CELLULAR AND MOLECULAR BIOLOGY

Development of an analytical method for determination of polyphenols and total tannins from leaves of *Syzygium cumini* L. Skeels

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Abstract: *Syzygium cumini* L. Skeels belongs to Myrtaceae family. This species has been recognized by its antidiabetic, anti-inflammatory, and antimicrobial activities. Despite ever-increasing scientific interest for this species there is no pharmacopeia method for characterization and standardization of *S. cumini* yet. So, toward this aim, the objective of this work was to develop an efficient analytical methodology able to determine polyphenols and tannins content from leaves hydroethanolic extract of *S. cumini* using Folin-Ciocalteu method by ultraviolet absorption spectrophotometry (UV-Vis). The analytical methodology was developed for the first time in the literature for leaves of this specie shown to be fast and low-cost with results expressed through tannic acid equivalent (TAE). Moreover, the methodology presented selectivity with maximum absorption at 706 nm wavelength, linearity with $R^2 > 0.99$; limit of detection 0.275 µg TAE mL^{-1} and 0.102 µg TAE mL^{-1}; limit of quantification 1.046 µg TAE mL^{-1} and 0.912 µg TAE mL^{-1} for total polyphenols and total tannins, respectively. Furthermore, the methodology was accurate with recover value greater than 98%, as well as exact, reproductive, and robust with coefficient of variation values less than 15% for both compounds. All the results are found within the fixed limits according to RDC 166/2017.

Key words: Polyphenols, *Syzygium cumini,* Tannins, UV-Vis.

INTRODUCTION

The antimicrobial resistance (AMR) serious economic and epidemiological implications are alarming the scientific community, turning their attention to new antimicrobial agents. For this reason, plant-derived antimicrobial agents used as primary therapeutic agents or adjuvants are viewed as one of the emerging solutions for AMR, due to their promising multi-target effects and natural origin (Park et al. 2006).

Syzygium cumini (L.) Skeels has as synonym *Syzygium jambolanum* (Lam.) DC. is commonly known as black olive or jambolan (Ayyanar & Subash-Babu 2012). This species has been known by its antidiabetic, anti-inflammatory and antimicrobial activities (Ayyanar & Subash-Babu 2012, Srivastava & Chandra 2013, Mundy et al. 2016, Chhikara et al. 2018). Its leaves have gallic acid, kaempferol, myricetin, ellagic acid, chlorogenic acid, quercetin, rutin, anthocyanins and tannins (Mahmoud et al. 2001).

In Brazil, *S. cumini* is included in the National List of Medicinal Plants of Interest to the Unified Health System (RENISUS) (Marmitt et al. 2018). In this list is including 71 vegetable species that have the potential to generate herbal medicines. Despite the potential of *S. cumini,* there is no pharmacopeia method for characterization and standardization of the species yet.

The standardizing of the extract, in other words, adjust of a defined content of one or more component responsible for the therapeutic activity, is indispensable to ensure quality, efficacy, and safety of the extract (Luo et al. 2016, Jagetia 2017, Huynh-Ba & Sassi 2018, Simmler et al. 2018). Extracts, and natural compound combinations have been shown to be more effective against bacteria and fungi than isolated constituents (Carmona & Pereira 2013, Mundy et al. 2016). Considering this fact, the combined quantification of more the one compound from an extract could be very advise for enhance efficiency and security of herbal medicines (Amorim et al. 2008, Dominguez-Rodriguez et al. 2017).

Tannins and flavonoids are phenolic compounds that can be determined by the Folin-Ciocalteu (FC) method. Many quantitative data have been reported based on the FC method, once it is a convenient and simple analytical technique with good reproducibility (Sánchez-Rangel et al. 2013). Accordingly, the total phenolic contents determination based on the FC method has been used extensively to characterize wines and spirits, fruit juices, plant tissues, sorghum grain and other similar products (Ramirez-Sanchez et al. 2010, Sánchez-Rangel et al. 2013, Chen et al. 2015, Baldissera et al. 2016).

Spectrophotometry (UV-Vis) is mostly used in the quality control by the pharmaceutical industry because is an analytical tool highly sensitive. The analytical methodologies using by this technique can provide fast and reliable results (Alves et al. 2010, Marques et al. 2014, Figueirêdo et al. 2015). Moreover, spectrophotometric methods for the determination of tannin and flavonoid levels in plant extracts are very popular for their speed and practicality, making them useful for various studies and techniques (Haddouchi et al. 2014, Ricci et al. 2017).

Toward this aim, this paper brings for the first time in the literature the development of a fast and efficient analytical method able to determine polyphenols and total tannins content from leaves hydroethanolic extract of *S. cumini* using FC method by ultraviolet absorption spectrophotometry (UV-Vis).

MATERIALS AND METHODS

Chemical products, glasses, and solvents

All the used solvents were of analytical grade: Phenol reagent (Folin-Ciocalteu ELL®), Sodium Carbonate (Na_2CO_3 P.A. Dinâmica®), Casein (P.A. Dinâmica®). Quartz Cuvette was used. As a standard for polyphenols, ultrapure tannic acid (Vetec®), purity ≥ 94% was utilized.

Equipment

Analytical balance BIOPRECISA® (type FA2104N), water bath with agitation Novatecnica® (type NT277), greenhouse with circulating air ETHIK TECHNOLOGY® (type 420-6TD), cutting mill SOLAB® (type SL-31), reactor by microwave CEM® (type Discover System) spectrophotometer SHIMADZU® (type UV/Vis-mini-1240) and spectrophotometer MICRONAL® (type AJX-1900), were used.

Plant material

Flowering branches of a single *S. cumini* were collected in the municipal district of Santo Agostinho's Cable (8º29'86.07"S and 35º06'45.29"W). The samples were identified by botany Rita de Cássia Pereira, of the Agronomic Institute of Pernambuco, under the number 90672.

Experimental procedures

Sample preparation

The samples of leaves were treated with alcohol to 70% (v/v). After, the sample was conducted to the greenhouse of air circulating for 40 h at 40 °C. Next, the dry leaves were pulverized by cutting mill with 20 mesh sieve (0.84 mm), obtaining a powdered dry plant material.

The hydroethanolic extract (HE-Sc) was prepared by microwave assisted extraction using a reactor according follow parameters: 200 W of power, 2 minutes of extraction time, and 30 °C of maximum temperature. Ethanol: water (1:1, v/v) was used as solvent. The ratio of 1 g powdered plant material to 20 mL of solvent, was used. After the end of the extraction, the liquid extracts were filtered in cotton.

Development of the analytical method for simultaneous quantification of Polyphenols and total tannins

The method was based on Amorim et al. (2008), which carried out the determination of total tannins and total polyphenols by applying the Folin-Ciocalteu reagent (FCR), calibration curve with increasing concentrations of tannic acid and reading at 760 nm. The calibration curve was prepared through a curve-test with 8 concentrations: 0.5; 2.0; 4.0; 6.0; 8.0; 10.0; 12.0 and 14.0 µg.mL^{-1} of tannic acid (TA). In the curve-test was added 1 mL of Na_2CO_3 solution (7.5%, p/v) and 700 µL FCR (10%, v/v). For evaluating the amount necessary to obtain a linear reading for polyphenols by spectrophotometer was used 500, 600, and 700 µL of FCR (10%, v/v).

After that, the aliquot of the extract (HE-Sc) for reading in the spectrophotometer was adjusted to 0.03 mL to obtaining a wavelength absorbance between 500 to 550 nm for total polyphenols. The residual polyphenol was originated by a complex of 6 mL of HE-Sc dissolved in 12 mL of distilled water and 1 g of casein.

To determine the ideal amount of casein, the total tannins levels were quantified using the following casein mass: 100; 250; 500; 750; and 1000 mg, maintaining the above parameters. The ideal time of complexation was defined by determination of total tannins levels obtained during 3 h, 1:30 h, and 30 min of time complexation. Results were expressed as follows: milligram tannic acid equivalent (TAE) per gram of dry extract (mg TAE g^{-1} ext).

The choice of the casein mass and time of complexation with the total tannins was defined by the response in the assay that presented a lower concentration of reagent and time, without resulting in a statistically significant difference between the analyzes.

All the tests were made in triplicate and the scanning was realized using 760 nm of wavelength by spectrophotometer.

Figures of merits evaluation

The specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness parameters were evaluated according to RDC 166/2017 (Anvisa 2017). These parameters were appraised the purpose of guarantee the credibility of the results displayed by analytical method. Results were expressed as microgram tannic acid equivalent (TAE) per milliliter of extract (µg TAE mL^{-1}). All the tests were realized in triplicate and the reliability of the parameters was checked by the coefficient of variation (CV%), not admitting values superior to 15% as determined in the Herbal Medicines legislation (Anvisa 2014). Furthermore, the results were processed statistically by variance analysis (ANOVA) *One-Way* or *Two-Way*, considering a significance level of 5%.

Preparation of extract stock solution

The extract stock solution (S-HE-Sc) was prepared using 10 mL of HE-Sc diluted to 100 mL with distilled water in volumetric flasks.

Specificity

The specificity of the method was established by means of the overlap of the sample spectrum in the 500 to 900 nm of range wavelength: standard solution (6 µg TA mL^{-1}) with 700 µL FCR, stock solution (0.06 mL of S-HE-Sc in 10 mL distilled water) with 700 µL FCR, and standard solution (6 µg TAE mL^{-1} in distilled water) without FC.

Linearity, limit of detection, and limit of quantification

Analysis of three authentic curves, each one with seven concentration levels of tannic acid (3 to 9 µg TA mL^{-1}) was assessed to prove the linearity of total polyphenols quantification method.

Analysis of three authentic curves, each one with seven concentration levels of tannins standard solution (3.54 to 7.98 µg TAE mL^{-1}) was assessed to prove the linearity of total tannins quantification method. This standard solution was developed by a stock solution of known tannins concentration (5.1 µg TAE mL^{-1}).

The curves were built using the rate values of the absorbance in function of the concentration of polyphenols and total tannins. The results were processed statistically by linear least-squares regression, in order to define the determination coefficient (R^2), embracing $R^2 >$ 0.98 as least value according to RDC 166/2017, also the angular coefficient significance was evaluated (Anvisa 2017).

The LOD and LOQ were estimated according to the equations: LOD = SD$_a$ x 3/IC e LOQ = SD$_a$ x 10/IC, in which SD$_a$ is the standard deviation of the intercept with the axis Y that were obtained by three linearity curves and IC is the angular coefficients rate (slope of the line) of their respective curves (Anvisa 2017).

Robustness

The assay to determine the robustness was performed by the variation of the brand of the spectrophotometer (Shimadzu® and Micronal®) and of the solution concentration of Na$_2$CO$_3$ 7.5%, 10%, and 15% (v/v) [27].

Precision

The precision was evaluated by repeatability and intermediate precision (Anvisa 2017). To calculate the repeatability, in one single day by a single analyst determined the concentration of six samples at 100% (6.0 µg TAE mL^{-1} for total polyphenols and 5.1 µg TAE mL^{-1} for total tannins) obtained from the six distinct extractive solutions. To intermediate the precision, two analysts in separate days assessed the concentration of the three samples at 100% obtained from the three distinct extractive solutions.

Accuracy

The accuracy was determined by recovery trials, where 1 µg mL^{-1} of tannic acid was added to samples containing the following concentrations: 4.8; 6.0; and 7.2 µg TAE mL^{-1} of total polyphenols and 4.2; 5.1; and 6 µg TAE mL^{-1} of total tannins. These concentrates are equivalent to 80, 100, and 120%, respectively. The recovery measures were expressed in percentages and calculated by the ratio of the mean concentrations determined experimentally (real value) and corresponding theoretical concentrations (Anvisa 2017).

RESULTS AND DISCUSSION

Development of the calibration curve

The FCR contains molybdenum VI, so phenolic compounds are determined based on the reduction of Mo^{6+} to Mo^{5+}, which is blue and can be measured optically at 730 nm. The FC method may be useful in characterizing and standardizing botanical samples. Likewise, FC method also may be used to quantify total tannins specifically providing that the sample be purified (Amorim et al. 2008).

Above all, the assays using protein precipitation are the method most advisable to determine tannins, once by definition are substance that has the property of associating and combining with proteins (Monteiro et al. 2005). Recognizing this, total tannins level corresponds to the difference between the value of total polyphenols and residuals polyphenols. This way, residuals polyphenols value corresponds to supernatant from reaction of total tannins complexation with a protein, as described by British-Pharmacopeia (2007).

Phenolic compound is defined as substance that has in its structure aromatic ring which one or more hydroxyl substitutes including its functional groups. Phenolic acid, cumarins, flavonoids, and tannins are classified as polyphenols. Polyphenols are secondary metabolites in plants and to date more than 8000 polyphenols have been identified (Khan et al. 2019). Flavonoids and tannins are the most highlighted for having wide range of therapeutic indications, such as anti-inflammatory, anticarcinogenic, cicatrizing, antifungal, antibacterial and antioxidant actions (Zhang et al. 2019). Moreover, tannins have been recognized by its potent antimicrobial activity (Baldissera et al. 2016, Zhan et al. 2017).

In view that *S. cumini* has tannins as majority phenolic components in its leaves (Ayyanar & Subash-Babu 2012). Herein the

standard calibration curve was development within of range concentration able to quantify total polyphenols (TP) and total tannins (TT). A standard calibration curve with larger and growing distribution of concentrations was necessary, once the value of total tannins level is obtained by difference of value of TP contents and residual polyphenols (RP) contents.

Standard curves built with 500 and 600 µL of FCR had not differences statistically significant among their absorbance value. However, their R^2 values were less than 0.99 not showing linearity within range chosen. The standard curve with R^2 satisfactory was it built with 700 µL of FCR, presenting $R^2 = 0.9924$ and $y = 0.0481x + 0.0445$ as straight line equation (Figure 1).

This result is associated with saturation of FCR by TP in the curves built with 500 and 600 µL of FCR, once FCR method depends on the selective oxidation of compounds to express the TP content. Thus, 700 µL of FCR was the concentration minimum necessary to provide linearity for standard curve.

Definition of Casein Mass and Complexation Time

The results of the TT content increased with increasing casein mass (Table I) showing the mass of casein 1000 mg as the ideal concentration. This fact indicates the saturation of casein by total tannins, once TT = TP - RP. Therefore, it is expected due to the presence of high concentration of tannins in the leaf extract of *S. cumini* (Verza et al. 2007, Baldissera et al. 2016). In addition, this result corroborates current data suggesting that microwave assisted extraction (MAE) has excellent performance in tannin extraction (Hoyos-Martínez et al. 2019).

Complexation time during 30 min, 1:30 h, and 3 h presented 2.972 mg TAE g^{-1} ext ± 0.009, 3.043 mg TAE g^{-1} ext ± 0.009, and 2.989 mg TAE g^{-1} ext ± 0.012 of TT levels, respectively. Nonetheless, there was
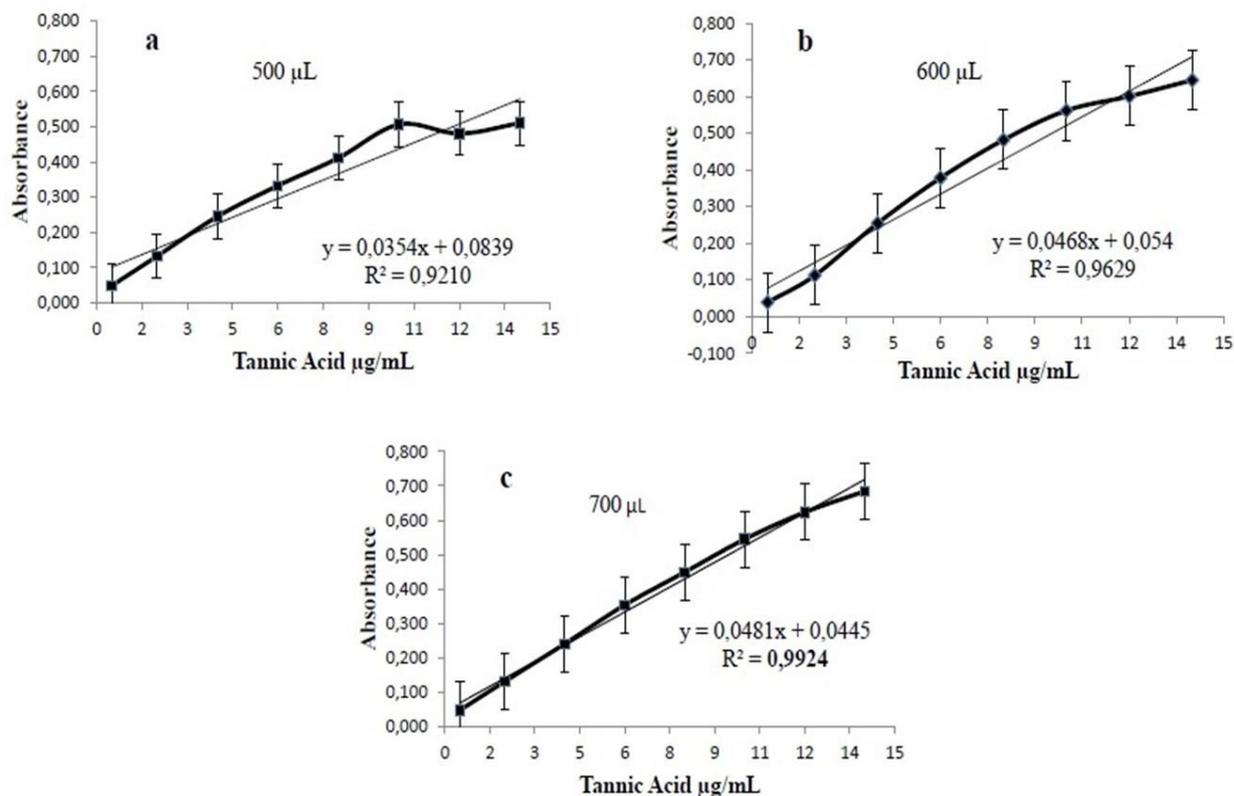
Figure 1. Standard curves built with 500, 600 and 700 µL of Folin-Ciocalteu reagent / Work data; a = Standard curve built with 500 µL of Folin-Ciocalteu reagent; b = Standard curve built with 600 µL of Folin-Ciocalteu reagent; c = Standard curve built with 700 µL of Folin-Ciocalteu reagent.

no difference significant statistical between the results, hence the complexation time of 30 min was selected to lead the experiments promoting a faster analytical answer.

Validation of the analytical method

Specificity

Specificity is the ability of measuring, exactly and specifically a substance of interest in a specific range of wavelength, even when other components are presents in the same sample.

According highlight in the Figure 2, the samples without FCR there were no absorbance in the wavelength range. On the other hand, in the presence of FCR one can see absorbance

in the wavelength range shown the maximum absorbance at 706 nm. Furthermore, there was overlap of the spectrum of the standard solution and S-HE-Sc when was added FCR in the respectively solutions. As expected, FCR method proved specificity in the total polyphenols determination.

The results of quantification of TP and TT showed tannins are the component majority representing about 85% of TP from hydroethanolic leaves extract of *S. cumini* by MAE. So, corroborating with current data whereas MAE is an excellent and may be specific method to extract tannins (Hoyos-Martínez et al. 2019). Furthermore, it is known that leaves of *S. cumini* have high concentrations of tannins, which has been associated a wide spectrum of action

Table I. TT quantification according to increase mass of casein.

Parameters	TT (mg TAE g^{-1} ext) ± SD	CV (%)
Casein 100 mg	2.378 ± 0.013	0.55
Casein 250 mg	2.403 ± 0.009	0.37
Casein 500 mg	2.622 ± 0.063	2.29
Casein 750 mg	2.701 ± 0.008	0.31
Casein 1000 mg	2.912 ± 0.006	0.19

Work data; CV = Coefficient of Variation; SD = Standard Deviation; TAE = Tannic Acid; Equivalent; TT = Total Tannins.

against fungi and bacteria. Once tannins have ability to complex with metal ions, and other molecules mainly proteins and polysaccharides, as well as it has antioxidant activity and free radical scavenger. (Chandrasekaran & Venkatesalu 2004, Bitencourt et al. 2017, Yadav et al. 2017).

Linearity, limit of detection, and limit of quantification

The linearity of a method proves that the absorbance value obtained by spectrophotometer has dependent correlation with concentration of sample.

The method showed linearity, homoscedastic and with independent residues values within the range chosen for TP, presenting R^2 of 0.996 (y = 0.0729x − 0.0396) and 0.998 (y = 0.0642x + 0.0172) to standard solution and extractive solution, respectively. The LOD and LOQ results highlight in the Table II, showed reliability and sensibility to detection and quantification of TP from leaves extracts of *S. cumini*.

The linearity for TT was verified from the extractive solution (S-HE-Sc) and standard

solution by analyzing three authentic curves. The method showed linearity, homoscedastic and with independent residues values within the range chosen for TT, presenting R^2 of 0.998 (y = 0.0716x − 0.0134) and 1 (Y=0.0729x − 0.0396) to standard solution and extractive solution, respectively. The LOD and LOQ results highlight in the Table II, showed reliability and sensibility to detection and quantification of TT from leaves extract of *S. cumini*.

According the results was possible observe the methodology using FCR by UV-Vis supplies reliable results with a high sensibility to detect and quantify TP and TT present in leaves extract of *S. cumini*.

Robustness

Robustness is defined as the capacity of the method to be unaffected by a small and deliberate modification of its parameters. For this reason, alkaline solution at pH ~ 10 is a critical step for method due to dissociation of a phenolic proton which leads to the formation of a phenolate ion reducing the FCR (Sánchez-Rangel et al. 2013). So, this pH is reached by adding sodium carbonate. Moreover, changes in pH may affect both the grade of ionization of the molecules and the solubility properties in the sample (Amorim et al. 2008). However, both the increase in sodium carbonate and the change of the equipment brand did not caused variation in the quantification results (Table III). So, the methodology developed provided robust quantification results of TP and TT.

Precision

The methodology presented repeatability shown 6.642 μg TAE mL^{-1} ± 0.858 (CV=12.91%) and 5.621 μg TAE mL^{-1} ± 0.829 (CV=13.24 %) for TP and TT, respectively. The rehearsal of intermediate precision demonstrated satisfactory results
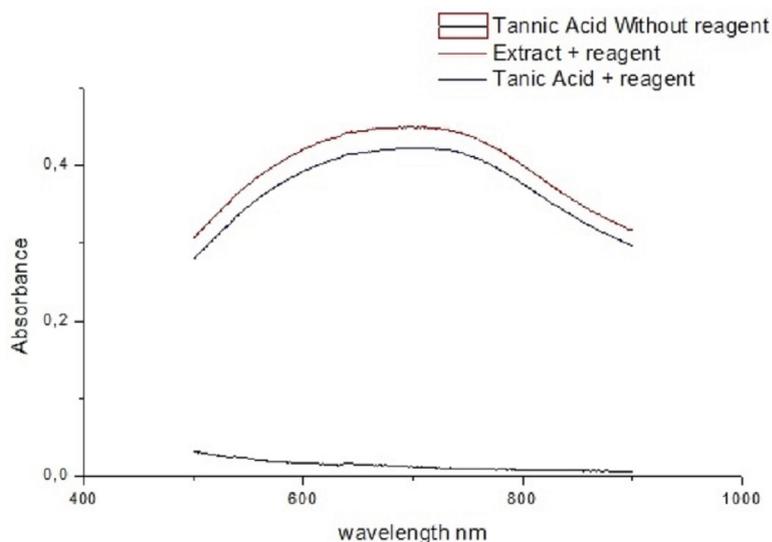
Figure 2. Scanning spectra used in the specificity analysis with and without the addition of Folin-Ciocalteu to determine polyphenols for *S. cumini*.

Table II. Linearity results.

	Merits evaluation	Standard Solution	Extractive Solution
TP	LOD	0.083 µg mL^{-1}	0.275 µg TAE mL^{-1}
	LOQ	0.314 µg mL^{-1}	1.046 µg TAE mL^{-1}
	Cochran`s Test	0.674 (p-value)	1 (p-value)
	Durbin-Watson Test	0.298 (p-value)	0.963 (p-value)
TT	LOD	0.100 µg mL^{-1}	0.102 µg TAE mL^{-1}
	LOQ	0.894 µg mL^{-1}	0.912 µg TAE mL^{-1}
	Cochran`s Test	1 (p-value)	0.709 (p-value)
	Durbin-Watson Test	0.963 (p-value)	0.961 (p-value)

Work data; LOD – Limit Of Detection; LOQ – Limit Of Quantification; TAE = Tannic Acid Equivalent; TP = Total Polyphenols; TT = Total Tannins.

Table III. Robustness results.

Parameters	TP level µg TAE mL^{-1} ± SD	TT level µg TAE mL^{-1} ± SD	F. calculated TP/TT	F. tabulated TP/TT
Sodium Carbonate 10%	6.194 ± 0.009	5.777 ± 0.118	1.189/ 1.997	5.318/ 5.591
Sodium Carbonate 15%	6.266 ± 0.146	5.652 ± 0.140		
Shimadzu® Equipment	6.365 ± 0.472	6.334 ± 0.212	3.572/1.847	7.709/ 7.709
Micronal® Equipment	6.732 ± 0.162	6.615 ± 0.289		

Work data; SD = Standard Deviation; TP = Total Polyphenol; TT = Total Tannins; TAE = Tannic Acid Equivalent; F. calculated = work data results; F. tabulated = expected results by ANOVA.

(Anvisa 2014) for TP and TT quantification highlight in the Table IV.

Accuracy

In the accuracy assay was obtained a rate percentage of recovery of 99.07% and 100% for polyphenols and total tannins, respectively (Table V). These data are within the range required by current legislation (80 to 120%) (Anvisa 2017).

In resume, this work presented an analytical methodology by UV-Vis advisable to determine TP and TT from hydroethanolic extract of *S. cumini*. Furthermore, the results confirming the presence of polyphenols in the leaves of *S. cumini*, among them, tannins showed to be as majority compound presenting about 85%

of TP. Moreover, the data suggest the MAE is an excellent technique to extract tannins.

The presented protocol showed to be fast, specific with peak maximum for polyphenols at 706 nm wavelength, linear ($R^2 > 0.99$), precise presenting 6 µg TAE mL^{-1} and µg TAE mL^{-1} as 100% of concentration for TP and TT, respectively. Furthermore, the methodology was exact with rate percentile of recovery of 99.07% and 100% for polyphenols and total tannins, respectively, as well as robust, cheap, and with high sensibility of detection (TP = 0.275 µg TAE mL^{-1}; TT = 0.102 µg TAE mL^{-1}) and quantification (TP = 1.046 µg TAE mL^{-1}; TT = 0.912 µg TAE mL^{-1}). The methodology could be recommended to determine TP and TT levels present in vegetable species, as well as standardization and characterizing vegetable extracts.

Table IV. Intermediate Precision.

Compund	Parameters	Level µg TAE mL^{-1} ± SD (CV%) (1)	Level µg TAE mL^{-1} ± SD (CV%) (2)	F$_{tabulated}$	F$_{calculated}$
TP	Days 1 e 2	7.169 ± 0.708 (9.87)	7.211 ± 0.119 (1.65)	5.317	1.222
	Analysts 1 e 2	6.233 ± 0.072 (1.16)	6.151 ± 0.103 (1.68)	3.466	0.020
TT	Days 1 e 2	6.280 ± 0.113 (1.80)	6.261 ± 0.829 (13.24)	5.317	0.001
	Analysts 1 e 2	5.695 ± 0.091 (1.59)	5.858 ± 0.080 (1.37)	7.708	5.467

Work data; SD = Standard Deviation; CV = Coefficient of variation; F. calculated = work data results; F. tabulated = expectated results by ANOVA; TAE = Tannic Acid Equivalent; TP = Total Polyphenols; TT = Total Tannins.

Table V. Recovery results.

Compound	Extractive Solution + TA	Theoretical Value	Real Value ± SD	Recovery
TP	4.80 µg TAE mL^{-1} + 1 µg TA mL^{-1}	5.80 µg TAE mL^{-1}	5.77 µg TAE mL^{-1} ±0.002	99.48%
	6.00 µg TAE mL^{-1} + 1 µg TA mL^{-1}	7.00 µg TAE mL^{-1}	6.97 µg TAE mL^{-1} ±0.005	99.57%
	7.20 µg TAE mL^{-1} + 1 µg TA mL^{-1}	8.20 µg TAE mL^{-1}	8.05 µg TAE mL^{-1} ±0.002	98.17%
TT	4.20 µg TAE mL^{-1} + 1 µg TA mL^{-1}	5.20 µg TAE mL^{-1}	5.20 µg TAE mL^{-1} ±0.017	100,00%
	5.14 µg TAE mL^{-1} + 1 µg TA mL^{-1}	6.14 µg TAE mL^{-1}	6.15 µg TAE mL^{-1} ±0.019	100.16%
	6.05 µg TAE mL^{-1} + 1 µg TA mL^{-1}	7.05 µg TAE mL^{-1}	7.05 µg TAE mL^{-1} ±0.019	100.00%

Work data; SD = Standard Deviation; TAE = Tannic Acid Equivalent; TA = Tannic Acid; TP = Total Polyphenols; TT = Total Tannins.

Acknowledgments

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