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Biological properties of *Eugenia uniflora* L. essential oil: phytochemistry composition and antimicrobial activity against gram negative bacteria

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ABSTRACT

According to the popular medicine, the infusion *Eugenia uniflora* L., popularly known as Brazilian cherry, has antimicrobial, antidiarrheal, diuretic, antireumatic, antipyretic, reducing cholesterol and uric acid in the urine. With base of this data, the work aimed to perform the phytochemical characterization of oil and assess the antibacterial activity *in vitro* on strains of *Enterobacter aerogenes* and *Salmonella typhimurium*. The collection of Brazilian cherry leaves was held during the month of February 2015, in São Lourenço do Sul, Rio Grande do Sul, Brazil. The chromatographic conditions were: initial temperature of 50ºC, rising to 10ºC/min, until achieved 280ºC, remaining in this temperature for 10 min; injected volume of 1 µl; 300ºC interface; 280ºC of temperature injector; helium as carrier gas; linear gas flow of 1.20 mL.min -1; split of 1:50; running scan mode; mass strip of 40 at 700 m/z and 70eV filament voltage. To evaluate the antibacterial activity, we used the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).The phytochemical characterization allowed us to identify 12 compounds: trans-β-ocimene, cis-β-ocimene, caryophyllene, allo-aromadendrene, β-copaene, α-muurolene, (+)-δ-cadinene, spathulenol, viridiflorol, Isolongifolene, 9,10-dehydro-, α-epi-cadinol and cis-Dihydro-α-copaene-8-ol.Oil yield obtained in the extraction process was 0.65% and inhibition action was found facing the strains of *E. aerogenes* and *S.typhimurium* with 3.125%. Thus, it can be concluded that the essential oil of Brazilian cherry is an alternative medical means with antibacterial action on *E.aerogenes* and *S.typhimurium* is considered possible natural source for the development of new drugs.

Propriedades biológicas do óleo essencial de *Eugenia uniflora* L.: composição fitoquímica e atividade antimicrobiana contra bactérias gram negativas

Palavras-chave
Eugenia uniflora
Concentração inibitória mínima
Sesquiterpenos

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RESUMO - De acordo com a medicina popular a infusão de *Eugenia uniflora* L., conhecida popularmente como pitangueira, apresenta atividade antimicrobiana, antidiarréica, diurética, antireumática, antifebril, controladora do colesterol e ácido úrico. Com base nestes dados, o estudo teve como objetivo a caracterização fitoquímicae avaliaçãoda atividade antibacteriana *in vitro* contra *Enterobacter aerogenes* e *Salmonella typhimurium*. A coleção das folhas de pitanga foi coletada durante o mês de fevereiro de 2015, em São Lourenço do Sul, Rio Grande do Sul, Brasil. As condições cromatográficas foram: temperatura inicial de 50ºC, 10ºC/min, até atingir 280ºC, permanecendo nesta temperatura por 10 min; foi injetado o volume de 1µl; 300ºC interface; temperatura do ejetor de 280ºC; gás carregado com hélio; gás linear de 1.20 mL.min-1; split de 1:50; modo de corrida escaneada; massa strip de 40 a 700 m/z e voltagem do filamento de 70eV. Para avaliação da atividade antibacteriana foi determinada a Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM) do óleo essêncial. A caracterização fitoquímica permitiu identificar 12 compostos: trans-β-ocimeno, cis-β-ocimeno, caryophileno, allo-aromadendreno, β-copaene, α-muurolene, (+)-δ-cadinene, spathulenol, viridiflorol, Isolongifolene, 9,10-dehydro-, α-epi-cadinol e cis-Dihydro-α-copaeno-8-ol. O rendimento do óleo obtido no processo de extração foi de 0,65%, verificando-se ação inibitória frente às cepas de *E. aerogenese* e *S. typhimurium* com de 3,125%. Dessa forma, pode-se concluir que o óleo essencial de pitanga constitui um meio medicinal alternativo com ação antibacteriana sobre *E. aerogenese* e *S. typhimurium*,sendo considerada uma possível fonte natural para o desenvolvimento de novos medicamentos.

1. Introduction

The use of plants for treating diseases is as old as the human (1). Egyptians, Assyrians, Mesopotamians and others civilizations could count on the traditional healer that indicated the correct plant species for many diseases. Ethnopharmacology provides evidences about potentially useful substances to the development of new drugs (2).

Although the progress in organic synthesis and industrial microbiology area, the pharmaceutical production of plant materials remains difficult, mainly to obtain synthetically molecules with the same stereochemistry. Achievement of the drugs that do not occur in nature, is facilitated upon the discovery of substances that could be employed as precursors at synthesis (3). Between natural products used in therapy, essential oils are reported as products with great pharmacological potential that exhibit different properties, such as its volatility, which makes them ideal for use at sprays, immersion baths or simply inhalations. The volatility and low molecular weight components allow it to be rapidly eliminated by the body (4).

The essential oils are noted for their biological properties as analgesic, antioxidant, antimicrobial and expectorant. In addition to possessing antimicrobial, anticancer and insecticide activity, being a great economic interest due to the use in general industry (5,6,7). It is not yet completely understood the antibacterial mechanism of essential oils, being probably linked to injuries on lipophilic compounds of bacterial membrane, similar the action of some semisynthetic products, like amoxicillin. The compounds can also have an effect on activation/inactivation of products, rupture or disintegration of bacterial cell membrane, with subsequent elimination of their enzymes and nutrients (8).

Various pathologies that affect public health are of microbial origin. Even with the wide variety of antimicrobials used in the combat of pathogenic microorganisms, studies are searching for an ideal antimicrobial agent that presents broader action spectrum, less toxicity, lower cost, and reduced cases of bacterial resistance (9).

In Brazil, gram negative bacterial were the major cause of nosocomial infections, as well as simpler infections in outcome patients (e.g. urinary tract infection (10,11).An important bacterium in hospital environment, *Enterobacter aerogenes*, is considered an opportunistic pathogen. This infects immunodepressive patients, causing a wide range of nosocomial infections, besides presenting high resistance to antibiotics. *Salmonella typhimurium* has a vast importance in public health, because the severity of gastroenteritis caused by this pathogen (12). Added to this, the emergence of multidrug resistant cases and the adaptation to human invasive mechanisms becomes alarming (13,14).

In view of the current situation, studies have demonstrated that various plant species are a source of secondary metabolites with potent antibacterial activity (15).However, there is little knowledge about the part of the majority of the species, especially some Myrtaceae's family, the example of the Brazillian cherry, with high potential for economic exploitation (16). Brazillian cherry (*Eugenia uniflora* L.), popularly known as Brazilian cherry, is a native tree from Brazilian Atlantic forest, found in the semi deciduous forest, in planalto and in the restingas from Minas Gerais to Rio Grande do Sul (8,17).The infusion of its leaves has application in folk medicine as astringent, anti-diarrheal, diuretic, anti-febrile, antimicrobial, reducer of cholesterol blood and controller of uric acid in the urine (8,3).

Considering the numerous medicinal indications of the mentioned specie, this study aimed the obtaining the essential oil of *E. uniflora* leaves, the chemical characterization of its compounds and research for antimicrobial activity *in vitro* against two gram negative bacteria, *E. aerogenes* and *S. typhimurium*.

Quality of life can be defined as a sense of comfort, well-being or happiness in physical, intellectual and psychic functions, both within the family, at work and in the community to which the individual belongs (1). In this context, born the palliative care, that is a group of measures directed toward the improvement of the quality of life of patients with terminal and severe diseases. This approach focus on prevention and suffer relief of patients and their family, through support and control of pain and other physical, psychosocial and spiritual issues (2,3). In Brazil, palliative care was introduced in the decade of 1980 and, in the last years, shown a significant growth due to the consolidation of previously established services such as the Brazilian Society for Pain Studies (SBED), the National Cancer Institute (INCA) and the Brazilian Association of Palliative Care (ABCP) and by the foundation of new specialized centers such as the National Academy of Palliative Care (ANCP) (4,5). Currently, the country counts with courses that allow healthcare professionals to assist in palliative care, however, teaching strategies directed towards this type of science is disperse and a resistance to debate this topic can be found in the academy (6).

Education in health is strictly connected to the promotion of health and, thus, it is essential the preparation of students and professionals to deal with aspects that relate to palliative care, once diseases and death phenomena are seen with frequency on their daily lives (7,8). In this scenario, the acquisition of knowledge and skills in palliative care are paramount and, as a consequence, the scarcity of education and training consists in a barrier for integration of palliative care in the healthcare system (9).

In general, palliative care has been considered as a critical field where the presence of healthcare professionals with higher instruction level and a multidisciplinary team has been showed ultimately necessary (10). Thus, this study aims to evaluate the student's knowledge about palliative care, in three undergraduate courses in the health area, taking into account the curricular analysis of the courses and the student's perception about the subject.

2. Material and methods

Plant Species and Essential Oil Production: The collection of *E. uniflora* leaves was held during the month of February2015, in São Lourenço do Sul, Rio Grande do Sul, Brazil (31º36'55.07''S and 51º98'50.00''). The taxonomic plant identification was confirmed and voucher specimen was deposited in the HerbarioPel, registered under number 25.930. For extraction process, the leaves were desiccated oven drying with air circulation at 37ºC, and then 100 g of the dried material were immersed in 1.5 L of distilled water. The essential oil extraction were performed at the Laboratório de Pesquisa de Produtos Naturais do Departamento de Química Orgânica from Centro de Ciências Químicas, Farmacêuticas e de Alimentos ofthe Universidade Federal de Pelotas (UFPel), by hydrodistillation process in Clavenger-type apparatus. The essential oil was conditioning in amber glass vial and kept refrigerated until the moment of evaluation of chemical composition and checking the biological activity.

Chemical Characterization: The identification of chemical compounds in the *E. uniflora* essential oil was performed using a gas chromatograph coupled to a mass spectrometer, GC/MS-QP 2010SE model (Shimadzu, Japan) equipped with auto-injector AOC 20i. The separation occurred on a RTX-5MS capillary column (Restek, USA) measuring 30 mm x 0.25 mm diameter x 0.25 µm thick. The chromatographic conditions were: initial temperature of 50ºC, rising to 10ºC/min, until achieved 280ºC, remaining in this temperature for 10 min; injected volume of 1 µl; 300ºC interface; 280ºC of temperature injector; helium as carrier gas; linear gas flow of 1.20 mL.min $^{-1}$; split of 1:50; running scan mode; mass strip of 40 at 700 m/z and 70eV filament voltage. The measurements were made at standard area and the compounds identification was made through mass spectrum using the NIST 8 library of the equipment.

Bacterial Strains: For study, standard Gram negative bacteria were used, *E. aerogenes* (ATCC 13048) and *S. typhimurium* (ATCC 14028). The strains were recovered in broth Brain Heart Infusion – BHI (Kasvi®) and incubated at 36°C for 24h. Afterwards, the colonies were subcultured on BHI inclined agar and incubated at 36°C for 24h to obtain a bacterial mass for the study of antibacterial activity. Analyses were performed in the Laboratório de Bacteriologia of the Department de Microbiologia e Parasitologia, Instituto de Biologia – Universidade Federal de Pelotas (UFPel).

Minimum Inhibitory Concentration: For determination of Minimal Inhibitory Concentration (MIC), it was used the microdilution technique broth according CLSI M07-A9 (18). To performed the test, a microtiter plate with 96 wells was used. The culture medium used was of MH broth with Tween80 – T80 (Vetec®) 1%, emulsifier employed to reduce the surface tension of the oil contact (apolar character) with the culture medium (polar characteristic). For the dilution procedure of Brazilian Cherry essential oil, dilutions were conducted serially ratio two, 25% at 0.19%. As negative control, we used culture medium and T80 and as a positive control was used culture medium + T80 + inoculum (evaluation of bacterial cell viability). In a cavity, it was utilized only the essential oil, to discard any possibility of contamination.

The suspension were made on 5 ml of sterile saline water 0.85% to achieve a concentration of 0.5 MacFarland Scale. Therefore, was performed a 1/100 dilution (50 µL of this added to 4950 µL of MH broth), then added 50 µL of suspension to all test wells, except the sterility control of the essential oil and culture medium. The final bacterial concentration was of approximately 10^4 CFU/mL^{-1}, the plate was incubated at 36°C for 24h and the experiment performed in triplicate. For reading the test 20 µL of 2, 3, 5 Triphenyl tetrazolium chloride 0.5% was added in all wells, the plate was incubated for 20 min at 36°C. The change in reagent color from clear to red was indicative of bacterial metabolic activity, proving the lack of inhibition of bacterial growth by the essential oil tested.

Minimal Bactericidal Concentration: The Minimal Bactericidal Concentration (MBC) is defined as the lowest concentration of essential oil where visible growth cannot be observed visible growth in the subculture. Following incubation and MIC reading, aliquots of 5 µL of removed from each well that showed inhibition of bacterial growth, these subcultured into a new BHI agar plate incubated at 36°C for 24h (19). The absence of bacterial growth in the culture medium was indication that the essential oil tested showed bactericidal activity.

3. Results and discussion

At the extraction process of *E. uniflora* essential oil, the yield obtained was 0.65% at 4h extraction. A study of this same botanical species (20) has demonstrated higher efficiency and selectivity for the Clevenger extraction method system, a yield of 2.4% in 1h, and this value increased to 0.6 after 5 hours. According to the authors, the extraction time is essential for the oil yield. Lower incomes are being found, demonstrating that variations in yield can be linked to the time the leaves are cropped, soil, nutrients and extraction time. Probst (2) also notes that several other factors can affect the performance; even variations associated the plant, such as development phase, pollination cycle, seasonal variations and stress conditions of the plant (8,21).

The chemical characterization allowed us to identify 12 of the 16 compounds in the essential oil of *E. uniflora*: trans-β-ocimene,cis-β-ocimene, caryophyllene, allo-aromadendrene, β-copaene, α-muurolene, (+)-δ-cadinene, spathulenol, viridiflorol, Isolongifolene, 9,10-dehydro-, α-epi-cadinol and cis-Dihydro-α-copaene-8-ol (Figure 1).

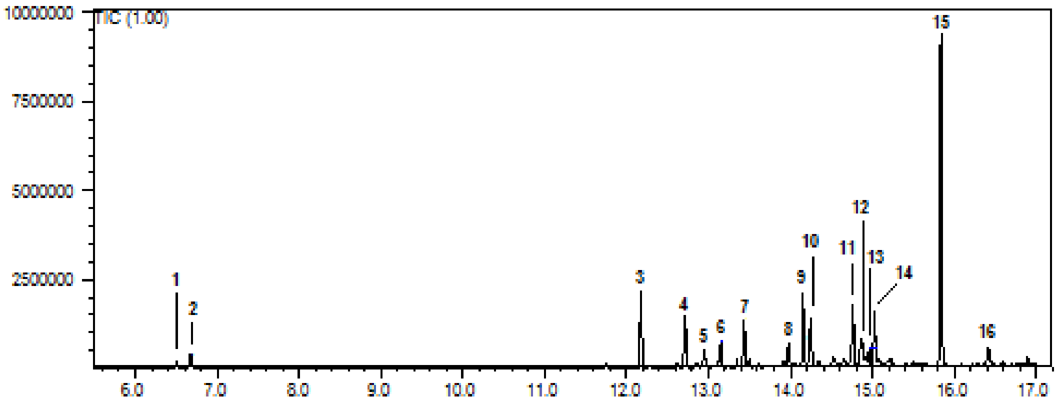

Figure 1. Representation of peaks obtained in the process gas chromatography coupled to mass spectrometry essential oil of *Eugenia uniflora* L.

In the analysis of chemical composition, it was observed that the identification a major compound was not possible, as peak 15 had concentration of 38.3% (Table 1). It can be seen that the identified compounds are monoterpenes, sesquiterpenes and terpenes.The terpenes and sesquiterpenes were the mainly compounds identified in volatile oils, which may be linked to the antibacterial activity (22, 23, 24).

It was also observed that most of the identified compounds are sesquiterpenes,caryophyllene for example, was often isolated in *E. uniflora* extraction (25, 26). According to Silva (16), regardless of origin or part of *E. uniflora* plant used in the extraction, the sesquiterpenes show to be the main class of compounds, which corroborates the results found in this paper.

Table 1. Chemical composition of the *Eugenia uniflora* L. essential oil.

Peak	Retention time (min)	Concentration (%)	Compound	Classification
1	6.508	0,650	trans-β-ocimene	monoterpenes
2	6.674	1,155	cis-β-ocimene	monoterpenes
3	12.173	8,182	caryophyllene	sesquiterpenes
4	12.713	5,658	allo-aromadendrene	sesquiterpenes
5	12.951	1,595	β-copaene	sesquiterpenes
6	13.150	1,891	α-muurolene	sesquiterpenes
7	13.431	5,192	(+)-δ-cadinene	sesquiterpenes
8	13.977	2,283	UC	-
9	14.157	7,712	spathulenol	terpenes
10	14.245	5,781	viridiflorol	terpenes
11	14.762	6,211	isolongifolene, 9,10-dehydro-	sesquiterpenes
12	14.871	5,084	α-epi-cadinol	terpenes
13	14.959	2,423	UC	-
14	15.027	5,242	hihydro-cis-α-copaene-8-ol	terpenes

15	15.839	38,281	UC	-
16	16.419	2,234	UC	-

UC: unidentified compound

Even though the GC-MS system constitutes a breakthrough, it should be borne in mind that the library of spectral data is usually obtained with other apparatus. The fragmentation patterns can show variations in peak intensity, and the comparison between most intense peaks of the sample and library spectra can be complicated. It is important to consider the retention data for a safe identification and, despite these possibilities, it is normal that often a few spikes cannot be traced, so other analytical methods available should be used to complement the analysis (3).

The MIC test (Figure 2) showed antibacterial activity of the essential oil against *E. aerogenes* and *Salmonella typhimurium* up to the concentration of 3.125% for both. Evidenced by MBC test, the essential oil tested showed bactericidal activity in the dilutions 25%, 12.5%, 6.25% and 3.125% of the bacteria tested. There are already descriptions of antimicrobial activity of *E. uniflora* in gram negative and gram positive bacteria, and also against yeasts (25, 26, 27).

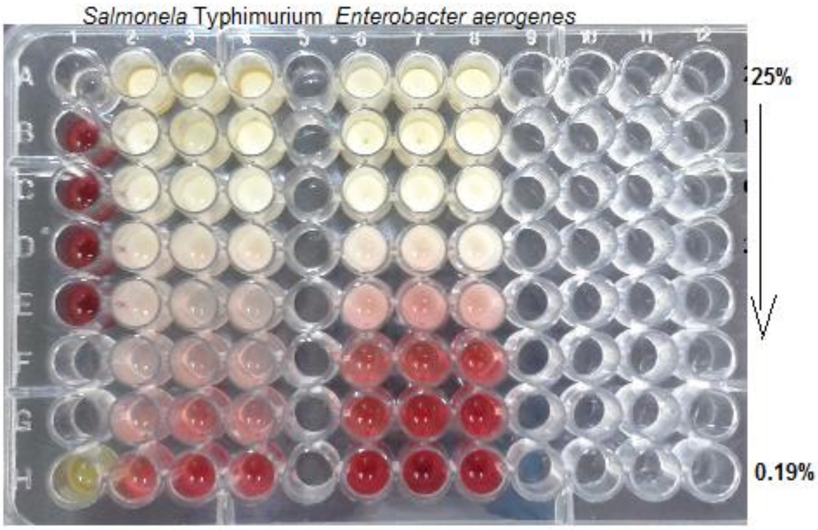

Figure 2 - Minimum Inhibitory Concentration Test (MIC) of *Eugenia uniflora* L. essential oil of against *E. aerogenes* and *S. typhimurium*. 1A – medium control negative; 1B-E – positive control strains; 1H – sterility essential oil control.

It is also reported, as well as in our research, the antibacterial activity of this essential oil against *E. cloacae* and *E. aerogenes* (28). Bouzada et al. (29) verified any antibacterial activity of the *E. uniflora* methanolic extract against *S. typhimurium*, and Barbosa et al. (30) registered in her research, the need for high concentrations of Brazillian cherry essential oil for inhibition of clinical and food isolates of *Salmonella enteric typhimurium* and *S. enteric* Enteretidis, respectively, unlike the data found in our study.

The antimicrobial activity performed by terpenes and derivatives has been described by research involving several species of plants and micro-organisms (2). Trombetta et al. (31) refer to some of the mechanisms by which these compounds act over bacterial cells. Monoterpenes can interfere with the permeability and to interact with cell membrane, results with an efflux of ions and ATP, causing an inhibition of respiratory chain. Studies that analyzed the mechanism of action of caryophyllene (a sesquiterpene) against bacterial specimens demonstrated mainly the inhibited histidine descarboxylase (in *E. aerogenes*) and the lyses of bacteria cells (32, 33, 34). Added to this, experimental research assays the inhibition or reduction of the bacterial toxins production after the treatment with essential oils (35).

When comparing studies with medicinal plants, is notoriously difficult to assess the results, since the variables ranging from edafic aspects - climate that influence the chemical composition, such as the stage of plant development at the time of the collect, part of the study plant, way to prepare the material for study, even the protocols followed in the experiments.

4. Conclusion

Based on the results obtained under test conditions, it can be concluded that the *E. uniflora* essential oil leaf showed that most of the identified compounds are sesquiterpenes. It was also shown antibacterial activity against the strains of *E. aerogenes* and *S. typhimurium*, indicating a potential use of this plant as a possible natural source for the development of new drugs.

Although all the experiments in vitro maintain limitations regarding the possible efficacy in vivo, the results of this study are promising with regard to *Eugenia uniflora* essential oil antibacterial potential, forming a solid foundation for future research.

Conflict of interest: The authors declare no conflict of interest.

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