



Chemical composition, antioxidant and antibacterial activities of essential oils from leaves and flowers of *Eugenia klotzschiana* Berg (Myrtaceae)

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ABSTRACT

Many essential oils (EOs) of different plant species possess interesting antimicrobial effects on buccal bacteria and antioxidant properties. *Eugenia klotzschiana* Berg (pêra-do-cerrado, in Portuguese) is a species of Myrtaceae with restricted distribution in the Cerrado. The essential oils were extracted through the hydrodistillation technique using a modified Clevenger apparatus (2 hours) and chemically characterized by GC-MS. The major compounds were α -copaene (10.6 %) found in oil from leaves *in natura*, β -bisabolene (17.4 %) in the essential oil from dry leaves and α -(*E*)-bergamotene (29.9 %) in oil from flowers. The antioxidant activity of essential oils showed similarities in both methods under analysis (DPPH and ABTS⁺) and the results suggested moderate to high antioxidant activity. The antibacterial activity was evaluated by determining minimum inhibitory concentrations (MICs), using the microdilution method. MIC values below 400 μ g/mL were obtained against *Streptococcus salivarius* (200 μ g/mL), *S. mutans* (50 μ g/mL), *S. mitis* (200 μ g/mL) and *Prevotella nigrescens* (50 μ g/mL). This is the first report of the chemical composition and antibacterial and antioxidant activities of the essential oils of *E. klotzschiana*. These results suggest that *E. klotzschiana*, a Brazilian plant, provide initial evidence of a new and alternative source of substances with medicinal interest.

Key words: Antioxidant activity, antibacterial activity, Brazilian pear, drying, *Eugenia klotzschiana*, sesquiterpenes.

INTRODUCTION

Essential oils from different plant sources carry out several biological activities, such as antibacterial, anticancer, anti-inflammatory, antimutagenic, antifungal, antioxidant and antiprotozoal ones. As a result, the vast arsenal of bioactive compounds found in essential oils has increasingly attracted researchers' intense attention in the last years (Raut and Karuppaiyl 2014).

The species *Eugenia klotzschiana* Berg (pêra-do-cerrado, in Portuguese), which is a species of Myrtaceae with restricted distribution in the Cerrado, yields fruits with nutritional potential that can be extracted from nature as raw material for the production of jams, jellies and juices. However, this rare species has been little studied (Oliveira et al. 1999). *E. klotzschiana* is a species found in tropical regions and that adapts well to drained and permeable soils, such as the ones in Goiás, the Federal District, Mato Grosso, Mato Grosso do Sul, Minas Gerais and southwestern Bahia (Oliveira et al. 1999, Takao et al. 2015). Recent studies have shown that this species can also be found in Bolivia (Villarroel and Proença 2013).

The Myrtaceae family, one of the most important of Brazilian flora, has representatives of great medicinal interest. This family presents pantropical and subtropical distribution, has about 130 genera and 4000 species. In Brazil there are 23 genera and approximately 1000 species (Paula et al. 2010). Species of the Myrtaceae family, such as *E. klotzschiana*, have stood out as rich sources of essential oils with important economic and health benefits (D'Angelis and Negrelle 2014).

Aromatic plants, mainly their essential oils, have been widely evaluated regarding their antioxidant and antimicrobial activities since they have been used in food and drink industries as natural antioxidants (Amiri 2012, Hellali et al. 2016). Their attributes are the result of the capacity some of their components, mainly phenols, have

to interrupt or mitigate oxidation. Well-known methods, such as DPPH and ABTS⁺, may be used for evaluating the antioxidant activity (Almeida et al. 2011).

The procedure, which yields the essential oil from the raw material, limits the phenolic content in the final matrix because many compounds are non-volatile. However, there are phenol-free essential oils which have antioxidant behavior due to the chemical structure of their constituents (Amorati et al. 2013). Consequently, there has been more interest in the use of natural substances as antioxidants not only because of toxicological worries related to the use of synthetic ones for long periods but also because of awareness-raising processes concerning the consumption of natural food (Singh et al. 2012).

Regarding antibacterial activity, several papers have reported the antimicrobial potential of essential oils (EOs) from plants against oral pathogens over the last decade (Sousa et al. 2015). EOs consist of mixtures of a variety of lipid-soluble and volatile compounds, such as monoterpenes, sesquiterpenes and phenylpropanoids, which can easily diffuse across cell membranes, a major advantage with regard to interactions with intracellular targets. Additionally, synergistic interactions among the components of EOs may happen and benefit their activities (Raut and Karuppaiyl 2014).

This study describes, for the first time, the chemical composition and the antioxidant and antibacterial activities *in vitro* of essential oils from leaves of *E. klotzschiana* (Myrtaceae), a species found in the Brazilian Cerrado in southwestern Goiás.

MATERIALS AND METHODS

COLLECTION AND IDENTIFICATION OF THE PLANT MATERIAL

Leaves and flowers of *E. klotzschiana* were collected in an area of Cerrado vegetation, located

in Portelândia (Goiás) (17° 23' S and 52° 38' W), in March 2014. The plant material was identified by the botanist Erika Amaral, Ph.D. Samples were stored at the Herbário Germano Guarim Neto, which belongs to the Universidade Federal de Goiás (no. HJ 7413 - leaves and no. HJ 7414 - flowers).

DRYING PROCESS OF THE PLANT MATERIAL

In order to evaluate the influence of drying on the content and composition of essential oils from leaves of *E. klotzschiana* Berg, they were submitted to three treatments: *i*) leaves *in natura*; *ii*) natural drying at room temperature; and *iii*) kiln drying by forced air circulation at 40 °C ± 2 °C.

In the natural drying process, 1000 g leaves *in natura* were placed in thin layers at room temperature, with no incident solar radiation, at the Laboratório de Química de Produtos Naturais, which belongs to the Instituto Federal Goiano, Campus Rio Verde. Gravimetric monitoring of reduction in water content was carried out throughout the process of leaf drying with the help of a 0.01 g resolution scale. Drying lasted 72 hours, up to 50 % reduction in the initial mass weight. Afterwards, leaves were submitted to hydrodistillation to have their essential oil extracted.

In kiln drying by forced air circulation, 1000 g leaves were stored in Kraft paper bags, identified and placed in the kiln at 40 °C ± 2 °C for 48 hours. The end of drying was achieved when there was 50 % reduction in the initial mass weight. Flowers were collected and immediately submitted to the extraction of their essential oil, i.e., no drying process was carried out.

ISOLATION OF ESSENTIAL OILS

Essential oils were extracted from leaves (100 g) and flowers (100 g) of *E. klotzschiana* by a modified Clevenger-type apparatus and 2-hour hydrodistillation. The oil was separated and dried over anhydrous sodium sulfate, stored in

hermetically sealed glass containers and kept under refrigeration at 5 °C until the analysis and antibacterial and antioxidant assays. Total oil yield was expressed as a percentage value (g/100 g of fresh plant material). All experiments were carried out in triplicate.

GC-MS ANALYSIS

GC-MS analysis was done on a Agilent Technologies 7820A GC and MSD 5975 using DB-5MS column (30 m x 0.25 mm, 0.25 mm in thickness). The carrier gas was He with pressure of 57.4 kPa and flow rate of 1.00 mL/min. The split ratio was 1/30, the injector temperature was 250 °C and the injected volume was 1 µL. Temperature programming was the following : 60 – 240 °C increasing in 3 °C/min. MS were recorded on electron ionization (EI) mode, with ionization energy of 70 eV (scan time: 2 scans/s). The identification of constituents was carried out based on the retention indices (calculated using from C₉ to C₂₂ alkanes) and by comparing the mass spectra with a computer databank (Wiley 7 and Nist 62) and with reference to published data (Adams 2007).

ASSESSMENT OF THE ANTIOXIDANT ACTIVITY

Quantification by the DPPH method was carried out in agreement with Rufino et al. (2007a), based on the capture of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) by antioxidant substances. Samples of essential oils of leaves and flowers (0.1 mL) at different concentrations (2000, 4000, 6000, 8000 and 10000 mg/L) were added to 3.9 mL DPPH solution and kept for 60 minutes in the dark. Absorbance was read at 515 nm wave length by a UV-Vis digital spectrophotometer Bel Engineering model UV-M51 in quadruple. A DPPH standard curve was constructed by using solutions whose concentrations ranged from 10 to 60 µM. The DPPH calibration equation obtained the following correlation coefficient: R² = 0.99. Results were

expressed as EC_{50} $\mu\text{g/mL}$ (amount of sample which was needed to decrease the initial concentration of the radical DPPH in 50 %), as g oil/g DPPH and as inhibition percentage of every concentration, as follows:

$$\%AA = \{(\text{Abs control} - \text{Abs sample}) \times 100\} / \text{Abs control}$$

The antioxidant activity assay was carried out by the Trolox equivalent antioxidant capacity (TEAC) method and the radical $ABTS^{+\cdot}$ (2,2-azino-bis-3-ethylbenzoline-6-sulfonic acid) was captured in agreement with the procedure described by Rufino et al. (2007b). Preparation of the radical $ABTS^{+\cdot}$ was based on the reaction between 5 mL $ABTS^{+\cdot}$ aqueous solution (7 mM) and 88 μL potassium persulfate solution (140 mM), which was left for 16 hours in the dark. Afterwards, the mixture was diluted with ethanol to reach absorbance of 0.70 $\text{nm} \pm 0.05$ at 734 nm. An aliquot of 30 μL of every dilution of essential oils from leaves and flowers (from 2000 to 10000 mg/L) was added to 3 mL of the radical $ABTS^{+\cdot}$ in test tubes and left for 6 minutes in the dark. Absorbance was read at 734 nm by a spectrophotometer whose blank was ethanol. A Trolox curve at different concentrations (from 100 to 2000 μM) was prepared and used as the standard. The $ABTS^{+\cdot}$ calibration curve obtained the following correlation coefficient: $R^2 = 0.99$. Results were expressed as Trolox micromole per gram of oil (μM trolox/g).

STATISTICAL ANALYSIS

Results of antioxidant analyses were submitted to the analysis of variance (ANOVA) whereas the Tukey's test ($p < 0.05$) was applied so that the means could be compared. Statistical calculations were carried out by Excel (version 2007) and Assistat.

MICROORGANISMS UNDER INVESTIGATION

Bacteria were acquired from the American Type Culture Collection (ATCC) and kept in the culture collection of the Laboratory of Research on Applied Microbiology (LaPeMA), which belongs to the Universidade de Franca, located in Franca, São Paulo, Brazil, at -80 $^{\circ}\text{C}$. The following microorganisms were used: *Streptococcus salivarius* (ATCC 25975), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49452), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus sobrinus* (ATCC 33478) and *Prevotella nigrescens* (ATCC 33563).

ANTIMICROBIAL ASSAYS

MIC values of essential oils were determined by the broth microdilution method in 96-well microplates, in agreement with the methodology described by CLSI (2006). Samples were dissolved in 125 μL tryptic soy broth (TSB) to yield concentrations between 50 and 400 $\mu\text{g/mL}$ of the compounds under investigation. The inoculum was adjusted to 625 nm for every microorganism by the spectrophotometer to obtain cell concentration of 5×10^5 colony-forming units (CFU/mL) (CLSI 2006). Chlorhexidine digluconate (Sigma-Aldrich), at concentrations which ranged from 0.115 to 59.0 $\mu\text{g/mL}$, was employed as the positive control. The microplates were incubated at 37 $^{\circ}\text{C}$ for 24 h; then, 30 μL 0.02 % resazurin (Sigma-Aldrich) aqueous solution was added to every well (Sarker et al. 2007). Resazurin is an oxireduction probe that enables microbial growth to be immediately observed. Blue and red colors represent the absence and the presence of microbial growth, respectively.

RESULTS AND DISCUSSION

The oil extracted from leaves and flowers of *E. klotzschiana* by hydrodistillation was yellow with high viscosity and strong odor. Essential oils from

leaves *in natura*, shade dried leaves, kiln dried leaves and flowers yielded 0.10 %, 0.17 %, 0.07 % and 0.09 %, respectively.

Thirty five components were identified in every essential oils under study; it represents from 80.8 to 92.9 % of the total composition of volatile essential oils (Table SI - Supplementary Material). Sesquiterpenes predominate in the composition of essential oils from *E. klotzschiana*. The following main terpenic components were identified: α -copaene (10.6 %), β -bisabolene (17.4 %), α -(*E*)-bergamotene (29.9 %) and germacrene-D (13.3 %).

Leaf drying led to modifications in the yield of secondary metabolites due to changes in temperature and the action of biotic and abiotic factors inherent to the development of the plants, as previously described by Oliveira et al. (2016). The essential oil from leaves submitted to shade drying had predominantly sesquiterpenic compounds, mainly β -bisabolene (14.0 %) and spathulenol (10.9 %), likewise the constituents found in the essential oil from leaves *in natura*.

Kiln drying by forced air circulation at 40 °C interfered in the chemical composition of the essential oil. Out of 35 substances, the ones that had the highest concentrations were: β -bisabolene (17.4 %), germacrene-D (13.3 %), α -caryophyllene (10.2 %) and α -(*E*)-bergamotene (10.1 %).

The method of drying medicinal and aromatic plants has been widely used for removing free water from cells and plant tissues, thus, preventing enzymatic degradation processes and keeping the quality of the chemical composition. Drying processes enable plant preservation since physical and chemical characteristics are kept intact. As a result, plants may be stored for a long time. However, the composition of essential oils was observed to undergo changes in post-harvest periods due to spontaneous reactions which happen continuously and alter the composition of essential oils (Oliveira et al. 2011).

The literature mentions that studies of leaves *in natura*, natural drying and kiln drying at 40 °C of leaves of long pepper (*Piper hispidinervum*) showed that the type and time of drying changed the chemical composition of its essential oil, e.g., it increased safrole, its main constituent in the industrial market. Only 78.2 % safrole was found in the oil extracted from the leaves *in natura* whereas percentages of safrole rose to 90.1 % and 92.6 % when the plant material was dried at room temperature and at 40 °C, respectively. Increases in the concentrations of some substances in the drying process may be explained by the reduction in the excess of humidity, a fact that damages the structures of plant cells and makes the extraction of essential oils easier (Machado et al. 2013).

Regarding the essential oil extracted from flowers of *E. klotzschiana*, its chemical constituents were observed to be similar to the ones of the essential oil extracted from leaves *in natura* and dry ones because they also had high content of hydrocarbon sesquiterpenes (64.2 %) (Table SI), mainly α -(*E*)-bergamotene (29.9 %), germacrene-D (12.1 %) and β -bisabolene (10.2 %). Oxygenated monoterpenes, such as geraniol (1.6 %), citronellol (1.4 %) and phenylpropanoid or eugenol (1.7 %), stand out but these three ones are only found in the essential oil from flowers.

The evaluation of the antioxidant potential of plant species by the DPPH and ABTS⁺ methods has drawn researchers' attention in their search for bioactive compounds (Almeida et al. 2011). Therefore, in order to measure the antioxidant activity of essential oils from *E. klotzschiana*, both free radicals DPPH and ABTS⁺ were used. Table II shows the capacity essential oils extracted from leaves (*in natura* and dry ones) and flowers of *E. klotzschiana* have to capture free radicals.

The essential oil from leaves *in natura* had high EC₅₀ = 29.77 µg/mL, followed by kiln dried leaves (EC₅₀ = 7.61 µg/mL), shade dried leaves

TABLE II
Antioxidant activity of essential oils from *E. klotzschiana*, evaluated by the methods DPPH and ABTS⁺.

Assays	Leaves <i>in natura</i>	Shade dried leaves	Kiln dried leaves	Flowers
Yields (%)	0.10ab	0.17a	0.07b	0.09b
DPPH - EC ₅₀ µg/mL	29.77±2.25a	6.48±0.69b	7.61±0.10b	5.70±0.37b
DPPH - g/g	3.25±0.24a	0.71±0.07b	0.83±0.01b	0.62±0.04b
ABTS ⁺ - µM trolox/g	57.81±4.80c	143.85±5.35a	106.27±4.75b	104.61±4.46b

Values represent the mean ± standard deviation of three samples. Means followed by equal letters in the same line do not differ among each other, by the Tukey's test ($p > 0.05$).

(EC₅₀ = 6.48 µg/mL) and flowers (EC₅₀ = 5.70 µg/mL).

In the literature, the most promising antioxidant activity of essential oils has varied from 8.3 to 278 µg/mL, depends on their chemical composition (Tian et al. 2014, Amiri 2012, Pandey et al. 2014). These values were higher than the ones found in essential oils from *E. klotzschiana*. The antioxidant capacity of the essential oils of *E. klotzschiana* may be attributed to the presence of the hydrocarbon sesquiterpene germacrene-D, which is a strong antioxidant because of the fact that its chemical structure has an extra cyclic methylene (Victoria et al. 2012).

The literature describes the high antioxidant activity of the essential oil from Indian clove since its majoritarian compound is eugenol, which has been known as a strong antioxidant. This phenylpropanoid was also identified in the essential oil from flowers of *E. klotzschiana* and has been related to its high antioxidant potential (Teixeira et al. 2013, Affonso et al. 2012, Silvestri et al. 2010).

Results of the measurement of the antioxidant capacity by an ability assay to kidnap the radical ABTS⁺ were similar to the ones obtained with DPPH. The essential oil from leaves submitted to natural drying was the one that had the best results by the ABTS⁺ (143.85 µM trolox/g), followed by artificially dried leaves (106.27 µM trolox/g), the essential oil from flowers (104.61 µM trolox/g) and dry leaves (57.81 µM trolox/g). The comparison between ascorbic acid (1593.6 µM trolox/g) and

BHA (1329 µM trolox/g), a synthetic antioxidant used in food, shows that the antioxidant activity of essential oils from *E. klotzschiana* ranges from moderate to high against the free radical. Damasceno et al. (2011) observed that the more the antioxidant activity increases, the higher the concentration of essential oils. In both methodologies, the essential oil from *E. klotzschiana* had antioxidant potential but the essential oil from the flowers had higher antioxidant activity.

The evaluation of the antioxidant potential of essential oils from *E. klotzschiana* showed that there was high correlation between both methods under analysis, the kidnapping activity of the free radical DPPH ($R^2 = 0.99$) and the Trolox equivalent antioxidant capacity ($R^2 = 0.99$). In their study of the species *Byrsonima gardneriana*, Rolim et al. (2013) stated that these data are evidence of the good antioxidant activity this plant has.

The antioxidant efficiency of essential oils from leaves and flowers of *E. klotzschiana* has been attributed mainly to its majoritarian components, such as β-bisabolene, germacrene-D, α-caryophyllene and α-(*E*)-bergamotene, even though it may also be caused by the synergic effect of minor components, as well as by the interaction among the compounds (Melo et al. 2011). Therefore, some essential oils stand out as natural antioxidants and, due to technological and nutritional issues, many of them have been studied to replace synthetic antioxidants (Silva et al. 2014).

The procedure which yields the essential oil from the raw material limits the phenolic content in the final matrix because many compounds are non-volatile. However, there are phenol-free essential oils which have antioxidant behavior due to the chemical structure of their constituents (Amorati et al. 2013). Consequently, there has been more interest in the use of natural substances as antioxidants not only because of toxicological worries related to the use of synthetic ones for long periods but also because of awareness-raising processes concerning the consumption of natural food (Singh et al. 2012).

To perform antibacterial activities, essential oils extracted from leaves *in natura*, shade-dried leaves and leaves dried at 40 °C were joined whereas the antibacterial activity of essential oils from flowers was carried out separately. The essential oils were tested against some aerobic/anaerobic oral microorganisms.

Table III shows that only the anaerobic bacterium *P. nigrescens* and the aerobic bacterium *S. mutans* were significantly inhibited by all essential oils under study, with MIC values ranging from 200 to 50 µg/mL. According to Holetz et al. (2002), in case MIC values are below 100 µg/mL, the antimicrobial activity can be considered good; from 500 to 100 µg/mL, it is moderate; from 1000 to 500 µg/mL, it is weak; and above 1000 µg/mL, it is inactive. Thus, essential oils from leaves and

flowers were the most effective ones against the anaerobic bacterium *P. nigrescens* (MIC = 50 µg/mL) and against the aerobic bacterium *S. mutans* (MIC = 50 µg/mL).

This result can be explained by the presence of secondary metabolites, such as sesquiterpenes and monoterpenes, including α -copaene, α -(*E*)-bergamotene, β -bisabolene and eugenol, which have antimicrobial activity against Gram-positive and Gram-negative human pathogens (Lang and Buchbauer 2012, Affonso et al. 2012, Silvestri et al. 2010). Essential oils from leaves and flowers of *E. klotzschiana* had good antibacterial activity against *P. nigrescens* and *S. mutans* whereas they had moderate activity against *S. salivarius*, *S. mitis*, *S. sanguinis* and *S. sobrinus*.

In the literature, action mechanisms have been proposed to explain biological activities of essential oils. Both mechanisms are associated with the hydrophobicity of monoterpenes and sesquiterpenes, which are often the main chemicals thereof. The hydrophobicity of terpenoids enables these compounds to permeate cell membranes easily and to cause the death of parasites or microorganisms by affecting their metabolic pathways or organelles (Knobloch et al. 1989).

Therefore, this study led to the conclusion that essential oils from *E. klotzschiana* showed a complex mixture of monoterpenes and sesquiterpenes, but consisted mainly of sesquiterpenic compounds.

TABLE III
Antimicrobial activity of essential oils from *E. klotzschiana* against oral bacteria under study.

Bacteria	Minimum inhibitory concentration (MIC) - µg/mL		
	Essential Oil - leaves	Essential Oil - flowers	CHD*
<i>Streptococcus salivarius</i> ^a	200	200	0.922
<i>S. mutans</i> ^a	50	50	0.922
<i>S. mitis</i> ^a	200	200	3.688
<i>S. sanguinis</i> ^a	400	400	0.922
<i>S. sobrinus</i> ^a	400	400	0.922
<i>Prevotella nigrescens</i> ^b	50	50	1.844

^aAerobic Gram-positive bacteria; ^bAnaerobic Gram-negative bacteria; *CHD: chlorhexidine dihydrochloride (positive control).

The essential oils from *E. klotzschiana* under evaluation by the DPPH and ABTS⁺ methods showed antioxidant potential, from moderate to high, a result which agrees with the presence of the compounds germacrene-D and eugenol whose antioxidant activity has been well-known even though the synergic effect of minor constituents was also taken into account.

Finally, essential oils from *E. klotzschiana* have promising antimicrobial activity against some cariogenic bacteria, such as *Streptococcus mutans* and *Prevotella nigrescens*. Moreover, the antimicrobial activity of essential oils from *E. klotzschiana* against oral pathogens makes them potential natural antimicrobial agents.

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SUPPLEMENTARY MATERIAL

Table SI - Chemical composition of essential oils from leaves and flowers of *E. klotzschiana*.