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OBTAINING EXTRACTS FROM LEAVES OF *MORUS NIGRA* (L.) WITH THE HIGHEST CONCENTRATION OF TOTAL POLYPHENOLS AND TOTAL FLAVONOIDS

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ABSTRACT

Currently, the extract from *Morus nigra* L. has been used to treatment the symptoms of menopause. The aim of this study was to obtain an extract from the leaves of *Morus nigra* L. with the highest concentration of total polyphenols and total flavonoids. The morphoanatomical analysis, moisture and particle size were performed to characterise the plant raw material for the extractive solutions, obtained by turboextraction using a hydroalcoholic solution; the parameters recorded were the dry residue, density, determination of total polyphenols and total flavonoids. Data found in the literature resembled morphoanatomical characterisation performed in this work. The moisture was within the acceptable limit. In relation to particle size, the average diameter was 0.420 mm. The dry residue ranged from 0.12 to 3.67 g/100 g and the density of 0.8098 to 0.9180 g/mL. Experimental values of the content of polyphenols totals varied from 7.92 to 201.68 mg of quercetin/100 g of extract. While for the content of total flavonoids, the variation was 34.88 to 80.41 mg of quercetin/100 g of extract. The parameters to obtain the best yield in total of polyphenols and total flavonoids were extract 60°GL, 20% of drugs, and an extraction time of 20 minutes.

Keywords: *Morus* spp. Polyphenols. Flavonoids. Blackmulberry.

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INTRODUCTION

Since ancient times, man has sought survival in nature, from water, soil, animals and plants (Junqueira, 2005). The use of plants for therapeutic, healing and prevention of diseases is the oldest form of medical practice (Junior, Pinto and Maciel, 2005). The use of medicinal plants for treating various diseases is deeply rooted in popular culture, widely adopted due to factors such as low cost, easy accessibility, minimal adverse effects, and proven therapeutic efficacy. In recent years, however, there has been a significant increase in interest in phytotherapy among users, researchers, and health services, driven by the demand for more natural and sustainable therapeutic alternatives (Ferreira, 2020).

Currently, the number of people who use this medical practice is increasing, stimulated carefully in an unusual manner by various media. However, many of the herbal medicines used do not have scientific studies proving their efficacy and safety, as well as quality assurance; however, there is no scientific validity, because there has been no research or pharmacological activity in the preclinical setting, and clinical scientific tests have not been clarified (Junior, Pinto and Maciel, 2005).

According to the World Health Organization (WHO), in the early 1990s, 65–80% of the population in developing countries relied on medicinal plants for basic healthcare needs due to limited access to medical services, diagnostic exams, and medications. Currently, most medicinal plants are sold in natural product stores and pharmacies; however, they do not always have quality certification (Junior, Pinto, & Maciel, 2005).

The blackmulberry has been widely cultivated as a food for the silkworm (Padilha *et al.*, 2009; Medina *et al.*, 2009). This plant is found in tropical and subtropical regions in the northern hemisphere. Blackmulberry cultivation in Brazil started with Japanese immigrants in the country (Padilha *et al.*, 2009). According to Hassimoto *et al*. (2007), *Morus nigra* is the most abundant species in Brazil.

Blackmulberry has several activities such as hypoglycaemic (Mahmoud *et al*., 2014), anti-inflammatory (Padilha *et al*., 2010), antioxidant (Memon *et al*., 2010), anti-nociceptive (Padilha *et al*., 2009), hepatoprotective (Mallhi *et al*., 2014) and molluscicidal activity (Hanif & Singh 2012). Its use has been growing due to prescription by gynaecologists to reduce the symptoms of menopause. However, not all of the statements had the desired effect, due to variability between batches and suppliers, a lack of standardisation of active content such as total polyphenols (PFT), and total flavonoids (FT), undermining the effectiveness of the same.

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Furthermore, the morpho-anatomical analysis for quality control of the raw material plant in the pharmaceutical industry, according to Di Stasi (1996), provides subsidies that contribute to the standardisation of inputs, allowing differentiation even among species that are botanically similar.

In addition, one must consider the seasonal variability, and genetic and taxonomic factors that can affect the active content of medicinal plants, so it is necessary for studies to establish quality criteria for plants (Yariwake *et al.*, 2005).

Thus, the objective of this work was to obtain an extract from *Morus nigra* L., in order to obtain higher concentrations of polyphenols and flavonoids through the leaves of *M. nigra* L.

MATERIALS AND METHODS

RAW MATERIAL

Morus nigra L. is a species in the family Moraceae, which is popularly known as blackmulberry, blackmulberry-silkworm, blackmulberry, and blackberry. In Xinjiang, China, this species is known as “Yao-Sang” and, in Brazil as a amora-miúra and amoreira-negra (Oliveira *et al.*, 2013; Pimenta, Zambrano and Venturini Filho, 2013; Volpato *et al.*, 2011; Hu *et al.*, 2011; Vanoni, 2006 ; Franzotti, 2006).

The leaves of *M. nigra* were collected in July 2014 in the mornings, in the garden of medicinal plants Profª Irenice Silva, located at the State University of Maringá.

A voucher specimen was deposited at the Herbarium of the State University of Maringá, (registration number HUEM 21405); the identification was performed by Professor Dr José Eduardo Lahoz da Silva Ribeiro the State University of Londrina (UEL).

MORPHOANATOMICAL ANALYSIS

The anatomical study was performed according to the techniques of Kraus and Ardruin (1997) and Oliveira (1991). The leaves of *M. nigra* were sectioned crosswise, yielding semi-permanent blades with free hand sections, and were stained with 1% Astra blue and 1% safranin. Permanent blades were made with sections obtained using a rotary microtome and stained with 1% toluidine blue. All sections were analysed and photographed in a microscope with a video camera digital microscope Moticam 2300, equipped with the Software MOTIC IMAGE 2.0 Plus; this had a Microscope Olympus CX31 coupled to it, with Achromatic lenses and optics UIS (correction to infinity).

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DETERMINATION OF MOISTURE

Samples of 2.0 g of ground vegetable drug were placed in petri dishes, and submitted to 115°C heating by infrared rays for a period of thirty minutes, until a constant weight was achieved. The infrared balance used was an OHAUS® model MB 200. The result was calculated in relation to 100 g, using the average of three determinations (%, w/w).

PARTICLE SIZE ANALYSIS BY SIEVING

A sample of 100 g of dried and ground plant drug was subjected to forced vibration, passing through pre-weighed sieves with nominal mesh openings corresponding to 1.00, 0.600, 0.350, 0.250 and 0.177 mm, provided with a lid and collector. The process was executed in a vibrating tamisador for 30 minutes. Then the samples were weighed, determining the amount of material retained on each sieve. Values represent the mean of the three determinations (n = 3) and are expressed in percentages (%, w/w). The results were transcribed into Excel® and submitted to analysis (F. Bras. IV, 1998).

OBTAINING THE EXTRACTS

The extracts were prepared from the leaves of blackmulberry, using the turboextraction process, by means of the ultraturrax Marconi model MA102/PLUS. The alcoholic contents were 60°, 77° and 94° Gay-Lussac (GL) with times of 10, 15 and 20 minutes of extraction and drug concentrations 10%, 15% and 20% (w/w), respectively. The extract solutions were filtered under reduced pressure and packaged in amber bottles.

DRY RESIDUE

Exactly 10 mL of heavy extractive solutions in petri plates was heated up. After evaporation of the solvent in a water bath, the plates were subjected to a temperature of 115°C for 30 minutes, until a constant weight was reached, on an infrared balance, OHAUS® model MB. The dry residue value was expressed for 100 g of extractive solution. The analysis of each extract was performed in duplicate, and the average of these values is presented in the results.

DENSITY

The density was determined by the picnometer method.

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OBTAINING ANALYTIC CURVE FOR TOTAL POLYPHENOLS

For the analytic curve, quercetin was employed as a reference chemical at five concentrations. Then, 0.0201 g of quercetin was weighed in a 20 mL volumetric flask, made up to the final volume with methanol, obtaining a stock solution with a concentration of 1 mg/mL. This solution was transferred in aliquots of 250 µL, 190 µL, 150 µL, 100 µL and 80 µL to 25.0 mL to volumetric flasks, to which 10.0 mL of distilled water and 2.0 mL of Folin-Ciocalteau reagent was added, making the volume 25.0 mL with 14.06% sodium carbonate solution. The reading was taken 30 minutes after the addition of carbonate solution against compensation solution (blank). All samples were assayed in triplicate.

DETERMINATION OF TOTAL POLYPHENOLS

The determination of total polyphenols of each sample was obtained according to colorimetric method described by Singleton & Rossi (1965). In a 25 mL volumetric flask, 100 µL of extract, 10.0 mL of water, and 2.0 mL of Folin-Ciocalteau reagent were added, and the volume was completed with 14.06% sodium carbonate solution. Then, 30 minutes after adding the last reagent, the absorbance was measured at 772 nm using the UV-visible double beam spectrophotometer (Shimadzu UV-1650 PC), employing 100 µL of extract, 10.0 mL of water and 14.06% sodium carbonate solution as the compensation solution. For quantification, a standard curve with solution of quercetin was used. All extracts n = 6.

OBTAINING AN ANALYTIC CURVE FOR TOTAL FLAVONOIDS

To obtain the analytical curve, a solution of quercetin with a concentration of 1 mg/mL was prepared in methanol. From this solution, 1.5 mL, 1.25 mL, 1.0 mL, 0.75 mL and 0.5 mL were transferred to 25.0 mL volumetric flasks completing the volume with methanol. An aliquot of 8.0 mL of each solution with 2.0 mL of 2% $AlCl_3$ was transferred to a 25.0 mL balloon, completing the volume with methanol and 5% glacial acetic acid. Thirty minutes after adding the last reagent, the absorbance was measured at 420 nm, employing 8.0 mL of each solution as a compensation solution. The volume was supplemented with methanol and 5% glacial acetic acid solution using the UV-visible double beam spectrophotometer (Shimadzu UV-1650 PC). All samples were assessed in triplicate.

The analytical curves were subjected to statistical analysis with linear regression, using Microsoft Excel 2007. The results were expressed by the coefficient of determination,

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straight skew, with the y-axis intercept. Total flavonoids content was expressed in mg quercetin/equivalent 100 mL of extract.

DETERMINATION OF TOTAL FLAVONOIDS

In 10.0 mL of the crude extract, 20.0 mL of acetone was added, along with 1.0 mL of hexamethylenetetramine to 0.5% (w/w) and 2.0 mL of hydrochloric acid (v/v). Solutions were heated in a water bath, under reflux, at 80°C, for 30 minutes. After cooling at room temperature, the solution was placed in a 50.0 mL volumetric flask, completing the volume with acetone. A 5.0 mL aliquot was passed to a separatory funnel, treated with 20.0 mL of distilled water, extracted with 15.0 mL of ethyl acetate. This operation was repeated for 3 more servings of 10.0 mL of ethyl acetate. The fraction in ethyl acetate has been filtered for a 50.0 mL volumetric flask, using cotton and anhydrous sodium sulphate and completed volume. Then, 10.0 mL of ethyl acetate was placed in a 25.0 mL volumetric flask, with 2.0 mL of a 2% (w/v) methanolic solution of aluminium chloride ($AlCl_3$) and the volume was completed with methanol to a 5% glacial acetic acid solution. Thirty minutes after adding the last reagent, the absorbance was measured at 420 nm using the UV-visible double beam spectrophotometer (Shimadzu UV- 1650 PC); this involved a 10.0 mL clearing solution of the ethyl acetate fraction, which was made up to a final volume with methanol and 5% glacial acetic acid solution. Six readings of each extract were performed. For the quantification of total flavonoids, quercetin was employed as a reference substance. The flavonoids content was calculated by the equation of the line and the graph of the analytical calibration curve.

STATISTICAL ANALYSIS

Data analysis was performed using Excel®. All tests were performed with a confidence level of 95%.

RESULTS AND DISCUSSION

MORPHOANATOMICAL FEATURES

Morus nigra L. is a medium-sized tree, which is about 4m high (Figure 1 - A).

Its leaves are characterised by being flexible, delicate, alternate, simple, petiolate, with dark green colouring on the adaxial face, and lighter green in the abaxial face. Limbo

has serrated margins, showing cordate base and acuminate apex, with reticulated nervation, which was lighter than the limbo (Figure 1 - B and C).

The specimen presented dorsiventral and hypostomatic leaves, in a cross-section, uniseriate epidermis with a thick cuticle, palisade parenchyma consisting of a layer of cells and a spongy two to three layers of cells (Figure 1 - D). The midrib is semi-convex with an open vascular bundle type. Also observed in the parenchyma are idioblasts containing druses (Figure 1 - E).

The epidermis in the adaxial face is composed of large isodiametric cells (Figure 2 - F) with cystoliths (Figure 2 - H and K). In the abaxial surface, the anomocitic stomata type (Figure 2 - G) and subestomatic chambers occur.

At the midrib, there are simple and unicellular non-glandular trichomes (Figure 2 - I) and capitate glandular trichomes with multicellular pedicel, consisting of three cells and with a head formed of four cells (Figure 2 - J).

The main purpose of botanical studies is the identification and authentication of species, to obtain comparative parameters that make it possible to detect the presence of other plant drugs or even dirt and/or other contaminant material during quality control.

Despite the interest and therapeutic use of blackmulberry, there is a shortage of studies with the anatomical description of the species.

Metcalfe and Chalk (1957) indicated the presence of cystoliths in the epidermis of plants in the family Moraceae, especially in *Morus* sp. feature confirmed in the species under study.

Padilha *et al*. (2010) reported on *Morus nigra* L. palisade parenchyma, which presents the same amount of cells as the spongy form. In the present study, the palisade parenchyma presents a single layer of cells while the 2 spongy forms presents the 3 layers of cells. The same authors highlight the presence of non-glandular trichomes, but make no mention of glandular trichomes, that were found in this study.

Sonibare *et al*. (2006) and Biasiolo *et al*. (2004) confirmed the presence of non-glandular trichomes. Thus, some elements can be highlighted as striking in this species and may assist in the diagnosis and preliminary analysis of drug quality vegetables, as the presence of glandular trichomes and cystoliths.

Padilha *et al*. (2010) did not notice the same amount of cells in the spongy and palisade parenchyma, and the presence of glandular trichomes, due to the habitat of the

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plant, because one must consider that humidity and intensity of sunlight are factors that can influence the development of a plant (Ribeiro, 2007).

FIGURE 1: *Morus nigra*. A and B: Habit. C: Leaves ± 13 cm. D: Cross-section of the mesophyll (Bar – 50 µm). E: Cross-section of the rib in the central region (Bar – 100 µm).

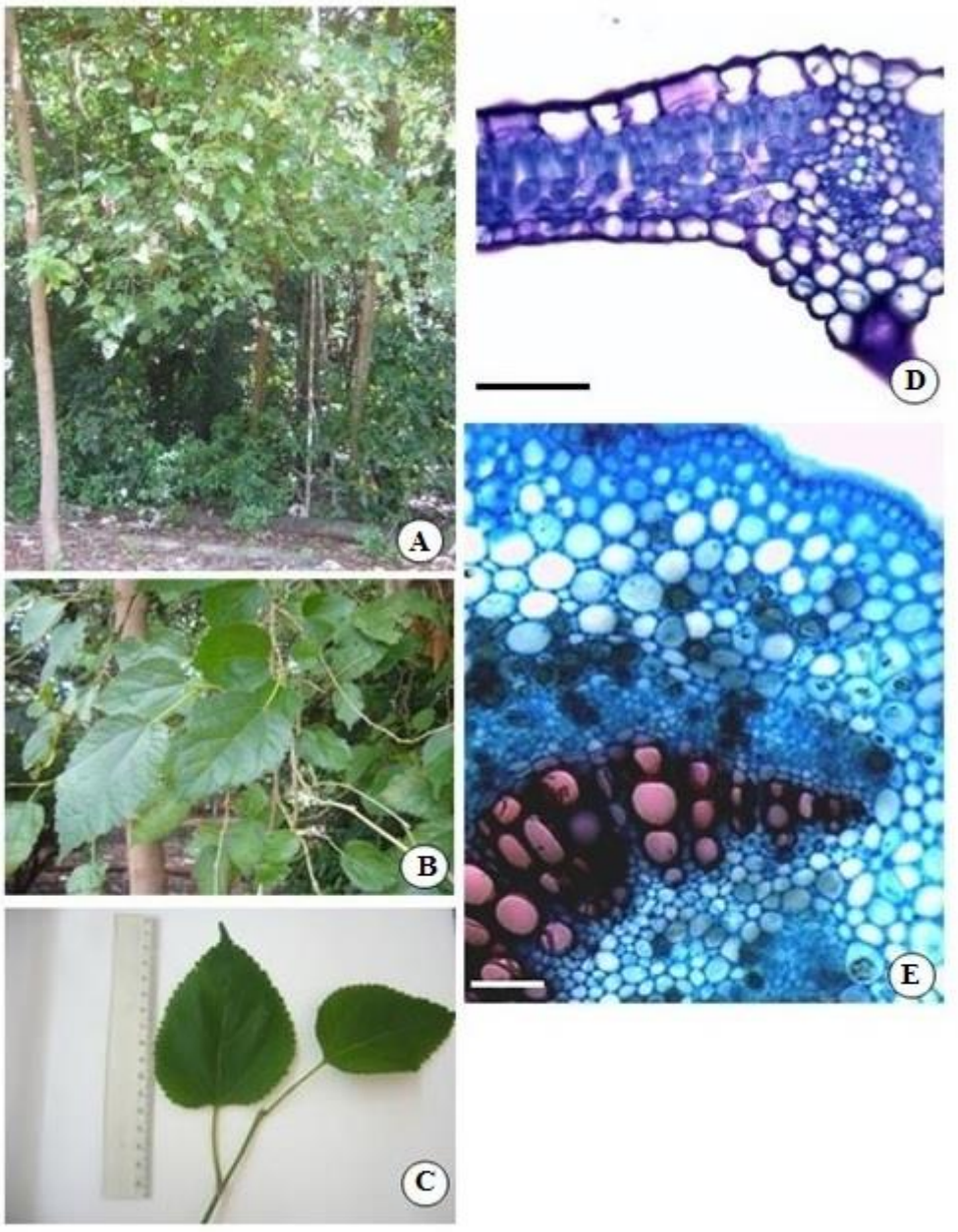

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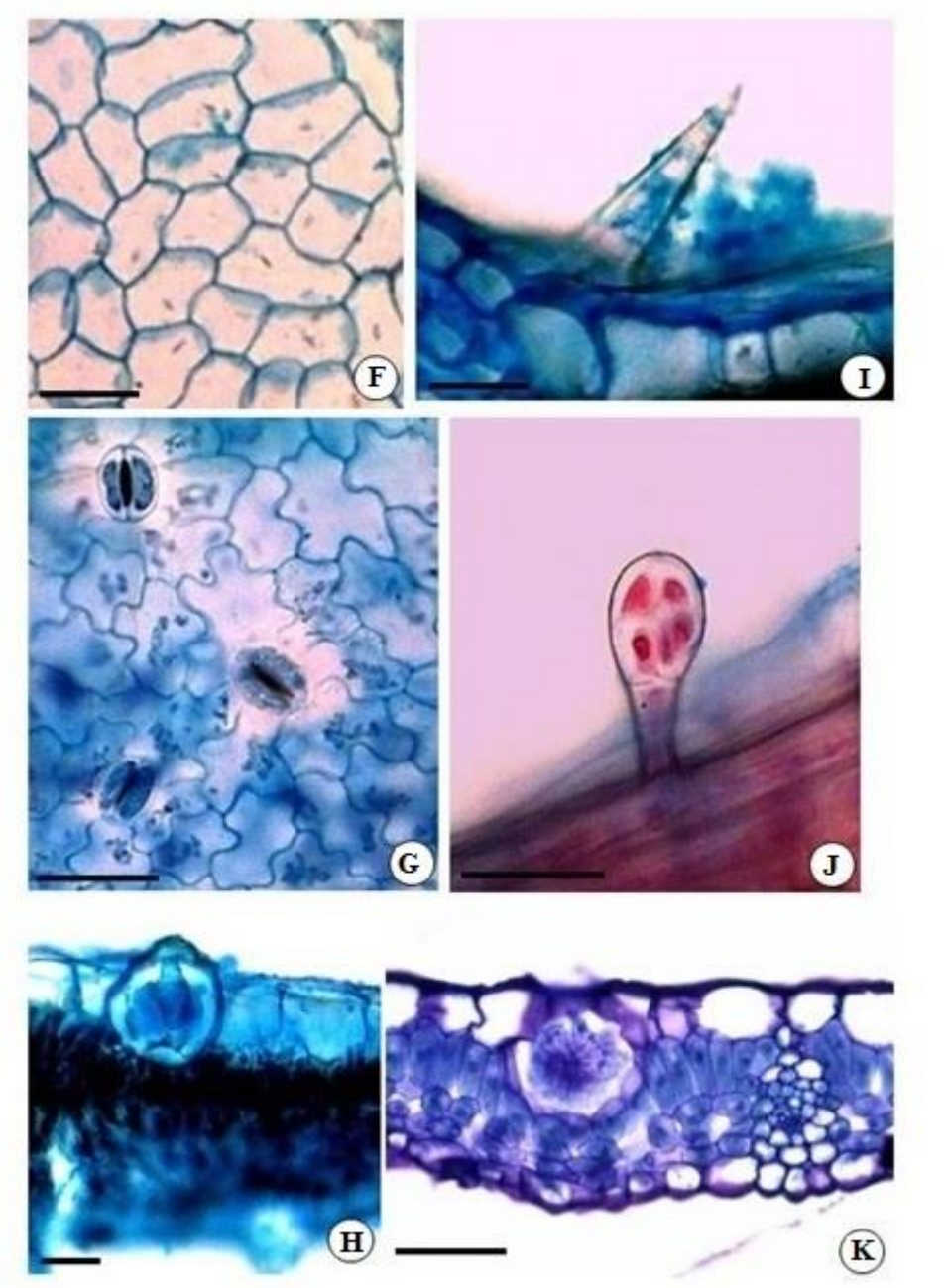
FIGURE 2: *Morus nigra*. F: Epidermis on the adaxial face. G: Epidermis abaxial; in detail, the stomata. H and K: Cystolith on the adaxial epidermis. I: Detail non-glandular trichomes. J: Detail glandular trichomes. (Bar - 50 µm).



Source: the author's personal collection

MOISTURE DETERMINATION FOR LOSS ON DRYING IN BALANCE WITH INFRARED

The presence of large amounts of water in a drug plant enables the development of microorganisms and insects and results in an increase in enzyme activity of its constituents, and thus its deterioration (F. Bras. IV ed. 1988; Costa *et al*., 2005). It is therefore necessary to establish boundaries of moisture to plant drugs. According to F. Bras. IV (1988), the moisture limit range is 8 to 14%.

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The average moisture of 3 samples obtained by infrared irradiation was 12%, with a standard deviation of ± 1 and a coefficient of variation of 8.33%.

Currently, despite the existence of official methodologies, there are several studies for the determination of moisture in both food and medicines, using balance linked to drying systems for infrared radiation, due to faster analysis compared to classic techniques (Borges *et al.*, 2005).

The basis of this methodology is the transformation of electromagnetic energy into kinetic energy, thus releasing heat energy (Borges *et al*., 2005).

PARTICLE SIZE ANALYSIS BY SIEVING

For the milling process to be more efficient, the particle size of the powder obtained from the reduction of the leaves of *M. nigra* L should be determined. The sieving is an accessory of the spraying operation, where there is determination of thinness and homogeneity of powders. This is a vital assessment. The degree of drug spraying exerts an influence on the process of mixing and extractive.

The particle size distribution is an essential factor in obtaining an extract, as some of the extraction is related to the surface area and thickness of the particles in contact with the liquid extractor (Cardoso, 1990).

The highest percentage of particles is in the range between 0.600 to 0.350 mm, according to the method of grinding.

The average particle size was determined by sieving processes, as the proportion of granulometric range, since it is not possible to determine a single particle dimension, as the grinding process obtains various sizes. The value of the average size was 0.420 mm, determined by the calculation described by Ansel *et al*., (2000).

OBTAINING THE EXTRACTS

Currently, there are several techniques for preparation of plant extracts. In this paper, the development of reproducible methods to evaluate extracts was proposed, in order to establish quality control criteria.

During the extraction process, two phenomena occur at the same time: the leaching of soluble substances of the disrupted cells and the dissolution or dissemination of soluble substances of the intact cells. In this work, the type of extraction used was turboextraction.

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In addition to this method, there is an efficiency in the simplicity, speed and versatility of the technique, which allows for ease of handling on both the small and medium scale (Sonaglio *et al*., 2003).

Alcohol concentration variation was used to create systems with different dielectric constants. The grain alcohol and water were chosen, since they are used in the preparation of commercial extracts, and present low toxicity to humans.

DRY RESIDUE

The value of the dry residue can be used as a measure to evaluate the efficiency of solvent extraction of the substances present as a whole in a particular species, not drawing specifically a substance or class (Cardoso, 1990). The values detected (Table I) demonstrated that the dry weight increases in proportion to the amount by which the plant drug was increased. The dry residue is just a feature of blackmulberry extract; it cannot be used to evaluate the chemical quality and therapeutic features of an extract.

Table I: Analysis of physicochemical characterization of extractive solutions of *M. nigra*

Extract type	DR ± *s* (g/100g) (*CV* %)	Density ± *s* (g/mL) (*CV* %)
1 ethanol 60 °GL, 10% drug, 10 min	1.5135 ± 0.0071 (5.26)	0.9098 ± 0.0057 (0.62)
2 ethanol 94 °GL, 10% drug, 10 min	0.1241 ± 0.0000 (0.00)	0.8098 ± 0.0076 (0.94)
3 ethanol 60 °GL, 20% drug, 10 min	3.3833 ± 0.0071 (2.33)	0.9114 ± 0.0048 (0.53)
4 ethanol 94 °GL, 20% drug, 10 min	0.4345 ± 0.007 (20.00)	0.8113 ± 0.0071 (0.88)
5 ethanol 60 °GL, 10% drug, 20 min	1.7729 ± 0.000 (0.00)	0.9110 ± 0.0086 (0.94)
6 ethanol 94 °GL, 10% drug, 20 min	0.1243 ± 0.000 (0.00)	0.8100 ± 0.0014 (0.17)
7 ethanol 60 °GL, 20% drug, 20 min	**3.6672 ± 0.0071 (2.12)**	0.9180 ± 0.0021 (0.23)
8 ethanol 94 °GL, 20% drug, 20 min	0.5573 ± 0.007 (15.56)	0.8248 ± 0.0029 (0.35)
9 ethanol 77 °GL, 15% drug, 15 min	1.5661 ± 0.0071 (5.26)	0.8612 ± 0.0006 (0.07)

CV: coefficient of variation; DR: dry residue; GL: Gay-Lussac; *s*: standard deviation; min: minutes.
Source: the author's personal collection

The extract which obtained the highest dry weight (3.6672 g/100g) was the extract with an alcohol content of 60°GL, drug concentration of 20% and extraction time of 20 minutes. This same extract was of a higher density (0.9180 g/mL) compared to others.

The most efficient extraction was achieved with a reduction of the alcohol content; that is, a concentration of 60°GL in ethanol. It is suggested that the extract contains substances that have high solubility in aqueous media in greater quantities. However, a decrease in ethanol concentration below 50% is not indicated, since this is more likely to show microbial contamination and degradation of the aqueous solution constituents (Cardoso, 1990).

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DENSITY

The evaluation of extractive solution density was established as another parameter for characterisation of the extract. There has been an increase in density, according to the increase in drug concentration, an increase in extraction time, and with a low alcohol content when preparing the extract (60° GL, 20% drug, 20 minutes). This increased density can be related to the dissolution of a greater quantity of water-soluble substances, and there was a saturation of the solvent within 20 minutes (Table I).

The density can be determined by several methods, with the most used being those obtained by hydrometers or picnometers. In both methods, substances can be extracted, which interfere by changing the density (Cardoso, 1990).

OBTAINING ANALYTIC CURVE FOR TOTAL POLYPHENOLS (TPF)

A standard curve was determined at 772 nm in a spectrophotometer, showing linearity between the absorbance and the concentration of polyphenols in quercetin in the range of concentration from 3.2 to 10 µg/mL.

Proof of the linearity of the quercetin standard curve was obtained by significant correlation ($\alpha < 0.001$) to the concentration of 3.2 µg/mL to 10 µ g/mL, with a coefficient of determination (r^2) greater than 0.9997, with an inclination (a) of 0.0959 and an intercept (b) 0.167156. The linearity of the method was also evaluated by analysis of variance (ANOVA); the F value obtained was 47746.43, P-value was 0.00 and the total sum of squares of the average was 0.790236. The value F of lack of fit obtained was 2.84, with an F list of 3.86 and P-value of 0.098.

The analysis of the coefficient of determination from analytical curves demonstrates a lack of linearity in the range of concentrations tested. The proposed mathematical model was capable of describing 99.97% of the results obtained.

DETERMINATION OF TOTAL POLYPHENOLS (TPF)

The method for the determination of total polyphenols was based on the spectrophotometric assay of complexes formed between polyphenols and Folin-Ciocalteau reagent. The formation of the blue colour is due to reduction of the reagent by phenolic hydroxyl (Cardoso, 1990).

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The method employed uses a wavelength of 772 nm, where the maximum absorption for extracts of *M. nigra* were found. The results of the determination of total polyphenols are found in Table II. The results are the average of 6 determinations.

Table II: Results of determination of total polyphenols and total flavonoids present of extractive of solutions *M. nigra*

Extract type	TPF ± *s* (mg/100g) (*CV* %)	TF ± *s* (mg/100g) (*CV* %)
1 ethanol 60 °GL, 10% drug, 10 min	75.77 ± 13.35 (17.62)	34.88 ± 6.16 (17.66)
2 ethanol 94 °GL, 10% drug, 10 min	7.92 ± 0.42 (5.30)	40.19 ± 0.46 (1.14)
3 ethanol 60 °GL, 20% drug, 10 min	166.45 ± 6.18 (3.71)	53.73 ± 1.23 (2.29)
4 ethanol 94 °GL, 20% drug, 10 min	21.22 ± 0.98 (4.62)	65.19 ± 2.91 (4.46)
5 ethanol 60 °GL, 10% drug, 20 min	87.54 ± 7.29 (8.33)	47.31 ± 4.50 (9.51)
6 ethanol 94 °GL, 10% drug, 20 min	11.11 ± 0.89 (8.01)	50.25 ± 4.60 (9.15)
7 ethanol 60 °GL, 20% drug, 20 min	**201.68 ± 8.39 (4.16)**	**79.80 ± 5.39 (6.75)**
8 ethanol 94 °GL, 20% drug, 20 min	30.85 ± 6.14 (19.90)	80.41 ± 3.33 (4.14)
9 ethanol 77 °GL, 15% drug, 15 min	98.95 ± 1.57 (1.59)	71.95 ± 4.15 (5.77)

TPF: total polyphenols; TF: total flavonoids; *s*: standard deviation; *CV*: coefficient of variation; GL: Gay-Lussac; min: minutes.
Source: the author's personal collection

As shown in Table II, the largest yield in TPF was obtained through the statement with an alcohol content of 60° GL, drug concentration of 20% and an extraction time of 20 minutes. The content of total polyphenols varied from 7.92 to 201.68 mg of quercetin per 100 grams of extract, according to the different levels of parameters investigated (Table II).

OBTAINING ANALYTIC CURVE FOR TOTAL FLAVONOIDS (TF)

The wavelength used for the reading was 420 nm, showing linearity between the absorbance and the concentration of flavonoids in quercetin. Quercetin concentrations obtained for the completion of the calibration curve were: 19.2, 16.0, 12.8, 9.6 and 6.4 µg/mL.

Proof of linearity of the quercetin standard curve was obtained by significant correlation ($\alpha < 0.001$) in the concentration of the 6.4 19.2 µg/mL, with the coefficient of determination (r^2) greater than 0.9999. With an inclination (a) of 0.0756 and an intercept (b) 0.0041. The linearity of the method was also evaluated by analysis of variance (ANOVA), the F value obtained was 114608.8, with a P-value of 0.00 and the total sum of squares of the average of 1.7552. The value F of lack of fit obtained was 1.9053, with an F list of 3.71 and P-value of 0.1927.

According to the proposed mathematical model and the coefficient of determination, 99% of the results obtained in the assay of TF could be explained.

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The probability (p-value) of the regression model was 0.0000, meaning that there was a statistically significant multiple regression relationship between the concentration of quercetin and absorbance.

The lack of adjustment, which measures the adequacy of the model, resulted in a significant value ($p > 0.05$) in terms of the response variable absorbance studied. This indicated that the model was accurate enough to predict variations of the response.

DETERMINATION OF TOTAL FLAVONOIDS (TF)

The content of total flavonoids in 60° hydroethanolics, 77° GL and GL, 94° GL, were obtained from the leaves of *Morus nigra* L.

The method is based on the production of a stable complex between the aluminium and the flavonoid in a methanolic solution. When analysed by spectrometry, light is absorbed at 420 nm.

The measurements were performed in sextuplicate, and the results are expressed in mg/100 g of extract. The results are shown in Table II.

The selected wavelength, 420 nm, corresponds to the absorption of quercetin complex band-aluminium. The use of quercetin as the standard is justified as this is the most common flavonoid in plants. Some substances, such as complex derivative flavones, do not absorb in this wavelength, which leads to an underestimation of mixtures rich in flavones. Therefore, this method is valid to establish quality controls (Woisky and Salatino, 1998).

According to the results presented in Table II, the extract which showed the highest content of flavonoids was the total extract with 94° GL, drug concentration of 20% and an extraction time of 20 minutes (80.41 mg/100 g). All extracts with higher alcohol concentrations (94° GL) achieved greater total flavonoid contents when compared with the alcohol concentration of extracts with 60° GL. Since the levels of total flavonoids extracts with an alcohol content of 94° GL were greater than the levels of total polyphenols, it was necessary to investigate the increase in absorbance. As the flavonoids are polyphenolic antioxidants, they should, therefore, have a lower content of total flavonoids in comparison with the total polyphenols (Hubinger, 2009).

As a result, because the alcohol content of 94° GL presents average polarity, substances that have non-polar solvent solubility were extracted. Therefore, an analysis was performed with carotenoids that were obtained through the super-critical fluid

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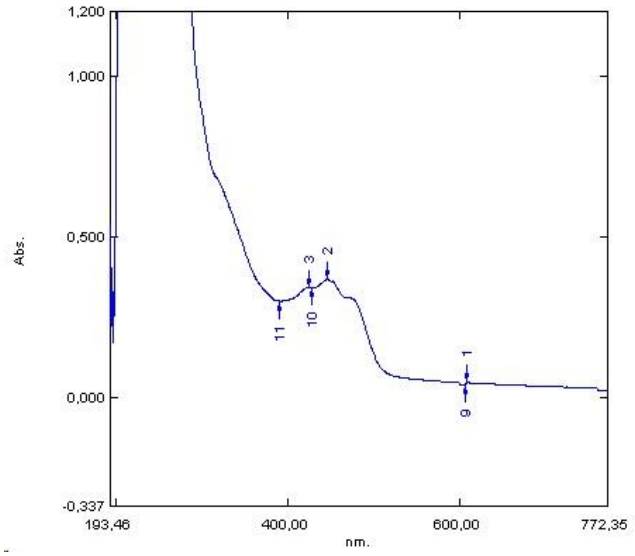
extraction. The substance was dissolved in methanol, and then complexed with $AlCl_3$; the reading was performed at 420 nm by a UV-visible spectrophotometer (Shimadzu UV-1650 PC).

Carotenoids are natural, fat-soluble pigments, found in most foods. They are reddish-orange, and play a role in human health, due to their high antioxidant power; also, a few of them present pro-vitamin A activity (Rodriguez-Amaya, 2001). Thus, carotenoids increase the absorption in the determination of total flavonoids and thus, the alcoholic extract of 94° GL, which gives a higher absorbance in relation to extracts with an alcohol content of 60° GL. This confirms that carotenoids react with aluminium chloride, and also absorb at the same wavelength, 420 nm, as depicted in Figure 3.

As a result, the colorimetric method using the aluminium chloride reagent was not effective to specifically quantify the content of total flavonoids, at least at this alcohol concentration.

Figure 3: Ultraviolet spectrum of carotenoid at 420 nm



Source: the author's personal collection

CONCLUSION

The presence of cystoliths and glandular trichomes was important for the quality control of raw material plants, *Morus nigra* L. It was possible to determine the best extract for greater income in polyphenols and flavonoids: 60° GL alcohol, a drug concentration of 20% and an extraction time of 20 minutes.

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With the current market demand, collections of blackmulberry leaves at different times of the year can influence in the development of the plant, interfering with their therapeutic effect.

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