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Antibacterial and antiproliferative activities of the fresh leaf essential oil of *Psidium guajava* L. (Myrtaceae)

E. A. J. Silva[a], E. B. B. Estevam[a], T. S. Silva[b], H. D. Nicolella[b], R. A. Furtado[b], C. C. F. Alves[a], E. L. Souchie[a], C. H. G. Martins[b], D. C. Tavares[b], L. C. A. Barbosa[c] and M. L. D. Miranda[d]*

[a]Instituto Federal de Educação, Ciência e Tecnologia Goiano, Rodovia Sul Goiana, s/n, Km 1, CP 66, CEP 75901-970, Rio Verde, GO, Brasil

[b]Centro de Pesquisa em Ciências Exatas e Tecnologia, Universidade de Franca – UNIFRAN, Av. Dr. Armando Salles Oliveira, 201, Parque Universitário, CEP 14404-600, Franca, SP, Brasil

[c]Departamento de Química, Universidade Federal de Viçosa, Campus Universitário, Av. Peter Henry Rolfs, s/n, 36570-900, Viçosa, MG, Brasil

[d]Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais, Av. Maria da Conceição Santos, 900, Parque Real, CEP 37550-000, Pouso Alegre, MG, Brasil

*e-mail: elizabethjosefi@yahoo.com.br

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Abstract

This study evaluated the antibacterial and antiproliferative activities of the essential oil of *Psidium guajava* leaves (PG-EO), traditionally used in folk medicine. The essential oil was obtained from fresh leaves by hydrodistillation, using a modified Clevenger apparatus. The major PG-EO chemical constituents were identified by GC-MS and GC-FID as being β-caryophyllene (16.1%), α-humulene (11.9%), aromadendrene oxide (14.7%), δ-selinene (13.6%), and selin-11-en-4α-ol (12.5%). The antibacterial activity of the essential oil of *P. guajava* leaves was determined in terms of its minimum inhibitory concentrations (MIC) using the broth microdilution method in 96-well microplates. PG-EO had moderate activity against *Streptococcus mutans* (MIC = 200 µg/mL), *S. mitis* (MIC = 200 µg/mL), *S. sanguinis* (MIC = 400 µg/mL), *S. sobrinus* (MIC = 100 µg/mL), and *S. salivarius* (MIC = 200 µg/mL). The antiproliferative activity was evaluated against different tumor cell lines: breast adenocarcinoma (MCF-7), human cervical adenocarcinoma (HeLa), and human gliobastoma (M059J). A normal human cell line (GM07492A, lung fibroblasts) was included. The antiproliferative activity was evaluated using the XTT assay and the results were expressed as IC_{50}. The essential oil showed significantly lower IC_{50} values against MCF-7 and M059J lines than that obtained for the normal line, showing selectivity. Our results suggest that the essential oil of *Psidium guajava* L. has promising biological activities and can be considered a new source of bioactive compounds.

Keywords: P. guajava, essential oil, oral pathogens, antiproliferative activity.

Atividades antibacteriana e antiproliferativa do óleo essencial das folhas frescas de *Psidium guajava* L. (Myrtaceae)

Resumo

Este estudo avaliou as atividades antibacteriana e antiproliferativa do óleo essencial das folhas frescas de *Psidium guajava* (PG-OE), tradicionalmente utilizadas na medicina popular. O óleo essencial foi obtido por hidrodestilação das folhas frescas, utilizando aparelho do tipo Clevenger. Os principais constituintes químicos de PG-OE identificados por CG-EM e CG-DIC foram: β-cariofileno (16,1%), α-humuleno (11,9%), óxido de aromadendreno (14,7%), δ-selineno (13,6%) e selin-11-en-4α-ol (12,5%). A atividade antibacteriana do óleo essencial das folhas de *P. guajava* foi determinada em termo de sua concentração inibitória mínima (CIM) utilizando o método de microdiluição de caldo em microplacas de 96 poços. PG-OE apresentou moderada atividade contra *Streptococcus mutans* (CIM = 200 µg/mL), *S. mitis* (CIM = 200 µg/mL), *S. sanguinis* (CIM = 400 µg/mL), *S. sobrinus* (CIM = 100 µg/mL) e *S. salivarius* (CIM = 200 µg/mL). A atividade antiproliferativa foi avaliada frente a diferentes linhagens de células tumorais como: adenocarcinoma de mama (MCF-7), adenocarcinoma cervical humano (HeLa) e gliobastoma humano (M059J). Foi incluída uma linhagem celular humana normal (GM07492A, fibroblastos pulmonares). A atividade antiproliferativa foi avaliada utilizando o ensaio XTT e os resultados foram expressos como CI_{50}. As linhagens MCF-7 e M059J mostraram valores significativamente mais baixos de CI_{50} do que os obtidos para a linhagem normal, mostrando seletividade. Nossos resultados sugerem que o óleo essencial das folhas frescas de *Psidium guajava* L. possui atividades biológicas promissoras e pode ser considerado como uma nova fonte de compostos bioativos.

Palavras-chave: P. guajava, óleo essencial, patógenos orais, atividade antiproliferativa.

1. Introduction

Essential oils are sources of natural substances with several biological activities with antioxidant, antimicrobial, anticancer, antinociceptive, antiviral, and antiphlogistic properties (Martins et al., 2015).

Tooth decay is an important public health problem that affects a large number of people in many countries in the world. More than 700 species of bacteria are identified in the oral cavity and some of them are responsible for caries and other periodontal diseases, among them we can highlight the bacteria of the genus *Streptococcus* (Melo et al., 2017).

Plant-derived materials such as plant extracts, essential oils, and pure compounds have antimicrobial effects against oral pathogens and these materials have attracted the interest of researchers worldwide. However, reports on the antimicrobial activity of natural products against oral pathogens are still scarce (Estevam et al., 2016).

Currently, cancer treatment is considered one of the most challenging problems in medicine and several experimental and epidemiological studies have shown that the use of some plants may promote chemopreventive and/or antineoplastic action (Oliveira et al., 2014). In this scenario, some essential oils extracted from different plants have promising antitumor potential, both *in vitro* and *in vivo* (Lesgards et al., 2014).

The species *Psidium guajava* L., common name guava, belongs to the family Myrtaceae, which is composed of more than 100 genera and 3800 species and is one of the most studied species of this family (Haida et al., 2015). The essential oils and leaf extracts of this plant have several biological activities, including antidermatophytic, anti-inflammatory, antibiotic, analgesic, hepato-protective and antioxidant properties (Bhushan et al., 2014; Ferdinand et al., 2014).

This study, continuing our line of research on chemical composition and the biological activities of essential oils (Lemes et al., 2017; Oliveira et al., 2017), analyzes the chemical composition and the antibacterial and antiproliferative activities of the fresh leaf essential oil of *P. guajava* L. collected in the southwest region of the state of Goiás.

2. Material and Methods

2.1. ***Plant material***

The experiment was conducted at the Laboratory of Natural Products Chemistry of the Instituto Federal Goiano - Campus Rio Verde, GO. In March 2014, fresh *P. guajava* leaves were collected at 4:00 p.m from a native population in the Rio Verde region. Collection took place at coordinates 17°48'12.006''S and 50°54'19.083''W, 715m altitude. Plant material was identified, and the samples were deposited as desiccated specimens in the Herbarium of the Universidade Estadual de Montes Claros, state of Minas Gerais, Brazil under identification number 4481.

2.2. Essential oil extraction

The essential oil was extracted from fresh leaves of *P. guajava* (100 g) ground in a knife mill by the hydrodistillation method using a Clevenger type apparatus at 100 °C for 4 h (Xavier et al., 2016). Thereafter, the hydrolate was subjected to liquid-liquid partition in a separatory funnel and three washes with three 10 mL portions of dichloromethane. Essential oil samples were stored at −4 °C until further chemical and biological tests.

2.3. Chemical analysis of essential oil

Essential oil chemical composition was analyzed at the Laboratory of Analysis and Synthesis of Agrochemicals of the Universidade Federal de Viçosa, Minas Gerais. Quantitative analysis of the essential oil components was performed in a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (FID) and SPB-5 fused silica capillary column (30 m × 0.25 mm; 0.25-µm film thickness). Nitrogen was used as the carrier gas (1.8 mL min^{-1}), split 1/10, and injector and detector temperatures were 220 and 240 °C, respectively. The initial column temperature was 40 °C isothermal for 4 minutes, followed by heating at 3 °C min^{-1} up to 240 °C; 1µL of the essential oil was injected for the analysis. Essential oil components were identified using a gas chromatograph (CG-EM Shimadzu, QP-5050A) equipped with a mass-selective detector, operating by electronic ionization (70 eV), with an RTX-5 fused silica column (30 m × 0.25 mm; 0.25-µm film thickness). Chromatographic conditions were identical to the conditions applied to the CG-FID, except for the carrier gas, which in this case was helium at a 1mL min^{-1} flow. The identification of constituents was based on the retention indices relative to C_9–C_{22} alkanes and by comparing the mass spectra with a computer databank (Wiley 7 and Nist 62) and with published data (Adams, 2007).

2.4. Antibacterial assays

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) at Universidade de Franca, state of São Paulo, Brazil, at -80°C. The following microorganisms were used: *Streptococcus salivarius* (ATCC 25975), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49452), *Streptococcus sanguinis* (ATCC 10556) and *Streptococcus sobrinus* (ATCC 33478).

Minimum Inhibitory Concentrations (MIC) of essential oils were determined by the broth microdilution method in 96-well microplates, following the methodology of CLSI (2006). Samples were dissolved in 125 µL tryptic soy broth (TSB) to yield compound concentrations between 50 and 400 µg/mL. The inoculum was adjusted to 625 nm for every microorganism in a spectrophotometer to obtain cell concentration of 5×10^5 colony-forming units (CFU/mL) (CLSI 2006). Chlorhexidine digluconate (Sigma-Aldrich), at concentrations from 0.115 to 59.0 µg/mL, was used as the positive control. The microplates were incubated at 37 °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.

2.5. Cell lines and culture conditions

In this study, we used three different tumor cell lines: human breast adenocarcinoma (MCF-7), human cervical adenocarcinoma (HeLa), and human gliobastoma (M059J). A normal human cell line (lung fibroblasts, GM07492A) was included to evaluate the possible selective activity of the natural product tested. The different cell lines were maintained as monolayers in plastic culture medium (HAM-F10 + DMEM, 1:1, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich), and 2.38 mg/mL Hepes (Sigma-Aldrich). The cells were incubated at 36.5 °C in a humidified 5% CO_2 atmosphere.

2.6. Antiproliferative assay

The antiproliferative activity was measured using the *in vitro Toxicology Colorimetric Assay Kit* (XTT; Roche Diagnostics) according to the manufacturer's instructions. For the experiments, the cells (10^4 cells/well) were plated onto 96-well microplates. Each well received 100 µL HAM-F10/DMEM medium containing essential oil at concentrations ranging from 3.91 to 500 µg/mL. Negative (no treatment), solvent (0.02% DMSO, dimethylsulfoxide, Sigma-Aldrich) and positive (doxorubicin, DXR, Pharmacia Brasil Ltda.,) controls were included. After incubation at 36.5 °C for 24 h, the culture medium was removed. The cells were washed with 100 µL of PBS (phosphate buffered saline) to remove the treatments and exposed to 100 µL of culture medium HAM-F10 without phenol red. Then, 25 µL of XTT was added, and the cells were incubated at 36.5 °C for 17 h. The absorbance of the samples was determined using a multi-plate reader (ELISA – Tecan – SW Magellan vs 5.03 STD 2P) at the wavelength of 450 nm and reference length of 620 nm. The antiproliferative activity was assessed using IC_{50}, the concentration able to inhibit 50% of cell line growth as a response parameter, which was calculated with the GraphPad Prism program by plotting cell survival against the respective concentrations of the natural product tested. One-way ANOVA was used for the comparison of means ($p \leq 0.05$). The experiments were performed in triplicate. The selectivity index was calculated by dividing the IC_{50} value of the essential oil obtained for GM07492A cells by the IC_{50} value obtained for the cancer cell line.

3. Results and Discussion

The essential oil of *P. guajava* fresh leaves is composed mainly by hydrocarbon sesquiterpenes (62.0%), followed by oxygenated sesquiterpenes (14.8%), hydrocarbons monoterpenes (1.8%), and oxygenated monoterpenes (1.2%) (Table 1). Seventeen compounds were identified in the essential oil of *P. guajava*, which corresponded to 94.1% of the total oil analyzed and the main components are: β-caryophyllene (16.1%), α-humulene (11.9%), aromadendrene oxide (14.7%), δ-selinene (13.6%), and selin-11-en-4α-ol (12.5%) (Table 1).

The chemical composition of the essential oil extracted from fresh leaves of *P. guajava*, collected in the state of Goiás, showed little similarity to other oils reported in the literature for species occurring in other countries. For example, the major constituents identified in the leaf essential oil of *P. guajava* from Nepal were *E*-nerolidol (35.6%) and *E*-caryophyllene (15.8%) (Satyal et al., 2015), whereas in Tunisia, the major constituents were viridiflorol (36.4%) and *trans*-caryophyllene (5.9%) (Khadhri et al., 2014) and, in Nigeria, the predominant constituents were limonene (42.1%) and β-caryophyllene (21.3%) (Ogunwande et al., 2003). The essential oil chemical composition of *P. guajava* occurring in Lavras, a municipality in the southern region of Minas Gerais, showed similarity with the chemical composition found in the present study, especially the compound selin-11-en-4α-ol was present in both (Lima et al., 2010). The chemical composition observed in the present study was similar to the chemical composition already described in the literature for other species belonging to the same genus and Myrtaceae family (Scur et al., 2016).

Table 1. Compounds identified in the leaf essential oil of *P. guajava* (Myrtaceae).

Compounds	***RI****	***RA*%***
Limonene	1024	1.8
1,8-Cineole	1026	1.2
α-Copaene	1374	1.3
β-Caryophyllene	1419	**16.1**
α-Humulene	1454	**11.9**
4,11-Selinadiene	1475	1.3
γ-Muurolene	1478	1.4
Aromadendrene oxide	1488	**14.7**
δ-Selinene	1499	**13.6**
α-Panasinsene	1517	1.3
trans-Nerolidol	1566	3.3
β-Caryophyllene oxide	1585	4.1
Humulene epoxide II	1612	1.4
Longipinene epoxide	1620	1.8
epi-α-Muurolol	1639	4.0
Selin-11-en-4α-ol	1645	**12.5**
α-Cadinol	1660	1.6
Total identified		94.1
Hydrocarbon monoterpenes		1.8
Oxygenated monoterpenes		1.2
Hydrocarbon sesquiterpenes		62.0
Oxygenated sesquiterpenes		14.8

***RI:** Retention index from the literature. %**RA**: Relative area (peak area relative to the total peak in the GC-MS chromatogram), average of three replicates.

Regarding the antibacterial activity, several papers have reported the antimicrobial potential of plant essential oils against oral pathogens over the last decade (Sousa et al., 2015). Table 2 shows the antibacterial activity of the fresh leaf essential oil of *P. guajava* against a representative panel of oral pathogens, with MIC values ranging from 400 to 100 µg/mL.

According to Holetz et al. (2002), the antimicrobial activity can be considered good when MIC values are below 100 µg/mL; moderate from 500 to 100 µg/mL; weak from 1000 to 500 µg/mL; and inactive above 1000 µg/mL.

The PG-EO showed moderate antibacterial activity against all bacteria of the genus *Streptococcus* assessed in this work. This activity can be explained by the chemical constituents of known antibacterial activity such as caryophyllene oxide (4.1%), β-caryophyllene (16.1%), and α-humulene (11.9%) present in the oil (Moreira et al., 2014).

Several mechanisms are proposed to explain the antimicrobial activity of essential oils. It is believed that microbial growth inhibition by essential oils is due to the direct damage to cell membrane integrity caused by their lipophilic components, which affects cell pH maintenance and inorganic ion balance. According to the literature, the inhibitory effects of essential oils are consistent with the action of monoterpene and sesquiterpene constituents on the cell membrane, and the damage to membrane produces different effects on microorganisms (Oliveira et al., 2016).

The PG-EO cytotoxicity was assessed against the GM07492A normal cell line, with an IC_{50} of 126.4 ± 11.8 µg/mL, and against the MCF-7, HeLa, and M059J tumor lines with IC_{50} of 96.9 ± 8.4; 128.7 ± 1.5; and 103.6 ± 5.1 µg/mL, respectively (Table 3). The IC_{50} of the lines MCF-7 and M059J were significantly lower than that of the normal line, with selectivity indices of 1.2 and 1.3, respectively. The IC_{50} values of PG-EO are lower and have higher antiproliferative activity than the IC_{50} values of the leaf essential oil of *Rosmarinus officinalis* against the cell lines MCF-7 (IC_{50} = 190.1 µg/mL) and LNCaP (IC_{50} = 180.9 µg/mL) (Hussain et al., 2010). Hussain et al., (2010) classified IC_{50} values < 10 µg/mL as potentially very toxic, IC_{50} from 10 to 100 µg/mL as potentially toxic, IC_{50} from 100 to 1000 µg/mL as potentially harmful, and IC_{50} > 1000 µg/mL as potentially non-toxic.

The evidence from this study, the high amount of terpenes, known for their anticancer activity such as β-caryophyllene (16.1%), aromadendrene oxide (14.7%), δ-selinene (13.6%), selin-11-en-4α-ol (12.5%), α-humulene (11.9%), β-caryophyllene oxide (4.1%), and α-cadinol (1.6%) (Table 1), suggests that *P. guajava* essential oil is a significant potential source of pure compounds with promising anticancer activity (Salvador et al., 2011; Quassinti et al., 2013; Fidyt et al., 2016; Guerrini et al., 2016). Besides, despite the low concentration of hydrocarbon monoterpenes (1.8%) and oxygenated monoterpenes (1.2%) in the essential oil of *P. guajava* (Table 1), this class of compounds deserves special attention for its important antitumor activity (Sobral et al., 2014). Furthermore, it is worth mentioning that the anticariogenic and antiproliferative activities of the leaf essential oil of *P. guajava* occurring in the southwestern region of Goiás can also be explained by the synergistic effect among all its chemical constituents (Lesgards et al., 2014; Casanova and Costa 2017).

Table 2. Antibacterial activity of essential oil of *P. guajava* leaves (PG-EO) against oral bacteria.

Minimum inhibitory concentration (MIC) - µg/mL		
Bacteria		**CHD***
Streptococcus salivarius[a]	400	0.922
S. mutans[a]	200	0.922
S. mitis[a]	200	3.688
S. sanguinis[a]	400	0.922
S. sobrinus[a]	100	0.922

[a]Aerobic Gram-positive bacteria. *CHD: chlorhexidine dihydrochloride (positive control).

Table 3. IC_{50} and selectivity index (SI) of essential oil of *P. guajava* leaves (PG-EO) against diferent cell lines.

Cell line	Treatment (µg/mL)			
	PG-EO		DXR	
	IC_{50}	SI	IC_{50}	SI
GM07492A	126.4 ± 11.8	-	0.5 ± 0.2	-
MCF-7	96.9 ± 8.4[a]	1.3	62.1 ± 2.0	-
HeLa	128.7 ± 1.5	-	5.3 ± 1.3	-
M059J	103.6 ± 5.1[a]	1.2	16.2 ± 2.5	-

Doxorubicin (DXR) was used as positive controls. GM07492A (human lung fibroblasts), MCF-7 (human breast adenocarcinoma), HeLa (human cervical adenocarcinoma) and M059J (human glioblastoma). The selectivity index is the ratio between the IC_{50} value of the PG-EO obtained for GM07492A cells and the value found for the tumor cell line. Values are mean ± SD, n = 3. [a]Significantly different from the normal cell line (GM07492A) ($p \leq 0.05$).

4. Conclusion

The GC-MS and GC-FID analysis of the essential oil of *P. guajava* leaves revealed the presence of seventeen compounds, among which β-caryophyllene, α-humulene, aromadendrene oxide, δ-selinene, and selin-11-en-4α-ol were the major components. Whit respect to testing the antibacterial activity, the essential oil showed moderate antibacterial activity against all bacteria of the genus *Streptococcus* assessed in this study. Regarding the anticancer activity, the essencial oil of *P. guajava* leaves was effective against different tumor cell lines tested.

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