

BIOLOGICAL ACTIVITY OF ESSENTIAL OIL OF PITANGA (*Eugenia uniflora* L.) LEAVES

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Many natural extracts present antioxidant and antimicrobial properties that can replace synthetic additives. This study aimed to extract essential oil from Pitanga (*Eugenia uniflora* L.) leaves and to evaluate its chemical composition, antioxidant and antibacterial activities. The essential oil was obtained by hydrodistillation with a yield of 1.34 ± 0.18 %. The total antioxidant activity was evaluated by the DPPH and phosphomolybdenum methods. The oil presented an IC_{50} value of $100.96 \mu\text{L mL}^{-1}$ and 1503.44 ± 29.48 mg α -tocopherol mL^{-1} of oil. The major compounds identified by the GC-MS were germacrone (13.57%), spathulenol (7.49%), curzerene (5.31%) and α -cadinol (4.70%). The oil was also evaluated for its antimicrobial activity and was effective against *Staphylococcus aureus*. These results show that essential oil of pitanga leaves have good antioxidant and antimicrobial activity, being an alternative source of bioactive compounds.

KEYWORDS: BRAZILIAN CHERRY; ANTIBACTERIAL; ANTIOXIDANT; HYDRODISTILLATION

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1 INTRODUCTION

The growing interest for natural foods and evidence that some synthetic additives may promote health side effects, such as allergies, hyperactivity (Polônio and Peres, 2009) and cancer (Botterwerck *et al.*, 2000) have increased the demand for natural compounds with similar activities. In this context, natural extracts have been studied as alternative food preservatives in the food industry due to their potential to act as antioxidant (Misharina *et al.*, 2009) and antimicrobial (Pisoschi *et al.*, 2018) agents. In addition, they have been widely applied in perfumery, cosmetic, pharmaceutical and textiles industries (Cavalcanti *et al.*, 2013). Natural extracts also provide beneficial human health effects (Ibañez *et al.*, 2003).

Plant extracts are products with high added-value and are composed of a complex mixture of numerous substances which are thermal degradable. The extraction methods considerably influence their quality (Alexandre *et al.*, 2017). The extraction of bioactive compounds from plants has been carried out by various methods, including alcoholics (Chan *et al.*, 2007), aqueous (Sivasothy *et al.*, 2011), hydrodistillation (Mesomo *et al.*, 2013) and supercritical fluids (Mesomo *et al.*, 2012).

Essential oils are obtained by the hydrodistillation method. The antioxidant and antimicrobial properties of these essential oils are determined by their composition. Among the phytochemicals capable of acting as antioxidants are minerals (zinc and selenium), vitamins (A, C, E and K), carotenoids and phenolic compounds (Nahak *et al.*, 2014). Many plant materials contain phenolic compounds and a significant quantity is available for human consumption (Kähkönen *et al.*, 1999).

Phenolic compounds include several groups of organic substances and the major classes are phenolic acids and flavonoids (Soares, 2002). They act as antioxidants by scavenging free radicals such as peroxide, hydroperoxide or lipid peroxyl, and thus preventing the oxidative reactions (Nahak *et al.*, 2014). Flavonoids are also iron chelators which improve the inhibition of lipid peroxidation (Van Acker *et al.*, 1996).

Essential oils with high levels of substituted phenols can reduce the oxidation processes of unsaturated aldehydes, even at low concentrations (Misharina *et al.*, 2009). In addition, plant-derived antimicrobials can inactivate microorganisms and enzymes without affecting the nutritional and sensory properties of food (Pisoschi *et al.*, 2018). The major components responsible for the antimicrobial properties of plants EOs are phenolics (flavonoids and non-flavonoids), terpenes, aliphatic alcohols, aldehydes, among others (Pisoschi *et al.*, 2018).

Fruits and vegetables are known sources of antioxidant and antimicrobial compounds. *Eugenia uniflora* L., popularly known as Pitanga, Surinam cherry or Brazilian cherry, belongs to the Myrtaceae family and it is native to Brazil and Argentina (Franzoni *et al.*, 2018). Its leaves are used in popular medicine as a tea and have application for the treatment of hypertension, gout, stomach disorders, rheumatism and yellow fever (Denardin *et al.*, 2015). It also has edible fruits which are widely appreciated in Brazil.

The essential oil obtained from Pitanga leaves is used by the cosmetics industry; however, its commercial application is still limited (Franzoni *et al.*, 2018). Victoria *et al.* (2013) showed that it has antidepressant and antioxidant properties. The authors suggested that it can be used for the development of phytomedicines due to the modulation of serotonergic and adrenergic systems. Additionally, the biological activities of *E. uniflora* as antihypertensive, anti-

rheumatic, antifungal, diuretic, among others, have been reported in the literature (Bezerra *et al.*, 2018).

Ethanol extract of *Eugenia uniflora* L. leaves characterized by HPLC showed that quercetin and quercitrin were the major components, while chlorogenic acid and gallic acid were the least abundant (Cunha *et al.*, 2016). The presence of polyphenolic compounds, such as quercetin, quercitrin, isoquercitrin, luteolin and ellagic acid, may be responsible for its beneficial effects as cytotoxic and antioxidative (Cunha *et al.*, 2016). These studies evidence the relevance of this material for pharmacological (Bezerra *et al.*, 2018) and food applications. Consequently, it is important to conduct more studies to identify alternative sources of extracts with high contents of bioactive compounds, such as Pitanga leaves.

The main objectives of this study were to evaluate the chemical composition, antioxidant activity and antibacterial activity of essential oil of Pitanga (*Eugenia uniflora* L.) leaves obtained by the hydrodistillation process.

2 MATERIAL AND METHODS

2.1 SAMPLE PREPARATION

The pitanga (*Eugenia uniflora* L.) leaves were collected in the region of Palmeira (State of Paraná, Brazil). They were dried in an air circulation oven at $303.15 \text{ K} \pm 2 \text{ K}$ until reaching a moisture content of $10.28 \pm 1.05 \%$. The dried leaves were ground in a food processor and stored in a freezer ($255.15 \text{ K} \pm 2 \text{ K}$) until extraction. The moisture content (H) of the raw material and the dried samples were measured by the toluene distillation method according to AOCS (1994), which can distinguish water from the volatile material. The moisture values were calculated as follow:

$$H = \left(\frac{V_{H_2O} \times \rho_{H_2O}}{w_{sample}} \right) \quad (1)$$

where V_{H_2O} is measured as the aqueous phase volume (mL), ρ_{H_2O} is the water density (g mL^{-3}) and w_{sample} is the sample mass (g).

2.2 HYDRODISTILLATION

The hydrodistillation technique was employed to extract the volatile oil from the dried sample using a Clevenger-type apparatus according to the method recommended by Brazilian Pharmacopeia (Farmacopéia, 1999). A total of 30 g of dried leaves were homogenized and placed in a 1000 mL round-bottom flask with 500 mL of distilled water. The preparation was boiled for 180 min at atmospheric pressure (93.50 kPa). The oil obtained was separated and dried over the minimum amount of anhydrous sodium sulfate to remove traces amount of moisture. The essential oil was stored at $278.15 \text{ K} \pm 2 \text{ K}$ until further analysis.

2.3 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

GC-MS analysis was carried out using a gas chromatograph (Agilent® 7890A) coupled to a mass selective detector (Agilent® 5975C) in EI mode at 70 eV with a nonpolar

capillary column VF-5MS (30 m, 0.25 mm and a film thickness of 0.25 μm , 5% phenyl-95% dimethylpolysiloxane). The carrier gas used was helium with injection volume 1.0 μL . Source and quadrupole temperatures were 503.15 K and 423.15 K, respectively. The mass spectrometer was programmed to detect the 40.0-1000.0 mass/charge (m/z) ratio. The initial column temperature was 473.15 K, maintained for 2 minutes, with heating 4 K min^{-1} to 573.15 K, kept for 4 minutes.

The active compound characterization was made by comparing retention time, Kovats retention indice (KI) calculated relative to n-alkanes (Alkane standard solution C8-C40 4070 Fluka) and recorded mass spectra with those stored in the NIST-11 mass spectral library of the GC-MS data system of the apparatus. The other data processing approach used was the AMDIS (Automated Mass Spectral Deconvolution and Identification System) software. For compounds confirmation, literature data were compared to values obtained by Equation 2:

$$IK_x = 100y + 100(z - y) \frac{t_{(r)x} - t_{(r)y}}{t_{(r)z} - t_{(r)y}}, \quad (2)$$

where y is the carbon number of the standard to the left of the peak of the evaluated compound, z is the carbon number of the standard to the right of the peak of the evaluated compound, $t_{(r)x}$ is the retention time of the subject compound (min), $t_{(r)y}$ is the standard retention time on the left (min), and $t_{(r)z}$ is the retention time of the right standard (min).

2.4 ANTIOXIDANT ACTIVITY

2.4.1 DPPH spectrophotometric assay

The antioxidant activity was determined following the procedure described by Mensoret *al.* (2001) using 1.1-Diphenyl-2-picrylhydrazyl (DPPH•) as the reactant. Solutions with final concentrations ranging from 25 to 125 $\mu\text{L mL}^{-1}$ were prepared from an ethanolic stock solution (2.5 mL mL^{-1}) of the oil. Aliquots (2.5 mL) of each oil solution were transferred to test tubes protected against the light, and 1.0 mL of a freshly prepared ethanolic solution of DPPH (0.3 mM) was added to each tube. This mixture was mixed well and left to react in a dark place for 30 min at room temperature. The absorbance (abs_{sample}) was then measured at 517 nm using a spectrophotometer (Bel Photonics, 2000UV). The blank was prepared by mixing 1.0 mL of ethanol with 2.5 mL of each oil solution. The negative control was prepared by mixing 1.0 mL of DPPH• solution and 2.5 mL of ethanol. The absorbance of these solutions was also measured (abs_{blank} , abs_{control}). The tests were carried out in triplicate, and the antioxidant activity (AA) was calculated using Equation 3:

$$AA(\%) = 100 - \left\{ \frac{[(abs_{\text{sample}} - abs_{\text{blank}}) \times 100]}{abs_{\text{control}}} \right\} \quad (3)$$

The antioxidant activity was also expressed as the half-maximal inhibitory concentration (IC_{50}), defined as the concentration ($\mu\text{L mL}^{-1}$) of oil required to inhibit the production of radicals by 50%.

2.4.2 Phosphomolybdenum reducing method

The total antioxidant activity was determined by the spectrophotometric method involving phosphomolybdenum reduction, as described by Prietro *et al.* (1999). It is based on the reduction of Mo^{6+} to Mo^{5+} with subsequent formation of a phosphate– Mo^{5+} complex, which presents maximum absorption at 695 nm. A 1.0 mg mL^{-1} aliquot of the solution (0.01 g of oil and 10.0 mL of ethanol) was combined with 1.5 mL of reagent solution (0.6 M sulfuric acid, 28.0 mM sodium phosphate, and 4.0 mM ammonium molybdate). The samples were incubated at 368.15 K for 90 min. After the samples cooled down to room temperature, they were centrifuged, and the absorbance of the solution was measured at 695 nm against a blank (1.5 mL of reagent solution and 0.02 mL of ethanol). The analysis was conducted in triplicate. The results were expressed as $\text{mg of } \alpha\text{-tocopherol mL}^{-1}$ of oil.

2.5 ANTIBACTERIAL ACTIVITY

The antibacterial properties of the oil obtained were tested by the agar well method (Bauer *et al.*, 1996). Two Gram-positive bacterial strains, *Staphylococcus aureus* (ATCC25923) and *Listeria monocytogenes* (ATCC19111), and three Gram-negative bacterial strains, *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella typhimurium* (ATCC14028) were tested.

The selected bacterial strains were inoculated into 10.0 cm^3 of trypticase soy broth (TSB) at 308.15 K for 24 h. Suspensions of bacteria ($50.0 \text{ } \mu\text{L}$) were prepared and adjusted by comparison according to the McFarland turbidity scale, standard No. 0.5, and seeded using sterile cotton swabs on trypticase soy agar (TSA) plates. Agar wells were prepared with a sterilized cork borer with a 7.0 mm diameter. The samples of extracts were dissolved in DMSO ($200.0 \text{ } \mu\text{L}$ of each sample in $600.0 \text{ } \mu\text{L}$ of DMSO), and $40.0 \text{ } \mu\text{L}$ of the solutions were added to different wells in the plates. The plates were kept at room temperature (295.15 K) for 3 h to allow diffusion of the solution in the culture medium before the growth of microorganisms and incubated in an upright position at 308.15 K for 24 h. $50.0 \text{ } \mu\text{L}$ of DMSO was used as the negative control. The antibacterial activity was assessed by the formation or not of inhibition zones around the wells. Assays were performed in triplicate, and the diameters of the inhibition zones (in mm) were measured.

3 RESULTS AND DISCUSSION

3.1 EXTRACTION YIELD

The fresh leaves of pitanga presented humidity of $60.00 \pm 1.22 \text{ wt}\%$ and were dried until $10.28 \pm 1.05 \text{ wt}\%$ of humidity. The dried leaves were used to obtain the essential oil (EO) by hydrodistillation. The yield of pitanga EO was $1.34 \pm 0.18 \text{ wt}\%$ on a dry basis.

3.2 CHEMICAL COMPOSITION

The compounds identified by GC-MS analysis of the pitanga leaves essential oil are presented in Table 1. 17 compounds were identified, which represent, on average,

approximately 55 % of the oil mass. The major compounds identified in the volatile fraction of the oil were germacrone (13.57 %), spathulenol (7.49 %), curzerene (5.31 %), α -cadinol (4.70 %), shyobunone (4.16 %), β and γ -elemene (6.15 % in total), cis-jasmone (2.91 %) and cubenol (2.92 %).

TABLE 1 - CHEMICAL COMPOSITION (%) OF THE VOLATILE FRACTION OF PITANGA LEAVES OIL OBTAINED BY HYDRODISTILLATION

Compounds	Rt (min)	RI ^a	RI ^b	Relative area (%)
Linalol	10.78	1099	1098	0.26
p-tertamyphenol	14.30	1224	1256	0.53
cis-jasmone	16.65	1311	1394	2.91
β -elemene	18.85	1396	1391	3.03
Caryophyllene	19.52	1423	1428	1.48
γ -elemene	19.80	1434	1433	3.12
α -Muurolene	20.57	1465	1477	1.30
Germacrene-D	21.07	1486	1480	1.06
curzerene	21.65	1510	1506	5.31
Shyobunone	21.97	1523	-	4.16
(-)- β -Cadinene	22.14	1531	1519	1.83
Spathulenol	22.56	1549	1576	7.49
cubedol	24.88	1650	1642	2.92
α - Cadinol	25.29	1668	1653	4.70
Germacrone	26.30	1715	-	13.57
Lanceol, cis	28.11	1800	1737	0.82
Ledene oxide -(II)	28.75	1832	1890	0.63
TOTAL				55.12

Rt, retention time.

RI, retention indices

^aCalculated retention index.

^bRetention index from Pherobase and Leffingwell.

A study by Claesonet *et al.* (1993) showed germacrone has anti-inflammatory activity. Zhong *et al.* (2011) demonstrated that germacrone present in Curcuma extract inhibited cell proliferation of human breast cancer (anti-tumor activity). Spathulenol is one of the main components in the essential oil of *Baccharissemiserrata* (Mendes *et al.*, 2008) and *Kundmannia* species (Paksoyet *et al.*, 2016). The latter authors stated that this compound has anticancer, antimicrobial and immunomodulatory effects. It can be applied to foods, medicines, cosmetics, personal care and cleaning products (Mendes *et al.*, 2008, Paksoyet *et al.*, 2016).

The chemical composition of Pitanga leaves EO was different from the supercritical extract obtained by Garmuset *et al.* (2014) and the EO characterized by Victoria *et al.* (2012). They obtained germacrenes and selina-1,3,7-(11)-trien-8-one as the major compounds. On the contrary, the present study obtained germacrone as a minor constituent and selina-1,3,7-(11)-trien-8-one was not detected. Similarly, Lagoet *et al.* (2011) did not identify selina-1,3,7(11)-trien-8-one in the pitanga leaves EO. Victoria *et al.* (2013) also found curzerene (4 %) as a major compound.

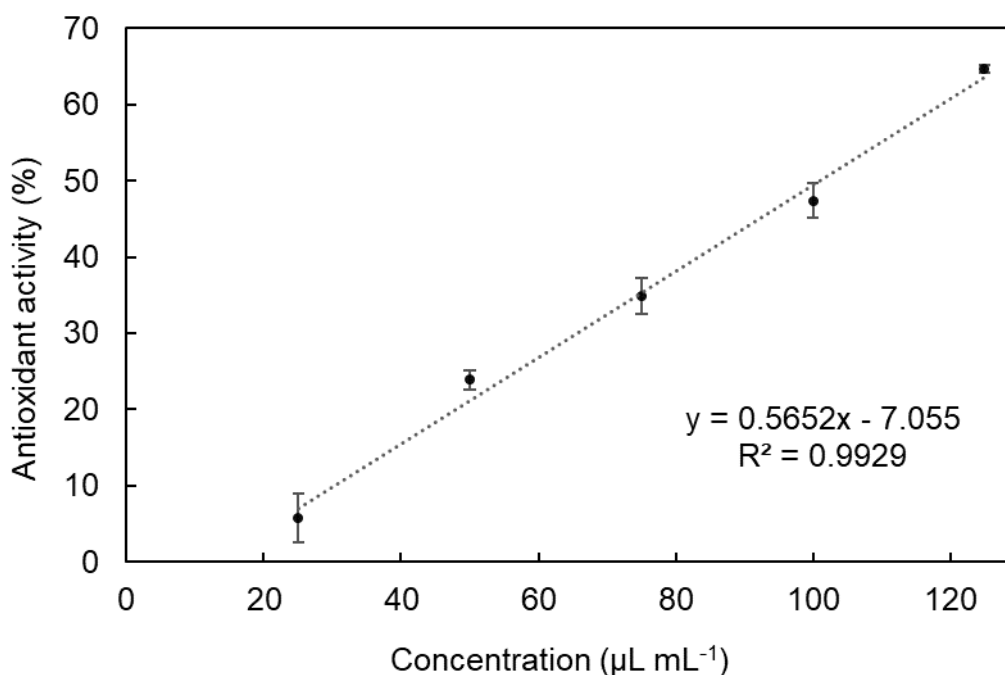
Previous research (Ogunwandeet *et al.*, 2005) detected low amounts of germacrone (2.60%), and spathulenol (1.50%) in pitanga leaves EO. Besides, curzerene (19.70%) was also a significant compound in the oil. According to Burt (2004), the chemical composition of essential oils from a plant species varies considerably, due to geographical origin and harvesting period. These differences are related to the plant chemotype and extraction method used (Garmuset *et al.*, 2014). Feippeet *et al.* (2011) found variability in antioxidant components at the intra-species

level and among different species of Uruguayan native fruits, such as Pitanga. They suggest that this information could be used for the breeding of commercial cultivars.

3.3 ANTIOXIDANT ACTIVITY

The highest antioxidant activity was 64.66 % obtained for oil at a concentration of 125 $\mu\text{L mL}^{-1}$, as shown in Figure 1. The antioxidant activity of the pitanga leaves EO was considered high. This result is due to the extraction of polar compounds which have high antioxidant activity (Mensoret *et al.*, 2001).

FIGURE 1 - ANTIOXIDANT ACTIVITY OF DPPH AS A FUNCTION OF THE ESSENTIAL OIL CONCENTRATION



The value of IC_{50} obtained from the linear regression was $100.96 \mu\text{L mL}^{-1}$ (Figure 1). Studies report that extracts which are considered very active present IC_{50} value $< 50 \text{ mg mL}^{-1}$, the moderately active extracts have IC_{50} of 50 to 100 mg mL^{-1} , slightly from 100 to 200 mg mL^{-1} and the ones considered inactive present an $\text{IC}_{50} > 200 \text{ mg mL}^{-1}$ (Reynertson *et al.*, 2005). Consequently, the EO was moderately active.

Additionally, the present EO was considered more active than reported by Victoria *et al.* (2012) and Victoria *et al.* (2013). They obtained an IC_{50} value of $833.3 \mu\text{g mL}^{-1}$ and $325.0 \mu\text{g mL}^{-1}$ for pitanga leaves EO, respectively. On the other hand, Garmuset *et al.* (2014) found $\text{IC}_{50} < 50 \mu\text{g mL}^{-1}$ for most extracts of pitanga leaves using a sequential extraction in fixed bed.

The total antioxidant activity determined by phosphomolybdenum reducing method was of $1503.44 \pm 29.48 \text{ mg tocopherol mL}^{-1}$ of oil. The value was higher when compared to ginger (*Zingiberofficinale* R.) extracts obtained using compressed propane and scCO_2 which ranged from 484.64 to $931.67 \text{ mg } \alpha\text{-tocopherol g}^{-1}$ of extract (Mesomoet *et al.*, 2012).

Therefore, the pitanga oil may be applied as an antioxidant in food products. It should be emphasized that the antioxidant compounds detected in each antioxidant method are not necessarily the same ones since the extracts are mixtures of compounds which can have either antagonistic or synergistic interactions (Martinez-Correa *et al.*, 2011).

3.4 ANTIBACTERIAL ACTIVITY

Table 2 shows the antibacterial activity of pitanga leaves EO against the five microorganisms tested. The essential oil showed inhibition only against bacteria of the genus *Staphylococcus aureus*, with an average diameter of the inhibition zones of 16.8 ± 2.0 mm. *S. aureus* is a Gram-positive bacterium which is more sensitive to the antibacterial action of essential oils, compared to Gram-negative bacteria (Nikaido and Vaara, 1985, Vaara, 1992). The other Gram-positive bacteria studied, *Listeria monocytogenes*, and the Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* did not form inhibition halos. The negative control with DMSO did not present inhibition, as expected.

TABLE 2 - ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF PITANGA LEAVES AGAINST SELECTED BACTERIAL SPECIES USING AGAR WELL METHOD

Bacterial species	Diameter of inhibition zone (mm)*	
	Pitanga leaves EO	Negative control (DMSO)
<i>Staphylococcus aureus</i>	16.8 ± 2.0	0.0 ± 0.0
<i>Listeria monocytogenes</i>	0.0 ± 0.0	0.0 ± 0.0
<i>Escherichia coli</i>	0.0 ± 0.0	0.0 ± 0.0
<i>Pseudomonas aeruginosa</i>	0.0 ± 0.0	0.0 ± 0.0
<i>Salmonella typhimurium</i>	0.0 ± 0.0	0.0 ± 0.0

*Mean \pm standard deviation of triplicate experiments.

These results agree with Victoria *et al.* (2012) findings for Pitanga leaf EO. They also did not observe antimicrobial activity against Gram-negative strains. The oil showed the antibacterial potential against *S. aureus* and *L. monocytogenes*.

Ogunwande *et al.* (2005) reported that Pitanga fruit oil had a stronger inhibition to the growth of *S. aureus* than Pitanga leaf oil. This oil also showed a weak antimicrobial effect against *E. coli* and *P. aeruginosa*. The authors concluded that the leaf oil had the stronger antibacterial effect against *Bacillus cereus* which was not tested in the present study.

Pereira *et al.* (2017) demonstrated that Pitanga leaves EO had antibacterial effect against *S. aureus* and *E. coli*. Similarly, the researchers observed that the oil did not have antimicrobial activity against *P. aeruginosa*. They stated that it was more effective against Gram-positive since Gram-negative bacteria is more resistant to the action of natural antimicrobials due to the external membrane which forms a protective envelope. Lago *et al.* (2011) also concluded that Pitanga leaves EO was active towards two Gram-positive strains, *Streptococcus equi* and *Staphylococcus epidermis*. Pitanga leaves essential oils used in the studies may result in antimicrobial activity against different microorganisms due to their chemical composition (i.e. compounds and quantities found).

Therefore, the application of the EO of pitanga leaves in foods may be interesting to prevent *S. aureus* growth, which is a bacterium that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms.

4 CONCLUSIONS

The essential oil of pitanga leaves presented high antioxidant activity and contained chemical compounds with proven antioxidant and antimicrobial properties. Additionally, it had an antimicrobial effect against the pathogenic bacterium *Staphylococcus aureus*. This oil has the potential to be applied in various products as a natural antioxidant and antimicrobial.

RESUMO

ATIVIDADE BIOLÓGICA DE ÓLEO ESSENCIAL DE FOLHAS DE PITANGA (*Eugenia uniflora* L.)

Muitos extratos naturais apresentam propriedades antioxidantes e antimicrobianas que podem vir a substituir aditivos sintéticos. Este estudo teve como objetivo extrair o óleo essencial das folhas de Pitanga (*Eugenia uniflora* L.) e avaliar sua composição química e atividades antioxidante e antibacteriana. O óleo essencial foi obtido por hidrodestilação com um rendimento de $1,34 \pm 0,18\%$. A atividade antioxidante total foi avaliada pelos métodos do DPPH e fosfomolibdênio. O óleo apresentou valor de IC_{50} de $100,96 \mu\text{L mL}^{-1}$ e $1503,44 \pm 29,48 \text{ mg de } \alpha\text{-tocoferol mL}^{-1}$ de óleo. Os principais compostos identificados pelo GC-MS foram germacrona (13,57%), spathulenol (7,49%), curzereno (5,31%) e α -cadinol (4,70%). O óleo também foi avaliado quanto à sua atividade antimicrobiana e foi efetivo contra *Staphylococcus aureus*. Estes resultados mostram que o óleo essencial das folhas de pitanga tem boa atividade antioxidante e antimicrobiana, sendo uma fonte alternativa de compostos bioativos.

PALAVRAS-CHAVES: PITANGA; ANTIBACTERIANO; ANTIOXIDANTE; HIDRODESTILAÇÃO

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