Baker (ML) and *Varronia curassavica* Jacq (VC) against oral pathogens associated with persistent dental root infections, under planktonic and biofilm conditions. Minimal inhibitory concentrations and minimal bactericidal/fungicidal concentrations were determined for the ML and VC fractions/extracts against *Enterococcus faecalis, Actinomyces israelii, Pseudomonas aeruginosa,* and *Candida albicans* using the microdilution method. The best results were chosen for subsequent biofilm assays. All tested ML and VC extracts/fractions demonstrated inhibitory activity against *E. faecalis* and *A. israelii*. The ML ethyl acetate fraction affected the growth of all microorganisms tested. *C. albicans* and *P. aeruginosa* were not affected by any VC extract/fractions. The ML ethyl acetate fraction eliminated *E. faecalis*, *A.israelii,* and *P. aeruginosa* biofilms after 24h. A similar result was observed for ML crude hydroethanolic extract and its hexane fraction for *A. israelii*. The VC hexane fraction was able to eliminate *A. israelli* biofilms. None of the tested extracts or fractions eliminated *C. albicans* biofilm. The *Mikania laevigata* ethyl acetate fraction is an efficient antimicrobial agent against oral pathogens and could be indicated for the treatment of persistent dental infections.

Keywords: Plant Extracts. Phytotherapy. Antimicrobial Activity.

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INTRODUCTION

The oral microbiota is formed by a complex ecosystem, which changes constantly throughout human life. Most of the microorganisms are commensal, however, they can cause disease in appropriate conditions. Dental caries and trauma allow the penetration of oral

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microorganisms into dentin tubules and the root canal system, causing pulp infections and, frequently, apical periodontitis with the formation of bone lesions (Persoon et al., 2017). Even after endodontic treatment, resistant microorganisms are trapped in the root canal system, requiring irrigants and dressings with a large spectrum of antimicrobial action (Siqueira & Rôças, 2008). Specific bacteria such as *Enterococcus faecalis* and *Candida albicans* can be found in cases of endodontic treatment failure (Cogulu et al., 2008) and *Actinomyces israelii* and *Pseudomonas aeruginosa* in refractory periapical lesions (Siqueira & Rôças, 2008).

Solvents such as water, butanol, hexane, and ethyl acetate have been used for the semi purification of the crude plant extract, from which various fractions have been obtained and studied for pharmaceutical purposes, including for controlling oral pathogens (Yatsuda et al., 2005; Michielin et al., 2009). *Mikania laevigata* SchultzBip. ex Baker, popularly known as "guaco", belongs to the *Asteraceae* family and the genus *Mikania* and is commonly found in southern Brazil. Several properties have been described for *Mikania laevigata*, including anti-inflammatory and antimicrobial potential (Yatsuda et al., 2005; Duarte et al., 2007; Michielin et al., 2009). Another plant with therapeutic effects is popularly known as "erva-baleeira". The classification of "erva-baleeira" has been widely discussed and is still controversial; the accepted name in World Plants – Catalogue of Life (Hassler, 2020) and Brazilian Flora (Jardim Botânico do Rio de Janeiro, 2020) is *Varronia curassavica* Jacq., belonging to the genus *Varronia* and family *Boraginaceae*, with *Cordia verbenacea* and *Cordia curassavica* as synonyms. However, according to The Plant List (2013) and International Plant Name Index (2012) the accepted name is *Cordia curassavica* (Jacq.) Roem & Schult. This species is native to Central and South America; and in Brazil it is commonly found in the Atlantic and Amazon Forests (Meccia et al., 2009). In the current study, we adopted the scientific name *Varronia curassavica* Jacq. based on its Brazilian origin, following the criteria of Brazilian Flora (Jardim Botânico do Rio de Janeiro, 2020). *Varronia curassavica* is widely used in popular medicine, mainly as an antimicrobial, anti-inflammatory, and analgesic agent (Matias et al., 2013). Thus, the aim of this study was to evaluate the antimicrobial activity of *Mikania laevigata* and *Varronia curassavica* crude hydroethanolic extracts and their aqueous, hexane, butanol, and ethyl acetate fractions against pathogens associated with persistent dental infections, under planktonic and biofilm conditions.

MATERIAL AND METHODS

Leaves of *Mikania laevigata* Schultz Bip. ex Baker and *Varronia curassavica* Jacq. were collected at the Medicinal and Toxic Plant Garden in the School of Pharmaceutical Science, UNESP, in Araraquara, São Paulo, Brazil (21°48'51.4"S and 48°12'05.1"W) and authenticated on location by Prof. Dr. Luis V. S. Sacramento. The leaves were dried in an oven at 40°C for 96h. A portion of 10g of dry extract was partitioned between 1:1 hexane: water, ethyl acetate, and n-butanol, in sequence. Fractions were separated and evaporated to dry. The solvents used to re-suspend the extracts and fractions were sterile distilled water for the crude extract, and aqueous, hexane, and butanol fractions and 5% DMSO for the ethyl acetate fraction.

The present study used the following microbial strains kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, Sao Paulo, Brazil): *Enterococcus faecalis* (ATCC 51299), *Actinomyces israelii* (ATCC 12102), *Pseudomonas aeruginosa* (ATCC 15442), and *Candida albicans* (ATCC 26790). Microbial suspensions were prepared from overnight cultures in Brain Heart Infusion broth (BHI, Difco Laboratories, Kansas City, MO, USA) for bacteria strains or Sabouraud Dextrose broth (SD, Difco) for *Candida albicans* and incubated at 37°C for 24h in a 5% CO_2 atmosphere.

MIC and MBC/MFC assays were conducted according to Balouiri et al. (2016). The final concentration of suspension in the wells was 5-10x10^5CFU/mL for bacteria and 2.5-5x10^3 CFU/mL for *Candida albicans*. First, the serially diluted plant extracts and fractions and microbial suspensions were inoculated in each well. The plates were incubated at 37°C for 24h in 5% CO_2. The colonies were counted, and the number of viable bacteria was determined in CFU/ml. The MBC/MFC was considered when the extracts/fractions killed (99.9%) of the

tested microbial culture. Chlorhexidine digluconate (CHX) and Amphotericin B (AB) were used as positive controls for the bacteria and *C. albicans,* respectively. Cultures without antimicrobial agents in MH broth or RPMI-1640 medium and 5% DMSO were considered as negative controls. All experiments were performed in triplicate.

Biofilm assays were performed with the extracts/fractions that presented 100% growth inhibition in the MBC/MFC tests, except for *C. albicans*, as described by Massunari et al. (2017). Briefly, 96-well microplates, U-shaped bottom, were pretreated with 200µl of artificial saliva per well for 4 h at 37°C in 5% CO_2 (coating phase). Next, 20µl of each microorganism suspension (1-5x10^6 CFU/mL) were inoculated in each well containing 180µl of BHI broth with 0.5% glucose for bacteria and SD broth with 0.5% glucose for *Candida*. The plates were incubated for 48h. Subsequently, 200 µl of plant extract or fraction solutions (5x or 10x MBC and MFC concentrations) were inserted in each well. After 24h of incubation, all wells were plated in Brain Heart Infusion agar for bacteria or Sabouraud Dextrose agar for *C. albicans* and incubated for 24h followed by microbial counting.

Data from planktonic growth (MIC assays) were converted to logarithmic scale (log10 (CFU+1) and the percentage (%) of microbial reduction compared to normal growth (control group) was calculated. Non-parametric data obtained in the biofilm assays were represented by box-whisker plots. Mann-Whitney tests were applied to compare one group with another and these with the positive controls (CHX or AB) and solvent controls (DMSO and water) for biofilm assays.

RESULTS AND DISCUSSION

Species of the family *Asteraceae* and *Boraginaceae* have been broadly studied for the treatment of various human illnesses due to their wide-ranging pharmacological properties, such as antimicrobial, anti-inflammatory, anthelmintic, and analgesic action, (Matias et al., 2015). There are records of the use of “erva-baleeira” by traditional communities in the treatment of inflammation, muscle pain, arthritis, rheumatism, stomach ulcers, and others (Passos et al., 2007; Roldão et al., 2008; Miranda & Hanazaki, 2008; Gandolfo & Hanazaki, 2011). Popularly, the plant leaves are used in the form of infusion, decoction, tinctures, hydroalcoholic extracts, and others (Hartwig et al., 2020). “Guaco” is widely used by traditional communities for the treatment of flu and cough, made from leaves in the form of syrup and infusion (Oliveira & Menini, 2012; Pinto et al., 2017). Due to the large number of ethnobotanical reports, a lot of research has been conducted to provide evidence of their pharmacological and biological properties (Matias et al., 2015).

The current study highlights the importance of investigating *Mikania laevigata* and *Varronia curassavica* as antimicrobial agents. Both plants are native to the Brazilian territory and listed among the 71 plants of the National List of Medicinal Plants of Interest to the Brazilian Unified Health System (Brasil, 2009). This list encourages the use of complementary therapies in the public health system and promotes research with these medicinal plants, guaranteeing their correct and safe use by the population (Marmitti et al., 2015).

The findings on the antimicrobial activity of *Mikania laevigata* plant extract and fractions in planktonic conditions are shown in Table 1. All *Mikania laevigata* (ML) extracts/fractions tested presented MIC values ranging from 250 to 4000 µg/ml and MBC/MFC values ranging from 1000 to >4000 µg/ml, showing inhibitory activity against *E. faecalis* (MIC 1000-4000 µg/ml) and *A. israelii* (MIC 250-2000 µg/ml), except for the ML aqueous fraction (MLAF) for *A. israelii*. The ML ethyl acetate fraction (MLEAF) affected the growth of all microorganisms tested and the ML hexane fraction (MLHF) affected *E. faecalis*, *A. israelli,* and *C. albicans.* MLEAF had the best effect compared to the other ML extracts/fractions. *A. israelii* was highly sensitive to MLEAF (MIC=250 µg/ml), MLCHE (MIC = 330 µg/ml), and MLHF (MIC=330 µg/ml). The ML extracts/fractions with the best results for MBC/MFC were chosen considering their performance against each microorganism for the biofilm assays: MLCHE for *A. israelii*, MLHF for *C. albicans* and *A. israelii,* and MLEAF for all microorganisms (Table 1).

Table 1. Minimum inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) of *Mikania laevigata* (ML) extract and fractions against resistant oral pathogens.

Extract/Fraction	Microorganisms	MIC	MBC/MFC
		(µg/ml)	(µg/ml)
ML crude hydroethanolic extract (MLCHE)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	330	2000
	C. albicans	>4000	>4000
ML Aqueous fraction (MLAF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	>4000	>4000
	C. albicans	>4000	>4000
ML Hexane fraction (MLHF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	330	1000
	C. albicans	4000	>4000
ML Butanol fraction(MLBF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	2000	>4000
	C. albicans	>4000	>4000
ML Ethyl acetate fraction (MLEAF)	*E. faecalis*	1000	1000
	P.aeruginosa	4000	4000
	A. israelii	250	4000
	C. albicans	4000	4000
Chlorhexidine digluconate (CHX)	*E. faecalis*	6.5	9,8
	P. aeruginosa	39	78
	A. israelii	1.2	1.2
Amphotericin B (AB)	*C. albicans*	0.16	0.49

All tested *Varronia curassavica* (VC) extracts/fractions demonstrated inhibitory activity against *E. faecalis* and *A. israelii*, with MIC values ranging from 410 to 4000 µg/ml and MBC/MFC values ranging from 500 to >4000 µg/ml. *C. albicans* and *P. aeruginosa* were not affected by any VC extract or fraction. VC ethyl acetate fraction (VCEAF) had the best effect compared to other VC fractions. *A. israelli* was highly sensitive to VCEAF (MIC = 410 µg/ml), VCHF (MIC = 500 µg/ml), and VCCHE (MIC = 500 µg/ml), and because of this effect, VCEAF, VCCHE, and VCHF were chosen for the biofilm assays (Table 2).

Table 2. Minimum inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) of *Varronia curassavica* (VC) extract and fractions against resistant oral pathogens.

Extract/Fraction	Microorganisms	MIC	MBC/MFC
		(µg/ml)	(µg/ml)
VC crude hydroethanolic extract (VCCHE)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	500	500
	C. albicans	>4000	>4000
VC Aqueous fraction (VCAF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	4000	>4000
	C. albicans	>4000	>4000
VC Hexane fraction (VCHF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	500	2000
	C. albicans	>4000	>4000
VC Butanol fraction (VCBF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	2600	>4000
	C. albicans	>4000	>4000
VC Ethyl acetate fraction (VCEAF)	*E. faecalis*	4000	>4000
	P. aeruginosa	>4000	>4000
	A. israelii	410	>4000
	C. albicans	>4000	>4000
Chlorhexidine digluconate (CHX)	*E. faecalis*	6.5	9.8
	P. aeruginosa	39	78
	A. israelii	1.2	1.2
Amphotericin B (AB)	*C. albicans*	0.16	0.49

Figure 1 indicates box-whisker plots of the antibiofilm activity of the plant extracts and fractions. MLEAF (5xMBC) eliminated *E. faecalis, A. israelii,* and *P. aeruginosa* biofilms after 24h of exposure. The same was observed for MLCHE and MLHF for *A. israelii.* None of the tested extracts or fractions eliminated the *C. albicans* biofilm. The 10x MLEAF obtained the best results against *C. albicans* biofilms, although without statistical difference between 5x MLHF, 10xMLHF, and 5x AB. VCCHE and VCHF significantly reduced the microbial growth on the *A. israelli* biofilms, however, only 10x VCHF was able to eliminate them.

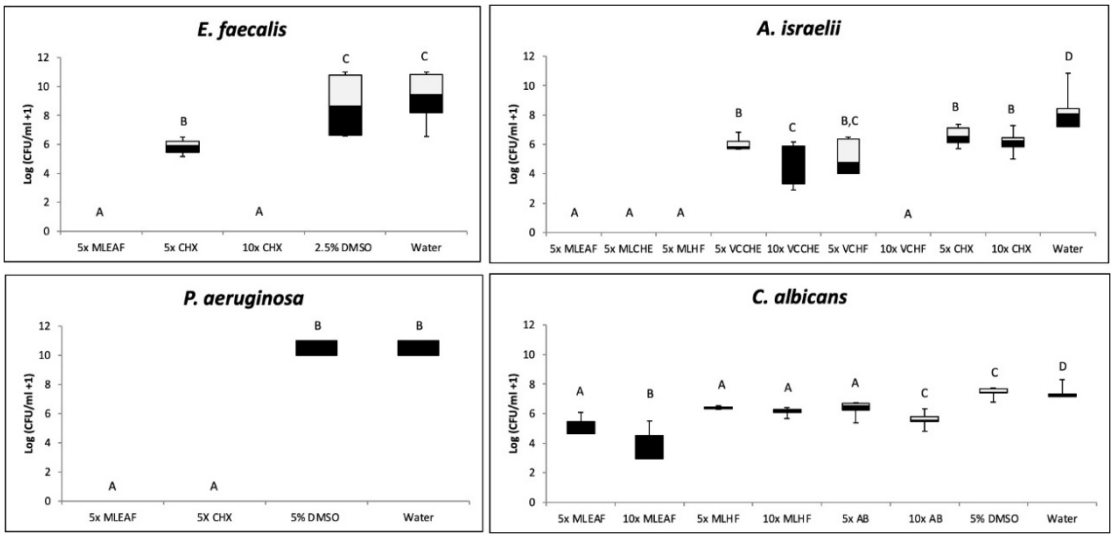

Figure 1. Box-whisker plots of the anti-biofilm activity of the extracts and fractions of *Mikania laevigata* and *Varronia curassavica* against pathogenic microorganisms. Different capital letters (A,B,C,D) show statistical difference among the groups, including the controls (DMSO and water), according to Mann-Whitney tests. Bars indicate minimum and maximum values. Black and white boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median MLCHE - *Mikania laevigata* crude Hydroethanolic extract, MLAF - *Mikania laevigata* Aqueous fraction, MLHF; *Mikania laevigata* Hexane fraction, MLBF - *Mikania laevigata* Butanol fraction, MLEAF - *Mikania laevigata* Ethyl acetate fraction, CHX - Chlorhexidine digluconate, AB - Amphotericin B, VCCHE – *Varronia curassavica* crude Hydroethanolic extract, VCAF - *Varronia curassavica* Aqueous fraction, VCHF - *Varronia curassavica* Hexane fraction, VCBF - *Varronia curassavica* Butanol fraction, VCEAF - *Varronia curassavica* Ethyl acetate fraction.

Following a similar antimicrobial methodology to that used in the current study, Yatsuda et al. (2005) found MIC and MBC/MFC values between 12.5 and 400 µg/ml for ML hydroethanolic extracts and hexane and ethyl acetate fractions against streptococci strains. The authors also observed that the extracts and fractions tested were able to inhibit the adherence of mutans streptococci cells to glass surfaces at sub-MIC levels (three times lower than their MIC values). The ML hexane fraction was the most effective antibacterial agent, displaying MIC and MBC values between 12.5 and 100 µg/ml. In the present study, the ML hexane fraction (MLHF) presented strong inhibitory activity against *A. israelii* (MIC = 330 µg/ml) and weak activity against *E. faecalis* (MIC = 4000 µg/ml) and *C. albicans* (MIC = 4000 µg/ml). The ML ethyl acetate fraction (MLEAF) was the most effective antimicrobial agent, showing activity for all microorganisms tested (MIC 250-4000 µg/ml). Although there are no standard values when considering the inhibition level of the plant materials, many authors have considered them as strong inhibitors (MIC lower than 500 µg/ml), moderate inhibitors (MIC between 600 µg/ml and 1500 µg/ml), and weak inhibitors (MIC above 1600 µg/ml) (dos Santos et al., 2006; Passari et al., 2014). Analyzing the ML composition through phytochemical screening, the major compounds isolated from ML extracts were coumarin, coumaric acid, terpenes, sesquiterpenes, diterpenes, and organic acids (Limberger et al., 2001). Coumarin 1,2benzopyrone is considered the main substance isolated from ML, and it can be used as a biomarker for pharmacological formulations (Sartoratto et al., 2004; Yatsuda et al., 2005). Secondary metabolites, such as the derivatives of cinnamic acid and kaurane diterpenes have also demonstrated synergic pharmacological effects (Sartoratto et al., 2004; Duarte et al., 2007).

One of the first studies to evaluate the antimicrobial activity of *Varronia curassavica* (VC) was developed by Carvalho et al. (2004) using the agar plate diffusion method. The authors found that VC essential oil was able to inhibit 88.8% of Gram-positive strains, 93.3% of yeast strains, including *Candida albicans,* and 20% of Gram-negative strains tested. VC essential oil inhibited *Enterococcus faecalis* growth with an MIC of 200 µg/ml (Meccia et al., 2009). In another study, *Pseudomonas aeruginosa* was sensitive, by the agar diffusion method, to 11 of

the 23 VC extracts, with the best results for the VC extract with 25% ethanol. In addition, the VC hydroethanolic extract inhibited *Pseudomonas aeruginosa* in MIC assays at a concentration of 1000µg/ml (Michielin et al., 2009). In the current study, *P. aeruginosa* and *C. albicans* growth were not affected by any of the VC extract/fractions tested, however, the VC crude extract and fractions exhibited effects on *E. faecalis* (MIC of 4000 µg/ml) and *A. israelii* growth (MIC between 410-4000 µg/ml). These discrepancies between our results and the literature might occur due to differences in the extraction methods and solvents used to obtain VC extracts. Other factors could be related to the collection site and season that the leaves were collected, which may influence the presence and concentrations of the plant metabolites in the extracts (Siqueira & Rôças, 2008). VC essential oil, obtained by the hydrodistillation method, showed inhibitory activity against *Staphylococcus aureus, Bacillus cereus, Candida albicans, Candida krusei, and Escherichia coli*, with MIC values between 64 and 512µg/ml (Rodrigues et al., 2012). Several investigators have studied the chemical composition of VC (Mello & Santos, 2002; Boussaada et al., 2008; Michielin et al., 2009), and the antibacterial activity of VC extracts has been associated with the presence of sesquiterpenes, such as bisabolol and farnesyl acetate 1, tannins, and flavonoids (Santos & Mello, 2002) or the presence of aromatic compounds, such as eugenol, detected in the VC ethanolic extract composition (Davino et al., 1989; Boussaada et al., 2008). A synergistic effect between bisabolol and eugenol could also explain the antimicrobial activity of VC (Boussaada et al., 2008; Michielin et al., 2009). In the present study, chlorhexidine digluconate and Amphotericin B were used as positive controls. CHX is the current gold standard for antibacterial tests (Gomes et al., 2013) and its effectiveness has been reported in biofilm assays (Matias et al.,2013). In addition, AB has been employed as positive control in studies on antifungal activity (Youssef et al., 2014).

Finally, considering the wide range of existing natural extracts native to South America, with diverse therapeutic properties (Tribess et al.,2015; Pereira et al., 2004), the current research shows the antimicrobial/antibiofilm effect and importance of two native plants. In addition, the selection of treatment using medicinal plants may be due to the availability of the local flora, but also because this type of treatment is considered healthier and more natural, with no adverse effects (Boccolini & Boccolini, 2020; Santos et al., 2011). However, further studies on the topic are still needed.

CONCLUSION

In summary, *Mikania laevigata* ethyl acetate fraction is an efficient antimicrobial/antibiofilm agent against resistant pathogens and could be indicated for the treatment of persistent dental infections.

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Author's contributions

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