Quantification of 3,5,7,3'-4'-pentahydroxflavona of the aerial parts of
p. Guajava

Quantificação da 3,5,6,3’-4’-pentahydroxflavona das partes aéreas da
p. Guajava

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ABSTRACT

Psidium guajava is popularly known as guava. It is a very common species in South America, found most often in tropical climate. It is a plant known for its anti-inflammatory property, used in folk medicine for combating intestinal discomfort, colic, colitis, among others. The leaves of P. guajava present flavonoids in their composition, with emphasis on quercetin. The flavonoid class has antioxidant properties and acts on the central nervous system through oxidative inhibition of the endoplasmic reticulum. Thus, the objective of this study was to quantify the quercetin of the aerial parts of guava. Therefore, ethanol extraction was performed, followed by fractionation with solvents of different polarities, obtaining the hexanic, dichloromethane and ethyl acetate fractions. Then, quercetin was identified in the ethyl acetate fraction by Thin Layer Chromatography (CCD), the total flavonoid content was quantified by spectrophotometry and quercetin by High Efficiency Liquid Chromatography (HPLC). The qualitative analysis in CCD of the ethyl acetate fraction revealed the presence of flavonoids. Quantitative spectrophotometry analysis showed 46.2 mg of total flavonoids per g of the ethyl acetate fraction, being 816 μg g⁻¹ of quercetin revealed by HPLC. With this study, it was possible to identify the presence of flavonoids, and about 2% correspond to the substance quercetin, present in the ethyl acetate fraction of P. guajava. Therefore, further studies can be carried out aiming at the isolation of quercetin for application in a pharmaceutical form acting on the central nervous system.

Keywords: flavonoids, TLC, HPLC, quercetin.

RESUMO

A Psidium guajava é popularmente conhecida como goiabeira. Trata-se de uma espécie bastante comum, encontrada com mais frequência em pomares e quintais. É uma planta conhecida por suas propriedades farmacológicas utilizada como anti-inflamatório, bem como para o combate à diarreia e vômitos. Além disso, estudos comprovam que ela possui compostos que agem no sistema nervoso central por meio da inibição oxidativa do retículo endoplasmático. Estudos mostraram que as folhas da Psidium guajava apresentam quercetina, um flavonóide com propriedades farmacológicas antioxidantes. Desse modo, o objetivo deste trabalho é quantificar a quercetina utilizando as partes aéreas desta planta. Para isso, primeiramente realizou-se extração com etanol e fracionamento com solventes de diferentes polaridades. Em seguida, avaliou-se a presença de quercetina na fração acetato de etila em Cromatografia em Camada Delgada (CCD), quantificação de flavonoides totais por espectrometria e identificação e quantificação da quercetina em Cromatografia Líquida de Alta Eficiência (CLAE). A análise qualitativa realizada por meio da CCD feita pela fração acetato de etila revelou a presença de flavonoides. A análise quantitativa realizada por espectrometria apresentou 46,2 mg · g⁻¹ de flavonoides totais por g de extrato de acetato de etila. Por meio da análise realizada no CLAE quantificou-se a quercetina isolando-a de outras substâncias presentes no extrato. Com esse estudo, foi possível quantificar a quercetina por diferentes métodos e comprovar que nas partes aéreas da folha Psidium guajava há a presença desse...
flavonoide. Com a confirmação desse composto, podem ser realizados novos estudos para aplicação do mesmo em uma forma farmacêutica que atue no sistema nervoso central.

**Palavras-chave:** flavonoides, CCD, CLAE, quercetina.

1 INTRODUCTION

*Psidium guajava* a native tropical American tree belonging to the Myrtaceae family which can grow up to 10m and produces eatable fruits. It is widely known by its diarrhea, dysentery, vomiting and sore throats control effects in popular medicine. It has opposite and petiolar leaves which are rich in flavonoids (KAMATH et al., 2008), a secondary metabolite constituted by a polyphenolic structure which are produced due its functions as signaling molecules, allopathic and antimicrobial defensive compounds as well as to provide protection against UV radiation (PANCHE; DIWAN; CHANDRA, 2016).

Quercetin is a pharmacological important flavonoid found in *P. guajava* leaves owing to its anticancer, antimicrobial, cardioprotective, anti-inflammatory, hepatoprotective, antioxidant and neuroprotective affects, among others. It has very effective anti-inflammatory function once its molecules can regulate reactive oxygen species (ROS) and reactive nitrogen species (RNS), reducing the endoplasmic reticulum stress, a factor that contributes to the inflammation and is associated to Alzheimer Diseases (AD). Moreover, it also modulates micro RNA expression, which regulates inflammation, proliferation, apoptosis, and neurodegeneration. Furthermore, quercetin increases heme-oxygenase (HO-1) expression via mitogen-activated protein kinase (MAPKs) (Benameur; Soleti & Porro, 2021) an important factor that induces antioxidant enzymes such as glutathione S-transferase pi (GSTpi) and NAD(P)H dehydrogenase [quinone] 1 (NQO1) (GBOTOSHO; KAPETANAKI; KATO, 2021).

Quercetin molecules are amphiphilic and present affinity to ethyl acetate, a polar molecule that is capable to extract quercetin from *Psidium guajava* leaves. A widely defunded method of extraction is maceration followed by organic solvents partition which contributes to dissolve and diffuse the dissolved solute. The maceration technique consists in the mechanical size reduction of the material and its insertion in an adequate solvent. The organic partition technique is chosen based in the molecule properties, which must be compatible with the solvent (VISHT; CHATURVEDI, 2012).
Thus, considering the common growth of *Psidium guajava* trees in Brazil and all the benefits of its leaves, this work aimed to analyze the flavonoids and quercetin content in the ethyl acetate fraction to future medicine applications.

2 MATERIALS AND METHODS

2.1 COLLECTION AND DRYING OF *Psidium guajava* LEAVES

The leaves of *Psidium guajava* were collected in the District of São Luiz do Oeste in Toledo - Paraná - Brazil (24°45'07.7"S 53°34'04.8"W). The plant material was dried in a forced air circulation drying oven, with a controlled temperature of 40 °C, for 12 hours, with periodic revolving. After drying, the leaves were manually reduced to smaller parts.

2.2 PREPARATION OF SAMPLES

The crushed leaves (61.39 g) were submitted to cold extraction with ethanol (1L) by maceration. The solvent was removed in a rotary evaporator with bath temperature between 35-40 °C in order to obtain the crude extract, which was resuspended with 200mL of H$_2$O:EtOH (1:1) and fractionated with 3x of 150mL of each solvent: hexane, dichloromethane and ethyl acetate. The solvents were rotaevaporated leading to the formation of the fractions: hexanic, dichloromethane, ethyl acetate and hydroethanolic.

2.3 QUALITATIVE TEST FOR QUERCETIN IDENTIFICATION

The fractions were analyzed by SILICA GEL CCD 60 F254. To that end, the chromatographic tank was previously saturated with the mobile phase steam, with the aid of filter paper. In order to verify the presence of quercetin in the ethyl acetate fraction, a AcOEt:MeOH 30% mobile phase was used and the CCD was analyzed in a UV light chamber in 365 nm, and also revealed with sulfuric anisaldehyde followed by heating at 100°C to identify the presence of quercetin in the fraction.

2.4 DETERMINATION OF TOTAL FLAVONOIDS

In order to quantify the total flavonoid content in the AcOEt fraction obtained from the leaves of *P. guajava*, a spectrophotometer (Biospectro SP-22) was used at wavelength 415 nm with AlCl$_3$ reagent. The standard curve was obtained from different concentrations of the quercetin standard (4 to 15 μg/mL) prepared in a MeOH/HA 5% solution. The analyzed samples reacted for 30 min with the solution of AlCl$_3$ 2% for
formation of the yellow-colored compound, following the same procedure of the standard curve. The concentration of total flavonoids was estimated using the line equation obtained by linear regression.

2.5 DETERMINATION OF QUERCETIN

For the identification of quercetin present in the AcOEt fraction previously analyzed by spectrophotometry, HPLC with UV-VIS detector (SHIMADZU) equipped with automatic injector and LC solution data reading software was used. Elution was isocratic, using acetonitrile 35% (A) and H$_2$O milli Q acidified with 1% acetic acid 65% (B), flow rate of 0.3 mL/min in Kromasil 100-5-C18, 5 μm, 4.6 x 150 mm column, kept in an oven with temperature at 40 °C. Samples of quercetin standard were injected at concentrations of 0.4 to 25μg/mL diluted in H$_2$O milli Q 30% and MeOH 70%, filtered in 0.45 μm micro pores and UV-VIS detection at wavelength of 256 nm. The linear equation was obtained by linear regression and used to quantify quercetin in the AcOEt fraction. Samples of the diluted extract, in H$_2$O milli Q 30% and MeOH 70%, were injected (20 μL) under the same conditions as the standard and identified the analyte comparing it with the retention time of the standard. The analysis of the detection limit (DL) and quantification limit (QL) was performed by the signal-to-noise method, as described by Ribani et al. (2003).

3 RESULTS AND DISCUSSIONS

CCD analysis revealed the presence of flavonoid quercetin in the AcoEt fraction through the retention factor (Rf) and fluorescence in ultraviolet light (365 nm). In reference to the literature (Sánchez & Calle, 2015), flavonoids tend to generate a luminous spectrum of color when placed in the presence of a UV light source, revealing, with greater content the colors purple, brown and yellow, predominant colors in plants rich in flavonoids. Regarding the analysis of CCD revealed with anisaldehyde-sulfuric followed by heating at 100 °C, a yellow color was observed, indicating the presence of flavonoids (Franco & Silva, 2019), reaffirming the test performed by UV light, proving the presence of quercetin and flavonoids in the fraction used by comparing the colors in the samples. After the qualitative analysis by CCD of the presence of quercetin, quantitative analyses of the samples were performed by spectrophotometry.
From the construction of the standard curve, the equation \( y = 0.1546 + 0.0503 \) and correlation coefficient \( (R) = 0.996 \) were obtained, meeting the parameter recommended by ANVISA \((R>0.990)\) (referenciar ANVISA, XXX). Thus, the equation of the line can be used to determine the flavonoid content, obtaining 46.2 mg/g of total flavonoids expressed in quercetin in the ethyl acetate fraction. Seo et al, (2014) presented similar results with both ethanol extraction and aqueous extraction, obtaining 50.0 mg/g of total flavonoids, while Wang et. al (2007) obtained 56.0 mg/g in the 95% ethanol extract. On the other hand, a significantly lower value was reported by Batubara, Suparto and Wulandar (2017) which performed exhaustive extraction with AcOEt followed by acid hydrolysis (11.5 mg/g) and Ademiluyi et, al (2015) by maceration with methanol and HCl for 24h (0.4 mg/g). Therefore, it is perceived that the concentration of total flavonoids expressed in quercetin by colorimetric analysis of the AcOEt fraction exceeds the values already reported and is equated with the values of non-fractionated ethanolic samples. Thus, it is evaluated that the content obtained depends on the extraction method. After quantification of the total phenolics analysis, it was performed a high efficiency liquid chromatography (HPLC) analysis to verify the quercetin content in the AcOEt fraction.

The limit of detection (LOD) consists of the minimum concentration of the analyte that can be detected in the sample and the limit of quantification (LOQ) is the smallest amount of analyte present in the sample that can be measured with precision and accuracy (ANVISA, 2017). In the method used, the LOD obtained was 0.0125 mg/L and LOQ 0.05 mg/L, Chen, Zhang & Jiannon (2000) expresses the LOD of quercetin as \( 0.225 \times 10^{-6} \) mol/L (approximately 0.06 mg/L) a similar value to that obtained in LOQ determination.

On the other hand, Carreri et al, (2003) found the values of 0.12mg/L and 0.35 mg/L as LOD and LOQ, respectively, and Stefova; Kulevanova & Stafilov (2001) expresses the values of LOD= 4 mg/L and LOQ= 13 mg/L. Thus, the greater sensitivity of the method used in the present study is observed when compared to those present in the literature, which means that it is able to detect a small difference in analyte concentration with great variation in signal value (AMARANTE et., al 2001).

The standard quercetin curve in HPLC with UV-VIS detector presented equation \( y=197027x - 2553.5 \) and correlation coefficient \( (R) = 1 \). This parameter indicates the lowest dispersion of the points and lower uncertainty of the estimated regression coefficients, and it can be considered as evidence of optimal data adjustment for presenting a value greater than 0.999 (RIBANI et. al., 2004).
From the peak area and the straight line equation, 816 μg of quercetin per gram of the ethyl acetate fraction were determined, which corresponds to 1.8% of the total flavonoids previously observed by the spectrophotometric method. Similar values were found by Batubara (2017) by sonication extraction (2.2%), while Rattanachaikunsopon & Parichat (2010) reports lower values (179.3 μg/g) of quercetin in methanol extract from dried leaves of *Psidium guajava*. Such disparities in the results found are due, in part, to factors such as seasonality, circadian rhythm, stage of development, temperature, water availability, ultraviolet radiation, available nutrients, air pollution, altitude and mechanical stimuli or attack of pathogens that influence the expression of secondary metabolites of the plants (GOBBO-NETO & LOPES, 2007). In addition, the different extraction methods and the different solvents used also influence the obtaining of metabolites.

4 CONCLUSIONS

The study of the aerial parts of *P. guajava* allowed the identification of total flavonoids in the ethyl acetate fraction by CCD and quantified (46.2 mg g⁻¹) by colorimetric reaction in spectrophotometer. Also, it was verified that the quercetin content (816 μg g⁻¹) in this fraction corresponds to about 2% of the total flavonoids. Due to the action on the central nervous system of quercetin, future studies contemplating the isolation of this substance in guava will contribute to the application of this plant species in pharmaceutical formulations, since this species is of wide occurrence throughout the national territory.
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