

***In vitro* evaluation of anticaries, antimycobacterial, antileishmanial and cytotoxic activities of essential oils from *Eremanthus erythropappus* and of α -bisabolol, their major sesquiterpene**

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Abstract

Interest in researches into medicinal plants and therapeutic effects of essential oils (EOs) on humans has increased over the last few years. *Eremanthus erythropappus*, known as *candeia*, is a Brazilian aromatic herbaceous plant whose α -bisabolol-rich oil has been used in several cosmetic preparations. This paper reports *in vitro* anticaries, antimycobacterial, antileishmanial and cytotoxic activities of EOs from *E. erythropappus* leaves (EL-EO) and stalks (ES-EO), besides α -bisabolol, their main sesquiterpene. EL-EO and ES-EO were extracted by hydrodistillation and analyzed by GC-FID and GC-MS. α -Bisabolol, *cis*- α -bisabolene and β -bisabolene were identified as their major constituents. Antibacterial activity of EOs was evaluated against eight standard strains of pathogens from the American Type Culture Collection (ATCC) by determining minimum inhibitory concentrations (MICs) with the use of the microdilution method. Antibacterial activity was evaluated against *Streptococcus mutans*, *S. mitis*, *S. sanguinis*, *S. sobrinus*, *S. salivarius*, *Mycobacterium tuberculosis*, *M. avium* and *M. kansasii*. EL-EO, ES-EO and α -bisabolol exhibited high leishmanicidal activity against promastigote forms of *Leishmania amazonensis*; IC₅₀ values were 9.22 μ g/mL, 6.00 μ g/mL and 3.12 μ g/mL, respectively. The 50% cytotoxic concentrations (CC₅₀) of EL-EO, ES-EO and α -bisabolol against mouse peritoneal macrophages were 24.65 μ g/mL, 8.87 μ g/mL and 1021.00 μ g/mL, respectively. These results suggest that EOs from *E. erythropappus* seem to be very promising for the development of new bactericidal and leishmanicidal agents.

Keywords: *Eremanthus erythropappus*, essential oils, genus *Mycobacterium*, α -bisabolol, *Leishmania amazonensis*, *Streptococcus mutans*.

Abbreviations: GC-FID - gas chromatography-flame ionization detector; GC-MS - gas chromatography-mass spectrometry; EOs - essential oils; MIC - minimum inhibitory concentration; DMSO - dimethylsulfoxide; NCCLS - National Committee for Clinical Laboratory Standards.

Introduction

The family Asteraceae, which has about 1,300 genera and 23,000 species, comprises mostly herbs, shrubs and small trees with leaves and stalks whose secretory structures, such as trichomes, secrete essential oils (EOs) (Rios, 2015). These EOs, especially the ones from *Eremanthus erythropappus* (Asteraceae), are rich in α -bisabolol, a fact that gives them great economic importance, because this sesquiterpene is used for the formulation of several cosmetic products (Scolforo et al., 2016). Extracts and EOs from *E. erythropappus* have been known for their important

biological activities, such as antimicrobial ones against several pathogens – *Alternaria carthami*, *Rhizoctonia solani*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Candida spp.* and *Cryptococcus spp.* –, besides antinociceptive, anti-inflammatory, schistosomicidal, antiedematogenic and antiulcerogenic ones (Hillen et al., 2012; Nascimento et al., 2007; Silvério et al., 2013; Santos et al., 2015; Sousa et al., 2008a; Almeida et al., 2012; Keles et al., 2010). However, anticaries, antimycobacterial and

antileishmanial activities of EOs from *E. erythropappus* have not been investigated yet.

Dental caries has been a major public health concern worldwide. This pathology is associated with a range of bacteria, including *Streptococcus mutans*, *S. mitis*, *S. sanguinis*, *S. sobrinus* and *Lactobacillus casei*, which adhere to the tooth surface. It is located on the hard tissues of teeth as the result of bacterial accumulation, which leads to biofilm development, and of its metabolism on tooth surfaces. It may systematically be explained by demineralization of the inorganic part of teeth (enamel) and by degradation of organic substances (dentin). It consists in an intermittent process that may advance as the result of several remission and recurrence phases which may result in total destruction of a tooth when the disease is not treated (Santiago et al., 2018).

Current antibacterial agents used for treating oral health problems have led to different side effects, such as diarrhea and vomit. Increase in bacterial resistance to drugs and the high cost of standard therapeutic procedures have also been causes for concern. Thus, new therapeutic agents must be explored and the search for bioactive natural products from distinct plant sources must be broadened. Therefore, the antibacterial potential of EOs extracted from different plant species has been recently highlighted and led to increase in researchers' interest in studies of EOs worldwide (Dagli et al., 2015).

Tuberculosis, which is caused by *Mycobacterium tuberculosis*, has been the major infectious and contagious disease of bacterial origin in the world, since it accounts for the death of two million people a year (Wang et al., 2010). In addition to *M. tuberculosis*, other nontuberculous mycobacteria, such as *M. avium* and *M. kansasii*, are also important because they affect lungs, lymph, skin and joints and lead to severe sequels if not treated properly (Alves et al., 2015). Plants have always been considered sources of effective chemotherapeutic agents against several infectious diseases. Since different plant species have shown significant *in vitro* antimycobacterial activity, more researches have been carried out all over the world to isolate new antimycobacterial agents from natural products. Among them, EOs have stood out due to their distinct biological applications (Hozoorbakhsh et al., 2016).

Leishmaniasis, which is a non-contagious infectious disease caused by protozoa of the genus *Leishmania*, has been endemic in 88 countries and has affected about 12 million people worldwide (Miranda et al., 2013). Treatment is based on pentavalent antimonials and pentamidines, which are not only toxic, expensive and hard to administer, but can also generate resistant parasites (Miranda et al., 2013). Thus, results achieved by these drugs have been considered unsatisfactory. Alternative treatments of leishmaniasis include amphotericin B and its lipid formulations, as well as paromomycin and miltefosine. However, high costs, toxic side effects and long treatment periods have limited the clinical use of these drugs (Ribeiro et al., 2014). As a result, EOs have become a new therapeutic alternative which has been broadly studied *in vitro* to reach this goal. Besides being natural alternatives, they use effective strategies and ensure their activities against different types of parasites, even against the causative agent of leishmaniasis (Pereira et al., 2017).

Considering that natural products play a significant role in therapeutics and are important targets for the development of new drugs, this study aimed to verify the chemical composition and *in vitro* anticaries, antimycobacterial, leishmanicidal and cytotoxic activities of EOs from *E. erythropappus* leaves and stalks, and of α -bisabolol, their major constituent.

Results and Discussion

EOs extracted from *E. erythropappus* leaves and stalks yielded $2.20\pm 0.35\%$ and $3.25\pm 0.36\%$ (w/w), respectively. GC-MS and GC-FID analyses identified eleven compounds in EOs from *E. erythropappus* leaves and sixteen compounds in EOs from stalks, i. e., 97.1% and 97.4% of total compounds, respectively (Table 1). Table 1 shows constituents with their retention indices, retention times and percentages. Oxygenated sesquiterpenes were the main constituents of EL-EO (69.1%) and ES-EO (73.2%). Major chemical constituents identified in EL-EO were α -bisabolol (65.7%), *cis*- α -bisabolene (8.6%) and β -bisabolene (8.0%), while in ES-EO, they were α -bisabolol (67.7%) and *cis*- α -bisabolene (7.9%) (Table 1). The high content of α -bisabolol in EOs from *E. erythropappus* has been recently validated by a quantitative analysis carried out by the ^1H NMR method (Cerceanu et al., 2016).

Previous reports of EOs extracted from other *E. erythropappus* specimens showed that terpenes predominate in the oils and that the chemical composition of EOs varies significantly, depending on the origin of the plant. For example, α -bisabolol concentration was found to be low in EOs extracted from *E. erythropappus* leaves and flowers found in Belo Horizonte, Minas Gerais state (MG), Brazil (Lima et al., 2013). Two studies of EOs from *E. erythropappus* collected in Juiz de Fora, MG, showed different chemical composition from the one found by this study. Four major constituents were identified in plants from Belo Horizonte – β -pinene, β -caryophyllene, β -myrcene and germacrene D (Sousa et al., 2008b) –, whereas, in plants from Juiz de Fora, α -bisabolol was found to be the major constituent in branches, but not in leaves (Silvério et al., 2013). Results of the study described by this paper corroborate a previous study which quantified and identified the main chemical compounds of volatile oils extracted from *E. erythropappus* grown in Pouso Alegre and Caeté, two cities located in MG, Brazil (Santos et al., 2015). However, a significant difference was found in relation to the major constituent δ -elemene, which was not identified in EOs from *E. erythropappus* in Aiuruoca, MG, Brazil.

This study investigated *in vitro* anticaries activity of EL-EO, ES-EO and α -bisabolol against the main cariogenic bacteria in terms of their minimum inhibitory concentrations (MICs), by comparison with chlorhexidine dihydrochloride (CHD, positive control). Table 2 summarizes results of MICs. EL-EO, ES-EO and α -bisabolol provided MICs which ranged from 50 to 250 $\mu\text{g}/\text{mL}$ against the main causative agents of dental caries. The lowest MICs of EL-EO, ES-EO and α -bisabolol were found against *Streptococcus mutans*, i. e., 62.5 $\mu\text{g}/\text{mL}$, 62.5 $\mu\text{g}/\text{mL}$ and 50 $\mu\text{g}/\text{mL}$, respectively. Against *S. mitis*, *S. sanguinis*, *S. sobrinus* and *S. salivarius*, EL-EO, ES-EO and α -bisabolol were considered moderately active, since they showed MICs between 100 and 250 $\mu\text{g}/\text{mL}$.

According to some authors, EOs whose MICs are above 1 mg/mL, are considered poorly active; MIC < 100 µg/mL means good activity; 100 < MIC < 500 µg/mL represents moderate activity; 500 < MIC < 1000 µg/mL shows weak activity; and MIC > 1000 µg/mL is inactive. Therefore, EOs whose MICs are below 100 µg/mL seem very interesting and promising in the search for new antimicrobial agents (Rios & Recio 2005; Dias et al., 2017). Taking into account all cariogenic bacteria under investigation, EL-EO (MIC = 62.5 µg/mL), ES-EO (MIC = 62.5 µg/mL) and α-bisabolol (MIC = 50 µg/mL) exhibited the lowest values of MICs against *S. mutans* (Table 2). It is a noteworthy result because very few natural compounds have been known to inhibit this microorganism, which is one of the primary causative agents of dental caries (Porto et al., 2009; Saleem et al., 2010).

Regarding antimycobacterial activity, since few studies mention the application of EOs to fight mycobacteria (Alvarenga et al., 2014), the study reported by this paper advocates that it is relevant to carry out *in vitro* evaluations of this biological activity. Therefore, *in vitro* antimycobacterial activity of EL-EO, ES-EO and α-bisabolol against *Mycobacterium tuberculosis*, *M. kansasii* and *M. avium* was investigated by determining minimum inhibitory concentrations (MICs, Table 3).

EL-EO, ES-EO and α-bisabolol showed MICs between 20 µg/mL and 1000 µg/mL (Table 3). EL-EO and ES-EO were active against *M. kansasii* (MIC = 250 µg/mL), moderately active against *M. tuberculosis* (MIC = 500 µg/mL) and inactive against *M. avium* (MIC = 1000 µg/mL). The constituent α-bisabolol was highly active against *M. tuberculosis* (MIC = 20 µg/mL) and active against *M. kansasii* and *M. avium* (MIC = 150 µg/mL). The literature reports that EOs whose MIC values are 500 µg/mL and 250 µg/mL are considered moderately active and active, respectively, whereas values from 1000 to 2000 µg/mL show that they are poorly active against mycobacteria under evaluation (Alves et al., 2015).

Promising anticaries and antimycobacterial activities of EL-EO and ES-EO found by this study can be explained by their high concentration of α-bisabolol. Antibacterial activity of α-bisabolol has been well known and reported by the literature. Besides, several other important biological activities have also been attributed to this sesquiterpene (Murungan and Mallavaparu 2013; Kamatou and Vilajoen 2010). Two isomers of bisabolol (β-bisabolol and α-bisabolol), which make up about 70% of EL-EO and ES-EO, were found. Bisabolol is a nontoxic substance that has anti-inflammatory, antibacterial and healing properties. Therefore, it has mainly been used by the pharmaceutical industry. Because of its anti-inflammatory properties, it reduces skin redness caused by excessive exposure to ultraviolet (UV) radiation. Due to its antibacterial activity, bisabolol has also been used for treating bromhidrosis and decrease bacterial concentration in affected areas (Martins et al., 2015a). In addition, antimycobacterial activity of α-bisabolol was previously examined and results showed that it exhibited high antibacterial activity against *Mycobacterium tuberculosis*, i. e., MIC = 16 µg/mL (Sieniawska et al., 2015). The mechanisms of action that explain biological activities of EOs may also be associated with the synergic effect and hydrophobicity of terpenoids, since they allow these compounds to permeate cell membranes easily and lead to

consequent death of parasites and microorganisms by affecting either their metabolic pathways or their organelles (Vieira et al., 2017).

The *in vitro* leishmanicidal potential of EOs has been well studied (Tariku et al., 2011) and EL-EO, ES-EO and α-bisabolol have exhibited high leishmanicidal activity when tested against *L. amazonensis* promastigote forms. Increase in parasite lysis was observed with increase in EOs concentration, at the following IC₅₀ values: 9.22 µg/mL (EL-EO), 6.00 µg/mL (ES-EO), 3.12 µg/mL (α-bisabolol) and 1.88 µg/mL (amphotericin B) (Table 4). EOs from *E. erythropappus* inhibited parasite growth in a concentration/dose-dependent manner. Amphotericin B (IC₅₀ = 1.88 µg/mL) was used as positive control. Due to its broad-spectrum antifungal activity, it has been used as a second-line drug against leishmaniasis, since it shows high toxicity against the host (Fernández-García et al., 2017).

Previous studies of antiparasitic activities have established that EOs can be highly active (IC₅₀ < 10 µg/mL), active (10 < IC₅₀ < 50 µg/mL), moderately active (50 < IC₅₀ < 100 µg/mL) or inactive (IC₅₀ > 100 µg/mL) (Estevam et al., 2017).

Not only EL-EO and ES-EO exhibit strong leishmanicidal activity, but also other species of the family Asteraceae stand out as producers of active EOs against parasites of the genus *Leishmania*: *Artemisia campestris* (Aloui et al., 2016), *Artemisia herba-alba* (Tamargo et al., 2017), *Artemisia absinthium* (Tamargo et al., 2017), *Artemisia abyssinica* (Tariku et al., 2010), *Vernonia brasiliensis* (Martins et al., 2015b) and *Vernonia polyanthes* (Moreira et al., 2017).

Leishmanicidal activity exhibited by EOs from *E. erythropappus* can be explained by the high concentration of α-bisabolol, which had also been previously identified in EOs from *Vanillosmopsis arborea*. Colares et al., (2013) tested pure α-bisabolol and reported its activity against *Leishmania amazonensis*, thus corroborating the findings of this study.

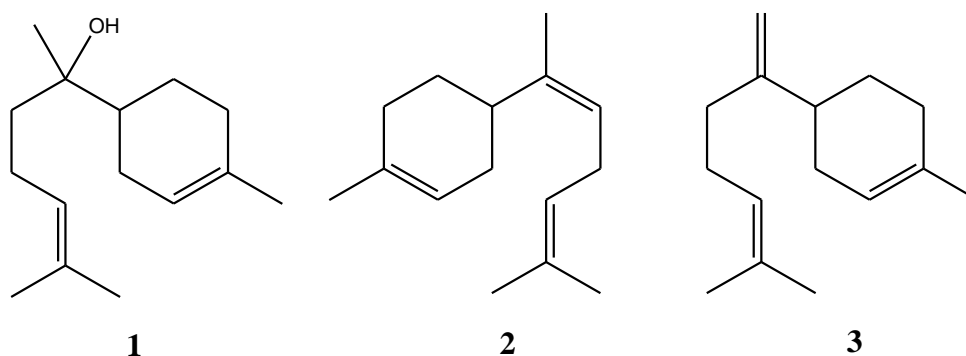
Table 5 shows *in vitro* cytotoxic activity of EOs from *E. erythropappus*, α-bisabolol and amphotericin B against peritoneal macrophages. EL-EO (CC₅₀ = 24.65 µg/mL) and ES-EO (CC₅₀ = 8.87 µg/mL) evaluated by this study showed high toxicity against mouse peritoneal macrophages, while amphotericin B was less toxic (CC₅₀ = 51.86 µg/mL) and α-bisabolol was nontoxic (CC₅₀ = 1021.00 µg/mL). Toxicity levels have been reported in the literature as highly toxic (CC₅₀ < 10 µg/mL), toxic (10 < CC₅₀ < 100 µg/mL), moderately toxic (100 < CC₅₀ < 1000 µg/mL) and nontoxic (CC₅₀ > 1000 µg/mL) (De Andrade et al., 2018).

In sum, the strong antileishmanial activity and the toxicity profile of EOs from *E. erythropappus* may be intrinsically related to the chemical constituents of EOs, which have no specific cellular targets. Hydrophobicity of EOs allows their constituents to pass through the cell wall and the cytoplasmic membrane easily; it affects the structure and leads to cell lysis (Andrade et al., 2016). Once constituents of the oil pass through the cell membrane, they can make the cytoplasm coagulate and interrupt specific metabolic pathways, including the biosynthesis of various lipids. Other findings include increased nitric oxide production in infected cells and depolarization of mitochondrial membranes, resulting in cell death due to necrosis and apoptosis (Rodrigues et al., 2013).

Table 1. Chemical composition of EOs from *E. erythropappus* (Asteraceae) leaves (EL-EO) and stalks (ES-EO).

RT (min)	Compounds	R _{l_{exp}}	R _{l_{lit}}	EL-EO	ES-EO
28.83	Eugenol	1364	1356	-	0.2
30.12	β-Elemene	1393	1391	-	0.3
31.92	α-trans-Bergamotene	1437	1434	0.7	0.6
32.97	α-Acoradiene	1463	1463	-	0.5
33.72	γ-Curcumene	1481	1479	1.7	2.5
33.86	α-Curcumene	1484	1481	0.7	-
34.08	β-Selinene	1490	1485	1.1	0.4
34.44	α-Selinene	1499	1494	0.4	0.9
34.97	β-Bisabolene	1513	1509	8.0	7.5
35.24	γ-Cadinene	1520	1516	4.6	1.8
35.85	trans-γ-Bisabolene	1535	1541	2.2	1.6
36.32	cis-α-Bisabolene	1548	1536	8.6	7.9
38.52	Caryophyllene oxide	1605	1606	-	0.2
40.60	β-Bisabolol	1674	1668	2.5	3.9
42.23	α-Bisabolol	1702	1683	65.7	67.7
44.88	Valerenol	1729	1736	-	0.2
45.13	Glaucyl alcohol	1731	1732	-	0.4
	Sesquiterpene hydrocarbons			28.0	24.0
	Oxygenated sesquiterpenes			69.1	73.2
	Others			-	0.2
	Total			97.1	97.4

R_{l_{exp}}: Retention index relative to *n*-alkanes (C₈-C₂₀) on the Rtx-5MS column; R_{l_{lit}}: Retention index found in the literature (Adams 2007). (-) = not detected.

**Fig 1.** Major chemical constituents identified in EOs from *Eremanthus erythropappus* (Asteraceae) leaves and stalks: α-bisabolol (1), cis-α-bisabolene (2) and β-bisabolene (3).**Table 2.** *In vitro* anticaries activity of EOs from *E. erythropappus* and α-bisabolol against aerobic oral bacteria.

Minimum inhibitory concentration (MIC - µg/mL)				
Microorganisms				
	EL-EO	ES-EO	α-bisabolol	CHD*
Aerobic				

Gram-positive bacteria; CHD: chlorhexidine dihydrochloride (positive control)

Table 3. *In vitro* antimycobacterial activity of EOs from *E. erythropappus* and α-bisabolol (MIC = µg/mL) against *Mycobacterium tuberculosis*, *M. kansasii* and *M. avium*.

	<i>M. tuberculosis</i>	<i>M. kansasii</i>	<i>M. avium</i>
EL-EO	500	250	1000
ES-EO	500	250	1000
α-bisabolol	20	150	150
Isoniazid*	0.06	1	> 1

*Positive control

Table 4. *In vitro* leishmanicidal activity of EOs from *E. erythropappus* and α -bisabolol against *L. amazonensis* promastigote forms. *Positive control (Amph. B = Amphotericin B).

	% of lysis \pm S.D /Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)					IC ₅₀ ($\mu\text{g}/\text{mL}$)
	50	25	12.5	6.25	3.12	
ES-EO	100	100	60.13 \pm 4.16	46.04 \pm 3.15	38.48 \pm 7.59	6.00
EL-EO	100	99.31 \pm 1.19	71.13 \pm 4.12	23.36 \pm 1.57	22.40 \pm 4.16	9.22
α -bisabolol	99.52 \pm 0.82	75.77 \pm 0.71	68.17 \pm 3.21	61.28 \pm 2.17	53.44 \pm 3.92	3.12
Amph. B*	100	50	25	12.5	6.25	IC ₅₀ ($\mu\text{g}/\text{mL}$)
	100 \pm 0	99.98 \pm 0.01	96.15 \pm 0.54	85.84 \pm 0.24	80.78 \pm 0.29	1.88

Table 5. Cytotoxicity of EOs from *E. erythropappus*, α -bisabolol and amphotericin B.

	Concentrations ($\mu\text{g}/\text{mL}$) \pm Standard Deviation					CC ₅₀ ($\mu\text{g}/\text{mL}$)
	50	25	12.5	6.25	3.12	
EL-EO	65.76 \pm 8.29	57.50 \pm 5.30	31.62 \pm 6.90	3.29 \pm 4.17	2.13 \pm 3.69	24.65
ES-EO	96.06 \pm 6.81	88.70 \pm 8.18	77.77 \pm 9.45	23.65 \pm 4.85	0 \pm 0	8.87
α -Bisabolol	5.63 \pm 3.75	4.46 \pm 3.72	1.87 \pm 3.25	0 \pm 0	0 \pm 0	1021.00
Amph. B*	52.54 \pm 3.04	51.44 \pm 1.90	50.64 \pm 2.21	19.76 \pm 0.08	9.73 \pm 0.38	51.86

*Positive control (Amph. B = Amphotericin B)

Materials and methods

Plant material

Plant material of adult *Eremanthus erythropappus*, i. e., leaves and stalks, was collected in June 2016, in Aiuruoca, MG, Brazil (21°58'23"S and 44°44'35"W). The plant material was identified by botanist Walnir G. F. Júnior. A voucher specimen (GERAES05) was deposited at the Machado Herbarium that belongs to the Biology Department, at the Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais, located in Machado, MG, Brazil.

Extraction of EOs

Samples of *E. erythropappus* leaves and stalks were subjected to hydrodistillation for 3 hours in a Clevenger-type apparatus. In order to carry out the analysis, 300 g plant material was divided into three 100-g samples and 500 mL distilled water was added to each sample. After manual collection of samples of EOs, traces of remaining water in oils were removed with anhydrous sodium sulfate; this process was followed by filtration. The extraction procedure was done in triplicate. Isolated oil was stored under refrigeration up to the analysis and test. α -Bisabolol (95% pure) was provided from Atina (Pouso Alegre, MG, Brazil). Yields (w/w) were calculated from the weight of leaves and stalks and expressed as the average of triplicate analysis.

Identification of chemical composition of EOs

Gas chromatography (GC) analyses were performed by a Shimadzu GC2010 Plus gas chromatograph equipped with an AOC-20s autosampler and fitted with a flame ionization detector (FID) and a data-handling processor. An Rtx-5 (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30-m x 0.25-mm i.d.; 0.25- μm film thickness) was used. Operation conditions were as follows: column temperature was programmed to rise from 60 to 240 °C at 3 °C/min and then hold at 240 °C for 5 min; carrier gas was He (99.999 %), at 1.0 mL/min; injection mode; injection volume

was 0.1 μL (split ratio of 1:10); and injector and detector temperatures were 240 and 280 °C, respectively. Relative concentrations of components were reached by peak area normalization (%). Relative areas were the average of triplicate GC-FID analyses. GC-MS analyses were carried out by a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column was an RTX-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary one (30 m x 0.25 mm i.d. x 0.25 μm film thickness). Electron ionization mode occurred at 70 eV. Helium (99.999 %) was employed as the carrier gas at a constant flow of 1.0 mL/min. The injection volume was 0.1 μL (split ratio of 1:10). Injector and ion-source temperatures were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC. Mass spectra were taken at a scan interval of 0.5 s, in the mass range from 40 to 600 Da. Identification of volatile components of *E. erythropappus* leaves and stalks (Table 1) was based on their retention indices on an RTX-5MS capillary column under the same operating conditions as the ones found in the case of GC, related to a homologous series of *n*-alkanes (C₈-C₂₀). Structures were computer-matched with the Wiley 7, NIST 08 and FFNSC 1.2 spectra libraries and their fragmentation patterns were compared with literature data (Adams 2007).

Bacterial strains and antimicrobial assays

In vitro antimicrobial activities of EOs from *Eremanthus erythropappus* leaves and stalks and α -bisabolol were determined by assays of MICs, which were based on the broth microdilution method (CLSI 2006). *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456) and *Streptococcus sanguinis* (ATCC 10556) were the standard strains used in the assays. Initially, bacteria were transferred to blood agar (Difco Labs, Detroit, MI, USA) and individual 24-h colonies were suspended in 10.0 mL tryptic soy broth (Difco). A spectrophotometer (Femto, São Paulo, SP, Brazil), at a wavelength (λ) of 625 nm, was used for standardizing suspensions of each microorganism so as to match the transmittance of 81,

equivalent to 0.5 in the McFarland scale (1.5×10^8 CFU/mL). Dilution of the standardized suspension generated the final concentration of 5×10^5 CFU/mL. EOs were dissolved in DMSO (Merck, Darmstadt, Germany) at 16.0 mg/mL. Concentrations ranging from 400 to 3.9 $\mu\text{g/mL}$ were achieved after dilution of EOs in tryptic soy broth (Difco). After dilution, DMSO concentrations were between 4 % and 0.0039 % (v/v). Negative controls, three inoculated wells with DMSO at concentrations ranging from 4 % to 1 % and one non-inoculated well, free of any antimicrobial agent, were included. An inoculated well helped to test whether the broth was adequate for microorganisms to grow. Positive control was chlorhexidine dihydrochloride (CHD) (Sigma-Aldrich, St. Louis, MO, USA) at concentrations ranging from 5.9 to 0.115 $\mu\text{g/mL}$, diluted in tryptic soy broth (Difco). Ninety-six-well microplates were sealed with parafilm and incubated at 37 °C for 24 h. After that, 30 mL aqueous solution with 0.02 % resazurin (Sigma-Aldrich, St. Louis, MO, USA) was added to each microplate well to indicate the viability of the microorganism (Palomino et al., 2002). The lowest concentration of the sample that inhibited microorganism growth (MIC value) was determined as the lowest concentrations of EOs that were able to prevent the resazurin solution from changing its color (Sarker et al., 2007). All assays were conducted in triplicate. Mycobacteria *Mycobacterium tuberculosis* H37Rv (ATCC 27294), *M. kansasii* (ATCC 12478) and *M. avium* (ATCC 25291) were provided by the American Type Collection (ATCC) and maintained at -80 °C. Antimycobacterial activity of EOs from *Eremanthus erythropappus* leaves and stalks and α -bisabolol was evaluated by the MIC broth microdilution method conducted on microplates. Resazurin was employed to reveal mycobacterial growth by the Resazurin Microtiter Assay (REMA) method (Palomino et al., 2002). EOs were serially diluted (two-fold) with Middlebrook 7H9 broth (DifcoTM, Detroit, MI, USA). The mycobacterium inoculum was then added to reach concentrations ranging from 250 to 2000 $\mu\text{g/mL}$. Isoniazid was used as positive control at concentrations ranging from 0.06 to 1.0 $\mu\text{g/mL}$, whereas Middlebrook 7H9 broth and the inoculum were used as solvent and negative control, respectively.

Antileishmanial assay

In order to evaluate leishmanicidal activity, *L. amazonensis* promastigote forms (MHOM/BR/PH8) were maintained in RPMI 1640 (Gibco) culture medium supplemented with 10% fetal bovine serum. Subsequently, about 1×10^6 parasites were distributed on 96-well plates. EOs and α -bisabolol were previously dissolved in 100% dimethylsulfoxide (DMSO, stock solution 10 mg/mL (Synth)) and added to cultures at concentrations from 3.12 to 50 $\mu\text{g/mL}$. Amphotericin B was previously dissolved in 100% DMSO at 1 mg/mL and, afterwards, diluted in stock solution 500 $\mu\text{g/mL}^{-1}$ in the culture medium (Synth) and added to cultures at concentrations from 6.25 to 100 $\mu\text{g/mL}$. Cultures were incubated at 25 °C in a BOD oven (Quimis) for 24 h. Leishmanicidal activity was determined by growth inhibition of promastigote forms by counting the total number of live promastigotes in a Neubauer chamber (Global Glass, Porto Alegre, Brazil), considering flagellar motility. RPMI 1640 medium (Gibco) containing 0.5% DMSO (Synth) (highest

concentration) was used as negative control and amphotericin B (Eurofarma, São Paulo, Brazil) at 1 $\mu\text{g/mL}$ was used as positive control. Results were expressed as the mean of lysis percentage relative to the negative control (0.1 % DMSO). Two experiments were performed in triplicate. Determination of 50% inhibitory concentration values (IC_{50}) was carried out by non-linear regression curves of a GraphPad Prism version 5.0 Windows software (GraphPad software, USA).

Cytotoxicity assay

In order to obtain peritoneal macrophages, BALB/c. mice were intraperitoneally injected with 500 μL 3% sodium thioglycolate. After 72 hours, mice peritonea were washed with 5 mL ice-cold phosphate buffered saline (PBS 1X) and cells collected during washing were centrifuged at 1000 rpm for 10 minutes at 4 °C. The supernatant was removed and 10 ml RPMI 1640 (Gibco) ice cold medium supplemented with 10% inactivated fetal bovine serum and 1% antibiotic (10,000 U/mL penicillin and 10,000 mg/mL streptomycin) was added to the pellet (cells). Cells were counted in a Neubauer chamber and adjusted to the concentration of 2×10^5 cells/mL. Cells were then seeded on a 96-well plate with supplemented RPMI 1640 medium (Gibco). Cultures were incubated at 37 °C with 5% CO_2 for 24 and 48 hours. Cell viability was determined by the colorimetric MTT metabolic activity assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which assesses the ability that metabolically active cells have to reduce MTT by converting their yellow salts into purple formazan crystals. EOs and α -bisabolol were analyzed at the same concentrations (from 3.12 to 50 $\mu\text{g/mL}$) as the ones of the assays on promastigote forms. Results were expressed as mean percent reduction in cell viability versus negative control (0.1% DMSO). Experiments were performed in triplicate. Finally, 50% cytotoxic concentration (CC_{50}) values were determined by means of non-linear regression curves with the use of GraphPad Prism version 5.0 Windows software (GraphPad software, USA).

Conclusions

Findings of this study show that EOs from *E. erythropappus* are effective against oral pathogens, mycobacteria and promastigote forms of the parasite *Leishmania amazonensis* with values of clinical relevance. EL-EO and ES-EO, despite showing relatively high cytotoxicity, exhibit promising antibacterial activity against some cariogenic bacteria, such as *Streptococcus mutans*, which is one of the main causative agents of dental caries. EL-EO and ES-EO were also active and moderately active against both mycobacteria *Mycobacterium kansasii* and *M. tuberculosis*, respectively. In short, this study suggests that the chemical constitution of these compounds and the synergistic effect found in EOs may contribute to increase their antibacterial and antiparasitic effects.

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