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ARTICLE

In vitro antimicrobial activity of essential oils from *Heterothalamus* Less. (Asteraceae) against clinically relevant bacterial and fungal species

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ABSTRACT: (*In vitro* antimicrobial activity of essential oils from *Heterothalamus* Less. (Asteraceae) against clinically relevant bacterial and fungal species). Essential oils are natural, complex, and volatile compounds produced by the secondary metabolism of plants, which often serve as a protection against fungal or bacterial attack. Two species of the Asteraceae family, *Heterothalamus alienus* (Spreng.) O. Kuntze and *Heterothalamus psiadioides* Less., are used in folk medicine and recognised for their production of bioactive substances. However, little evidence of their antimicrobial properties currently exists. The aim of this study was to examine the *in vitro* antimicrobial activity of essential oils from *H. alienus* and *H. psiadioides* against clinically relevant bacterial and fungal species. The major chemical components of the essential oils used are terpene substances, of which β-pinene is the major component. Analysis of antibacterial activity was conducted using the disc diffusion method against 22 bacteria (18 gram-positive strains, including 13 enterococci strains resistant or multiresistant to antibiotics, and 4 Gram--negative strains) and 9 fungi (6 yeasts and 3 filamentous fungi). The results indicated that essential oils from *Heterothalamus* species have a strong antimicrobial effect against gram-positive bacteria and yeasts, variable activity against filamentous fungi, and no activity against gram-negative bacteria. The most prominent results were observed in the sensitivity of *Enterococcus faecalis* (antibiotic- and multidrug-resistant strains) to the essential oils. In conclusion, this study demonstrates the potential for essential oils from *Heterothalamus* species for use as both an antiseptic and disinfectant in future approaches to control of clinically relevant gram-positive bacteria and fungi.
Keywords: antimicrobial activity, essential oils, β-pinene, strains resistant to antibiotics, sensitivity.

RESUMO: (Atividade antimicrobiana *in vitro* de óleos essenciais de *Heterothalamus* Less. (Asteraceae) contra espécies bacterianas e fúngicas clinicamente relevantes). Óleos essenciais são compostos naturais, complexos e voláteis produzidos no metabolismo secundário de plantas, os quais muitas vezes servem como proteção contra o ataque de fungos ou bactérias. Duas espécies da família Asteraceae, *Heterothalamus alienus* (Spreng.) O. Kuntze e *Heterothalamus psiadioides* Less., são utilizadas na medicina popular e reconhecidas por suas substâncias bioativas. Entretanto, existem poucas evidências sobre suas proprieda--des antimicrobianas. O objetivo deste estudo foi avaliar a atividade antimicrobiana *in vitro* dos óleos essenciais de *H. alienus* e *H. psiadioides* contra cepas clinicamente relevantes de bactérias e fungos. Os principais componentes químicos dos óleos essenciais utilizados são as substâncias terpênicas, da qual o β-pineno é o componente majoritário. A análise da atividade antimicrobiana foi conduzida usando a técnica de difusão em disco contra 22 bactérias (18 gram-positivas, incluindo 13 enterococos resistentes ou multirresistentes a antibióticos, e 4 gram-negativas) e 9 fungos (6 leveduras e 3 fungos filamentosos). Nossos resultados indicaram que os óleos essenciais das espécies de *Heterothalamus* possuem forte atividade antimicrobiana contra bactérias gram-positivas e leveduras, atividade variável contra fungos filamentosos e nenhuma atividade contra bactérias gram-negativas. O resultado mais proeminentes observado foi a sensibilidade de *Enterococcus faecalis* resistentes e multirresistentes aos óleos essenciais. Em conclusão, este estudo demonstra o potencial dos óleos essenciais das espécies *Heterothalamus* para serem usados como antissépticos e desinfetantes, abordando o controle de bactérias gram-positivas e fungos de importância clínica.
Palavras-chave: β-pineno, atividade antimicrobiana, cepas resistentes a antibióticos, óleos essenciais, sensibilidade.

INTRODUCTION

Asteraceae is a widespread family of angiosperms which contains about 25 000 species. Plants of this family produce a large diversity of secondary metabolites that act as chemical defences, and have been extensively studied for their chemical composition and biological activity (Verdi *et al.* 2005, Funk *et al.* 2009). Among these compounds are the essential oils, natural aromatic substances extracted from plants which consist of volatile compound mixtures, of which the major chemical components include terpenes and their derivatives. Essential oils are used by humans for their antiseptic and healing properties, fragrance, for food preservation (Bakkali *et al.* 2008), and for their well-documented activities against microorganisms (Kim *et al.* 1995, Burt & Reinders 2003, Fisher & Phillips 2006, Sandasi *et al.* 2008, Cândido *et al.* 2010, Cavalcanti *et al.* 2011, Jadhav *et al.* 2013).

Heterothalamus Less. species are shrubs of Asteraceae family which are recognized as major producers of essential oils with potential biological activity (Schmidt-Silva *et al.* 2011). *Heterothalamus alienus* (Spreng.) O. Kuntze occurs in Southern Brazil, Uruguay, and Argentina, and is locally known as "*alecrim-do-campo*". Their leaves have traditionally been used as an antipyretic, stimulant, and

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antifungal (Rücker *et al.* 1996, Pacciaroni *et al.* 2008). *Heterothalamus psiadioides* Less. grows in southern Brazil and Uruguay, and is known as "*alecrim-do-campo*", "*vassoura*" or "*erva-formiga*." In folk medicine, it is used as an antipyretic, an antidote for snake venom, and an anti-inflammatory (Suyenaga *et al.* 2004, Biavatti *et al.* 2007). Previous research has indicated that the main chemical compound of essential oils from *Heterothalamus* species is β-pinene (Suyenaga *et al.* 2004, Duschatzky *et al.* 2007). Monoterpenes, such as α-pinene and β-pinene, are among the most widely distributed compounds in plants and represent the major components of various essential oils. These phytochemicals have been extensively studied, and are known to exhibit similar antimicrobial activity against gram-positive bacteria and fungi (Canillac & Mourey 2001, Leite *et al.* 2007, Moreira *et al.* 2007).

Plant products have received renewed interest in their use as an alternative source of antimicrobial compounds (Dormam & Deans 2000). The possibility exists for such phytochemicals to one day be incorporated into antimicrobial drugs prescribed by physicians (Cowan 1999). Such phytochemicals have been associated with popular knowledge regarding the use of medicinal plants, which contributed to the advancement of research in new natural plant products. However, according Newman & Cragg (2012), there is a current decline in the discovery of new natural compounds in the last thirty years. This decline is due to new techniques allowing for the modification of compounds already used for all diseases world-wide, which is of concern when considering the increased incidence of infections caused by antibiotic-resistant microorganisms throughout the world.

In Brazil, numerous studies have been conducted to evaluate the inhibitory activity of essential oils from plants against microorganisms, but few reports evaluating the activity of *H. alienus* and *H. psiadioides* against bacteria and fungi to date. Currently, only the essential oil from *H. psiadioides* has been tested against *Listeria monocytogenes* strains (Ellwanger *et al.* 2015), and the methanol extracts from the leaves and roots of *H. alienus* were tested against moulds (Pacciaroni 2008). The aim of this study is to evaluate the antimicrobial activity of the essential oils from these two species against gram-positive and gram-negative bacteria, as well as yeast and mould strains.

MATERIAL AND METHODS

Plant material and essential oil

Leaves from *H. alienus* were collected in April 2008 in Bagé City (31° 19' S 54° 6' W), Rio Grande do Sul, Brazil, and leaves from *H. psiadioides* were collected in April 2012 in Porto Alegre City (30° 3' S 51° 7' W), Rio Grande do Sul, Brazil. The specimens have been deposited in the ICN Herbarium of the "Universidade Federal do Rio Grande do Sul" (UFRGS) under the voucher numbers ICN 153825 and ICN 175007, respectively. Fresh leaves were dried at room temperature. The essential oils of *H. alienus* and *H. psiadioides* were obtained by hydrodistillation using a modified Clevenger apparatus for 4 hours. The oil obtained was dried on anhydrous sodium sulphate and stored at -80 °C prior to use.

The chemical characterization of essential oils used in this study was determined by GC/MS – GC/FID analysis. The chemical composition analysis of essential oils from *H. alienus* and *H. psiadioides* was performed by Schmidt-Silva (2012) and Lazarotto (2014), respectively. Table 1 describes the main constituents of these essential oils.

Bacterial and fungal strains

A total of 22 clinically relevant bacterial strains were tested, including: nine reference strains (*Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212, *Staphylococcus epidermidis* ATCC 35984, *Streptococcus agalactiae* ATCC 13813, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* INCPS 0031, *Pseudomonas* sp. DEMIC 01, and *Salmonella* sp. DEMIC 02) and 13 antibiotic-resistant *E. faecalis* strains isolated from food, enviromental, and clinical samples (Tables 2 and 3). Additionally, six yeasts (*Candida albicans* ATCC 18804, *Candida glabrata* ATCC 40136, *Candida tropicalis* 24P, *Candida krusei* ATCC 6258, *Candida famata* RL08, and *Trichosporon asahii* TAH05) and three filamentous fungi (*Microsporum canis* 33, *Microsporum gypseum* 45, and *Trichophyton rubrum* 42) were tested. The bacterial and fungal strains were obtained from the collection of the Microbiology Department of UFRGS and Prof. Dr. Alexandre Meneguello Fuentefria from UFRGS School of Pharmacy, respectively. The microorganisms were individually stored in brain heart infusion broth (BHIB, Oxoid) or sabouraud broth (SB, HiMedia) containing

Table 1. Chemical composition of essential oils from leaves of *Heterothalamus alienus* and *Heterothalamus psiadioides* by GC-MS and GC-FID.

	Heterothalamus alienus[2]	***Heterothalamus psiadioides***[3]
Compounds[1]	**Area (%)**	**Area (%)**
α-pinene	4.24	4.41
β-pinene	40.8	44.65
Myrcene	1.09	1.91
Limonene	6.86	6.50
Trans-ocimene	5.37	2.30
Compounds identified		
Monoterpene hydrocarbons	58.51	63.17
Oxygenated monoterpenes	12.33	3.27
Sesquiterpene hydrocarbons	17.55	10.85
Oxygenated sesquiterpenes	2.94	0.92
Total identified	**91.33**	**78.21**

1. Compounds with more than 1% relative percentage. 2. Chemical composition performed by Schmidt-Silva (2012). 3. Chemical composition performed by Lazarotto (2014).

Table 2. Antimicrobial activity of essential oils from *Heterothalamus alienus* and *Heterothalamus psiadioides* against reference strains of microorganisms by disc diffusion assay. *Sensitivity classifications: (-) not sensitive = for diameters less than 8 mm; (+) sensitive = diameters of 9 –14 mm; (++) very sensitive = diameters of 15 – 19 mm; and (+++) extremely sensitive = diameters greater than 20 mm. ND: No determinate.

Reference strains	Sensitivity to essential oil of *	
	H. alienus	*H. psiadioides*
Gram-positive bacteria		
Enterococcus faecalis ATCC 29212	+ +	+ + +
Staphylococcus epidermidis ATCC 35984	ND	+ +
Bacillus cereus ATCC 14579	+ + +	+ + +
Streptococcus agalactiae ATCC 13813	+ +	+ + +
Listeria monocytogenes ATCC 7644	ND	+ + +
Gram-negative bacteria		
Pseudomonas sp. DEMIC 01	-	-
Salmonella sp. DEMIC 02	-	-
Escherichia coli INCPS 0031	-	-
Acinetobacter baumanii ATCC 19606	-	-
Yeasts		
Candida albicans ATCC 18804	+ +	+
Candida glabrata ATCC 40136	+ + +	+ +
Candida tropicalis 24P	+ +	+
Candida krusei 6258	+ + +	+ +
Candida famata RL08	+ + +	+ +
Trichosporon asahii TAH05	+ + +	+
Filamentous fungi		
Microsporum canis 33	-	-
Microsporum gypseum 45	-	+ + +
Trichophyton rubrum 42	+ +	-

50% (v/v) of glycerol and maintained frozen at -20 °C.

Prior to each experiment, an aliquot of frozen bacterial cells was recovered onto brain heart infusion agar (BHIA, Oxoid). For fungus an aliquot was recovered on agar sabouraud (AS, HiMedia) with 2% glucose and 0.4% chloramphenicol. Bacteria and yeast were incubated at 37 °C for 24 h and filamentous fungi were incubated for seven days at room temperature. For the experimental procedures, a loopful of BHI or AS cultured with each isolate was dispersed in sterile 0.9% saline solution (w/v) until matched to the 0.5 McFarland turbidity standards (approximately $1x10^8$ CFU/mL).

Determination of antimicrobial activity

Antimicrobial activity of essential oils from *H. alienus* and *H. psiadioides* was investigated by the agar disc diffusion method, as described by Ponce *et al.* (2003). The inoculums adjusted to a 0.5 MacFarland standard were uniformly spread on the surface of Müeller-Hinton Agar (MH, HiMedia) plates for bacteria, and on MH with 2 % glucose and 0.4 % chloramphenicol for fungi. Sterile filter paper discs of 6 mm, impregnated with 20 µL of pure essential oil from *H. alienus* or *H. psiadioides*, were placed on the surface of the culture medium at the centre of the dish. The plates were subsequently incubated at 37 °C for 24 h for bacteria and yeasts, and for seven days at room temperature for filamentous fungi. Following the

Table 3. Antimicrobial activity of the essential oils from *Heterothalamus alienus* and *Heterothalamus psiadioides* against antibiotic-resistant *E. faecalis* strains by disc diffusion assay. 1. Ba (Bacitracin); Cp (Ciprofloxacin); Cl (Chloramphenicol); Eri (Erythromycin); St (Streptomycin); Gn (Gentamicin); Nf (Norfloxacin); Tt (Tetracycline); Vn (Vancomycin). 2. Sensitivity classifications: (+) sensitive = diameters of 9 –14 mm; (++) very sensitive = diameters of 15 – 19 mm; and (+++) extremely sensitive = diameters greater than 20 mm. ND: No determinate.

E. faecalis strains	Source	Resistance profile[1]	Sensitivity to essential oil of[2]	
			H. alienus	*H. psiadioides*
ATCC29212		-	+ +	+ + +
A8	Food	Er	+ + +	+ + +
I8b	Food	Ba, Tt	+ +	+ + +
G12	Food	E, Gn, Tt	ND	+ + +
LB	Food	Cl, St, Er, Nf, Tt	ND	+ +
114	Food	Ba, Tt	+ +	+ + +
378	Food	Tt	+ + +	+ + +
3.3	Environment	Er, Tt	+ +	+ +
4.16	Environment	Cl, Er, Tt	+	+ + +
3.20	Environment	Cp	ND	+ + +
488	Clinical	Cp, Te	ND	+ + +
593	Clinical	Er, Nf	+	+ +
612	Clinical	Cp, Cl, Er, Nf	+	+ +
2389	Clinical	Vn	ND	+

incubation period, antimicrobial activity was evaluated by measuring the inhibition zone. Each assay was carried out on two independent occasions. The sensitivity to oils was classified by the diameter of inhibition as: not sensitive (-) for diameters less than 8 mm; sensitive (+) for diameters of 9 – 14 mm; very sensitive (++) for diameters of 15 – 19 mm; and extremely sensitive (+++) for diameters greater than 20 mm.

Ten antimicrobials commonly used in the treatment of clinical infection and agricultural procedures were used to confirm the antimicrobial resistance profiles of enterococci isolates (concentrations are expressed in µg ml^{-1} and units): vancomycin (30), erythromycin (15), tetracycline (30), ciprofloxacin (5), norfloxacin (10), chloramphenicol (30), gentamicin (120), bacitracin (0.041U), and streptomycin (300). Sterile distilled water was used as negative control.

RESULTS AND DISCUSSION

Tables 2 and 3 outline the antimicrobial activity of essential oils from *H. alienus* and *H. psiadioides* against the microorganisms tested. The results obtained in this study indicate that, of the organisms evaluated, essential oils exhibited *in vitro* activity against eighteen gram-positive bacteria, six yeasts, and two filamentous fungi. *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 14579, *Streptococcus agalactiae* ATCC 13813, *Listeria monocytogenes* ATCC 7644, *Microsporum gypseum* 45, and eight antibiotic-resistant and multiresistant enterococci strains were found to be very or extremely sensitive to the essential oil of *H. psiadioides*, with inhibition zone diameter ranges of 16 - 50 mm. Moreover, *Bacillus cereus*

ATCC 14579, *Candida glabrata* ATCC 40136, *Candida krusei* 6258, *Candida famata* RL08, *Trichosporon asahii* TAH05, and two resistant enterococcus strains (A8 and 378), were very or extremely sensitive to the essential oil from *H. alienus*, with a range of inhibition zone diameter ranges of 17 - 29 mm. *Staphylococcus epidermidis* ATCC 35984 and multiresistant *E. faecalis* strains (LB, 3.3, 593, and 612) were very sensitive to the action of *H. psiadioides* essential oil. The fungi *Candida albicans* ATCC 18804, *Candida tropicalis* 24P, and *Trichophyton rubrum* 42 were very sensitive to the essential oil of *H. alienus*. The vancomicyn-resistant strain (VRE) 2389 was sensitive to the essential oil of *H. psiadioides*. However, the essential oils of both species studied did not exhibit any activity against *Pseudomonas* sp. DEMIC 01, *Salmonella* sp. DEMIC 02, *Escherichia coli* INCPS 0031, *Acinetobacter baumannii* ATCC 19606, and *Microsporum canis* 33.

The most prominent result was observed against gram-positive strains, particularly in the inhibition of antibiotic-resistant or multiresistant *E. faecalis* strains by the essential oils tested. The majority of tested strains were classified as being either very or extremely sensitive to the essential oils. Enterococci, Staphylococci, and Streptococci are important human pathogens associated with a number of opportunistic infections in hospitalised patients. In fact, enterococci and staphylococci are often isolated from medical devices such as intravenous catheters (Otto 2010, Kiss *et al.* 2013). One of the most important results obtained in this study was that the enterococcus vancomicyn-resistant strain 2389 was sensitive to the essential oil of *H. psiadioides*. This represents a very significant result, since vancomycin is considered to be the last alternative for the treatment of infections caused by multidrug-resistant gram-positive bacteria such as enterococci and staphylococci**.** Vancomycin-resistant enterococci are a major cause of infection in the hospital setting. Infection with VRE affects patients in intensive care units, those with intravascular or bladder catheter devices, immunosuppressed patients, those experiencing prolonged hospitalization. Treatment of diseases caused by resistant gram-positive bacteria requires the appropriate use of available antibiotics and management to prolong their effectiveness (Rivera & Boucher 2011).

Other gram-positive bacteria which were very sensitive to the essential oils tested were *B. cereus* and *L. monocytogenes*. These bacteria have been categorized as food spoilage and foodborne pathogens, respectively (Gandhi & Chikindas 2007, Sandasi *et al.* 2010). The essential oils tested in this study may provide alternatives to conventional antimicrobial additives in foods. These findings are in accordance with Ellwanger *et al.* (2015), which demonstrated that the essential oil of *H. psiadioides* could prevent the growth of different serovars of *L. monocytogenes* via the agar disc diffusion method.

In comparison to some reported literature, the results of this study indicate that gram-negative bacteria were more resistant to essential oils than gram-positive bacteria. Some authors report that gram-negative bacteria are somewhat less sensitive to essential oils due to their highly hydrophilic surface, which blocks the penetration of hydrophobic compounds and prevents the accumulation of essential oils into the cells membrane . The lipoteichoic acids of gram-positive cell membranes facilitate the entry of hydrophobic compounds (Canillac & Mourey 2001, Bezic *et al.* 2003). Other reports suggest that gram-negative bacteria are susceptible to essential oils with high amounts of phenolic compounds (Burt & Reinders 2003, Bilge *et al.* 2010, Cândido 2010), which are not present in the essential oils of *Heterothalamus*.

All yeasts tested were somewhat sensitive to essential oils, and the inhibition zones were slightly larger for the essential oil of *H. alienus* when compared to *H. psiadioides*. Some studies have reported that the possible target of action of essential oil components against fungi is the cell plasma membrane. Ahmad *et al.* (2011) demonstrated that essential oils can cause changes in the cytoplasmic membrane of *Candida* cells and also decrease the production of ergosterol, resulting in injury to the membrane and cell death. Other research with essential oils and their components against *Candida* sp. suggests a decrease in metabolic activity in standard and clinical strains (Grumezescu *et al.* 2012, Palmeira-de-Oliveira *et al.* 2012, Silva *et al.* 2012).

The filamentous fungi *T. rubrum* 42 and *M. gypseum* 45 were very and extremely sensitive to the essential oils from *H. alienus* and *H. psiadioides*, respectively. In contrast, *M. canis* 33 was unaffected by the essential oils. Moreira *et al.* (2007) demonstrated that filamentous fungi can be inhibited by essential oils, of which β-pinene is one of its major compounds, even after 14 days of incubation. This divergent data for filamentous fungi suggests that perhaps not only the chemical composition of the essential oil may influence the antimicrobial activity, but also the characteristics of the strains evaluated may have an influence as well.

In accordance with previous studies, the essential oils from *Heterothalamus* evaluated in this study contain mainly monoterpenes, such as β-pinene (Suyenaga *et al.* 2004, Duschatzky *et al.* 2007). In plants, these compounds possess a broad spectrum of biological functions, including chemical defense and allelopathy (Ding *et al.* 2010). Leite *et al.* (2007) demonstrated that α-pinene and β-pinene inhibited the growth of species of gram-positive cocci. These two phytochemicals represent the main components of *Heterothalamus* essential oils, indicating that these phytochemicals may be responsible for the antimicrobial activity of *Heterothalamus*. Monoterpenes have been known to interfere with the cell membrane functions in bacteria, penetrating into the interior of cells and interacting with the intracellular sites, eventually cause cell death (Oliveira *et al.* 2010). Although the chemical composition is similar between both *Heterothalamus* essential oils, variations in the concentrations of the components may have influenced the results found in this initial screening – especially when comparing inhibition halos for the same microorganism.

In conclusion, this study demonstrated that the essential oils of *H. alienus* and *H. psiadioides* have a potential use in the control of some important pathogens, and that these substances could become promising alternatives for the current antimicrobials.

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