

Physico-chemical quality control and dosage of total polyphenols, flavonoids of *Morus alba* Leaves (MORACEAE)

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ABSTRACT: To establish parameters for quality control of *Morus alba* leaves were done tests of purity by determining the loss on drying, foreign matter, total ash, acid insoluble ash, swelling index, bitterness index, yield of crude extract, pH and organoleptic properties. The tests were done with samples collected on March, June, September and December according to Brazilian Pharmacopeia and World Health Organization. The percentage of loss on drying found are according to the established value of 14 %, the highest content was on September 12.89 ± 0.1400 %. The indices of foreign matter found are agreement with the amount allowed by Brazilian Pharmacopeia, which establishes a maximum of 2 % for most of the herbal drugs. The collect on March showed the highest content of total ash and acid insoluble ash, respectively, 20.48 ± 0.3517 % and 5.68 ± 0.1057 %. The swelling index showed higher values in samples collected on September 7.95 ± 0.2186 mL and on December 7.1 ± 0.8544 mL. The bitterness index was higher in the first two collections, 1.037 units/g in both. The yield produced was 18 % on March, 29.61 % on June, 26.47 % on September and 24.35 % on December. The pH of aqueous extract was neutral (pH 7.0) on March and slightly acid (pH 6.0) in other months. The four collect showed small variations in relation the organoleptic properties. It was done dosage of total polyphenols and flavonoids with 70% ethanol extracts of *M. alba* leaves. The content found of polyphenols were 6.467 ± 0.012 ; 5.503 ± 0.007 ; 6.016 ± 0.020 ; 6.943 ± 0.046 milligrams of gallic acid per gram of dried plant on March, June, September and December, respectively. The content of flavonoids found were 4.345 ± 0.008 ; 3.782 ± 0.016 ; 4.362 ± 0.005 ; 4.893 ± 0.013 milligrams of rutin per gram of dried plant.

Descriptors: *Morus alba*; Moraceae; Quality Control; Leaves.

Controle de qualidade físico-químico e dosagem de polifenóis totais e flavonóides nas folhas de *Morus alba* (MORACEAE)

RESUMO: Visando estabelecer parâmetros para o controle de qualidade das folhas de *Morus alba* foram realizados ensaios de pureza através das determinações de umidade, matéria estranha, cinzas totais, cinzas insolúveis em ácido, teor de mucilagem, índice de amargor, rendimento do extrato bruto, propriedades organolépticas e pH. Os ensaios foram realizados com amostras coletadas em março, junho, setembro e dezembro, de acordo com a Farmacopéia Brasileira e a Organização Mundial de Saúde. A perda por dessecação apresentou teor de umidade de acordo com o valor estabelecido de 14 %, o valor mais elevado foi em setembro $12.89 \pm 0,1400$ %. Os índices de matéria estranha encontrados estão de acordo com a quantidade permitida pela Farmacopéia Brasileira, que estabelece um máximo de 2% para a maioria das drogas vegetais. A coleta de março apresentou o maior teor de

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cinzas totais e cinzas insolúveis em ácido, respectivamente, $20,48 \pm 0,3517$ % e $5,68 \pm 0,1057$ %. O índice de intumescência mostrou valores mais elevados nas amostras coletadas em setembro $7,95 \pm 0,2186$ mL e dezembro $7,1 \pm 0,8544$ mL. O índice de amargura foi mais elevado nas duas primeiras coletas 1,037 unidades/g em ambas. O rendimento produzido foi 18 % em março, 29,61 % em junho, 26,47 % em setembro e 24,35 % em dezembro. O pH do extrato aquoso foi neutro (pH 7,0) em março e levemente ácido (pH 6,0) nos outros meses. As quatro coletas apresentaram pequenas variações em relação as propriedades organolépticas. Foi realizada dosagem de polifenóis e flavonóides com o extrato etanólico 70 % das folhas de *M. alba*. O valor de polifenóis encontrado em março, junho, setembro e dezembro foi $6,467 \pm 0,012$; $5,503 \pm 0,007$; $6,016 \pm 0,020$; $6,943 \pm 0,046$ miligramas de ácido gálico por grama de planta seca, respectivamente. O valor de flavonóides encontrado foi $4,345 \pm 0,008$; $3,782 \pm 0,016$; $4,362 \pm 0,005$; $4,893 \pm 0,013$ miligramas de rutina por grama de planta seca.

Descritores: *Morus alba*; Moraceae; Controle de qualidade; Folhas.

Introduction

The use of medicinal plants in therapy requires quality of herbal drugs, which begins on the correct identification of species, planting, harvesting, preparation of plant extracts and medicines. Therefore, it is necessary to obtain parameters of quality for pharmaceutical purposes, which are set out in Pharmacopeias and Officers Codes.

Family Moraceae occurs in tropical and subtropical regions and subdivided into approximately 60 genera, which comprise about 1400 species.¹ The genus *Morus* is distinguished by its inflorescence spike amentacea type. Inflorescence is formed during months of July and August, with small flowers, often single-sex. They have corolla and a cup formed by four free sepals.² Due to diversity of species of fruits, they are known as white-mulberry (*M. alba*), blackberry (*M. nigra*), cranberry-red (*M. rubra*) and bramble-rose (*M. rosea*). The main cultivated are white-blackberry and blackberry because they don't have thorns. They are adapted to different soil types, except those with trend water logging.³⁻⁶ The Moraceae family leaves are rich in protein, fiber, minerals and vitamin C, they also show a type of glycoprotein, called moran A, which is attributed an antidiabetic effect.⁷ The genus *Morus* has a variety of phenolic compounds including isoprenyl flavonoids, coumarins, chromones and xanthenes. Many of these compounds exhibit biological properties such as antiinflammatory, diuretic and hypotensive effects.¹ The leaves, bark and branch of *M. alba* (Fig. 1), are used in traditional medicine as antihyperglycemic, to treat fever, to protect the liver, to improve eyesight and to strengthen joints.⁸⁻⁹ Due to lack *M. alba* in monographs, this work aims to establish standards of quality and purity of plant drug by physico-chemical analysis and evaluation of total polyphenols and flavonoids in four collections during the year. The physico-chemical analysis carried out were: determination of loss on drying, foreign matter, yield of ethanol extrac, total ash, sulphated ash, swelling index, bitterness index, pH and organoleptic properties.

Figure 1 — Morus alba leaves.
(Font: http://www.cirrusimage.com/tree_white_mulberry.htm)



Material and methods

Plant material

The *Morus alba* leaves were collected in March, June, September and December 2010, in Santa Maria city, Rio Grande do Sul State, Brazil. A sample of plant material was identified by Dr^a Jumaida Maria Rosito and voucher specimens were deposited in the Herbarium of Biology Department of the Federal University of Santa Maria (UFSM) as voucher specimen SMDB 13079. The plant material was dried at 40 °C with air circulation and grounded in a knife mill.

Preparation of ethanol extract

The extracts were obtained by cold maceration of powdered plant drug (80 g) with drug solvent ratio 20 % (w/v) in 70 % hydroethanolic solvent during 30 days, after this, they were concentrated, lyophilized and calculated yield.

Physical-chemistry quality control

Loss on drying

The water determination was performed according to Brazilian Pharmacopeia.¹⁰ It was weighed exactly 1 g of powdered plant drug and it underwent heating in an oven at 105 °C during 5 hours. The loss of drying weight was calculated considering the air-dried sample. The analysis was performed in triplicate.

Foreign matter

Macro e microscopy examination was conveniently employed to determine the presence of foreign matter in powdered plant drug according to Brazilian Pharmacopeia.¹⁰ In the macroscopy examination, 25 g of plant drug was spread on a thin layer from which the foreign matter was selected by inspection through the magnifying less. The content of foreign matter was calculated considering the air-dried sample. The analysis was performed in triplicate.

Total ash

The analysis of total ash was conducted according to Brazilian Pharmacopeia.¹⁰ The powdered plant drug (3g) was accurately weighed into a crucible that has been previously ignited, cooled and weighed. Then the sample was ignited by gradual heating to 450 °C, cooled in a desiccator and weighed. For obtaining carbon-free ash, the residue was moistened with 2 mL of water after cooling the crucible. The remaining material was dried on a water-bath and then placed on a hot-plate, being ignited again until constant weight. The residue was cooled in a desiccator for 30 minutes and weighed. Total ash content was calculated considering the dry material. The analysis was performed in triplicate.

Acid insoluble ash

The analysis of acid insoluble ash was conducted according to Brazilian Pharmacopeia.¹⁰ In the crucible containing the total ash, it was added 25 mL of hydrochloric acid (70 g/L), covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 mL of hot water and then this liquid was added to the crucible. The insoluble matter was collected on an ashless filter-paper and washed with hot water until the filtrate was neutral. Thereafter, the filter-paper containing the insoluble matter was transferred to the original crucible, which was dried on a hot-plate and ignited to constant weight. After cooling the residue in a desiccator, the crucible was weighted and the content of acid-insoluble ash calculated considering the dry material. The analysis was performed in triplicate.

Swelling index

The analysis of swelling index was conducted according to Brazilian Pharmacopeia.¹⁰ One gram of powdered plant drug was introduced into a 25 mL glass-stoppered measuring cylinder for the verification of the volume occupied by the sample. Next, 25 mL of water was added and the mixture was shaken thoroughly every 10 minutes for about 1 hour. After that, the mixture was stood for 3 hours at room temperature, and finally the volume in ml occupied by the plant material was remeasured. The test was performed in triplicate.

Bitterness index

The determination of the index of bitterness was performed according to Brazilian Pharmacopeia.¹⁰ The bitter properties of plant material were determined by comparing the

threshold bitter concentration of the extract from the material with the concentration from a dilute solution of quinine hydrochloride. Drinking-water was the vehicle to the extraction of plant materials and for the mouth-wash after each tasting. It's was prepared a stock solution (St) of *M. alba* at 1 % (w/v). St was transferred to 10 tubes with volumes from 1 to 10 mL. The standard solution of quinine hydrochloride R was distributed in nine tubes from 0.042 to 0.058 mg/10 mL. The birtteness value was expressed in units equivalent to the bitterness of a solution containing 1 g of quinine hydrochloride in 2000 mL.

Yield

The analysis of yield was conducted according to Costa.¹¹ The yield was determined using the equation: Yield (%) = $M_f / M_i \times 100$, where M_i is the initial mass of sample (g) and M_f is the final mass of the dry extract (g). Crude extracts were obtained by cold maceration of powdered plant drug (80 g) with drug solvent ratio 20% (w/v) in 70 % hydroetanolic solvent during 30 days and the yield determined. Botanical studies of yield must be performed as an indicative of correct amount of material to be collected for production of extracts and fractions used in pharmacological tests.

Organoleptic properties and pH

The organoleptic features analyzed were odour, flavor and color. The odour was checked by pressing of a small portion of leaves on the palm of the hand, inhaling slowly and repeatedly. The color of the leaves was analyzed with fresh plant under diffuse daylight. The flavor was tasted through the instillation of few drops on tongue of an aqueous solution 1 % (w/v) of the powdered plant drug heated on an eletric-plate and brought to be boiling for 5 minutes.¹² pH was verified by potentiometry with the above solution, according to Brazilian Pharmacopeia.¹³

Dosage of total polyphenols

The dosage of polyphenols was performed through the Folin-Ciocalteu, with some modifications.¹⁴⁻¹⁵ Based on the solution of 0.04 % of the ethanol extract of *M. alba*, solutions was prepared in volumetric flasks at concentrations of 5,10,20,30 µg/mL. To this end, we used the required amount of solution of 0.04 % with addition of 1 mL of Folin-Ciocalteu for 5 minutes, 2 mL of sodium carbonate (Na_2CO_3) 20 % for 10 minutes. The solutions were homogenized, capped and protected from light and kept at room temperature. The absorbance was measured at 730 nm using water as a blank. The test was performed in triplicate. The standard curve was obtained using standard solutions of gallic acid at the same concentration of the sample. The content of total polyphenols was expressed as milligram of gallic acid equivalent per gram of dried plant.

Dosage of flavonoids

The dosage of flavonoids was performed according to a modified methodology from Rio¹⁶. Based on the solution of 0.04 % of the ethanol extract of *M. alba*, solutions was prepared in volumetric flasks at concentrations of 10,20,50,100,150 µg/mL . To this end, we used the required amount of solution of 0.04 % with addition of 75 µL of 5 % methanol (MeOH) solution of AlCl₃, 3.9 mL of methanol 70 % for 30 minutes. The solutions were homogenized, capped and protected from light and kept at room temperature. The absorbance was measured at 425 nm using MeOH 70% as a blank. The test was performed in triplicate. The standard curve was obtained using standard solutions of rutin at the same concentration of the sample. The flavonoids content was expressed as milligram of rutin equivalent per gram of dried plant.

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using One-way ANOVA followed by Tukey test using Graph Prism 5.0 software (Graph Pad Software, San Diego, CA, USA) and the results were considered significant when $p < 0.05$.

Results and discussion

Physico-Chemical Quality Control

The loss on drying of *M. alba* leaves showed a moisture content according to the established value of 14 %, which is the maximum limit recommended of moisture for plant drugs.^{12;13;17} According to Simões¹⁸ the water founded in the plant drug is directly related to its storage and microbial contamination or enzymatic degradation by action of the chemical constituents. The excess of water in the raw material enables the development of fungi and bacteria.¹⁹ Therefore, preliminary operations employed in the leaves were effective in standardizing of moisture content to established values. Through statistical analysis were checked significant differences in the loss on drying in the four collections (table 1).

Table 1 — Loss on drying, mean ± standard error of mean (SEM) of *Morus alba* leaves.

Month	Mean (%)	SEM
March	9.73	± 0.1253
June	11.15	± 0.2118
September	12.89	± 0.1400
December	8.56	± 0.0808

Indices of foreign matter found in *M. alba* on March and June are agreement with the amount allowed by Brazilian Pharmacopeia,¹⁰ which establishes a maximum of 2 % for most of the herbal drugs (table 2). Collections made during September and December didn't show

foreign matter. There weren't significant differences in the indices of foreign matter in the four collections. The amount of foreign matter may be related to the fact of leaves have been pre-selected and milled.²⁰ Analysis for determination of foreign matter has the purpose to identify portions or products not specified in the definition and description of the drug, besides impurities from mineral or organic nature, don't related to the drug.¹³

Table 2 — Foreign matter, mean \pm standard error of mean (SEM) of *Morus alba* leaves

Month	Mean (%)	SEM
March	0.0200	± 0.0121
June	0.0126	± 0.0063

The determination of contenting total ash is important for quality control, since its aim is to check the presence of non-volatile inorganic impurities that may contaminate the plant drug.^{10;21} The colletec that had the highest content of total ash was done on March (table 3). This can be explained by the higher amount of inorganic salts present in the first collection.¹⁷

In the analysis of acid insoluble ash was possible to observe a large discrepancy in values. In the gathering done on March and December, the content found was 5.68 % and 3.50 %, respectively. Through statistical analysis were checked significant differences in the content of total ash and acid insoluble ash in the four collections. Determination of ash insoluble in acid chloridric is intended to detect constituents of silica and siliceous constituents, wich above of the established for the plant drug indicates contamination by excess soil or sand.¹³ There is not maximum value for total ash and acid insoluble ash officially established for the species in study.

Table 3 — Determination of total ash, acid insoluble ash, mean \pm standard error of mean (SEM) of *Morus alba* leaves.

Month	Total ash (%)	Acid insoluble ash (%)
March	20.48 \pm 0.3517	5.68 \pm 0.1057
June	9.57 \pm 0.4908	0.75 \pm 0.0333
September	11.88 \pm 0.2408	1.67 \pm 0.0738
December	17.86 \pm 0.4020	3.508 \pm 0.0601

Analyzing the swelling index, *M. alba* leaves showed the highest values in samples collected on September and December (Table 4). The significant differences were checked only in results obtained on September versus March and September versus June. Banderó²² found a quantity of swelling index of 2.03 mL to *Glechon spathulata*. This test serves to measure the volume (mL) occupied by plant matter plus mucilage or other material attached.^{12,13,17}

Table 4 — Swelling index, mean \pm standard error of mean (SEM) of *Morus alba* leaves.

Month	Mean (mL)	SEM
March	5.83	± 0.1202
June	5.73	± 0.0882
September	7.95	± 0.2186
December	7.1	± 0.8544

Pharmacopeias and specialist books on herbal drugs include certain types of sensation like flavour. Analysis of the bitterness index is determined by comparing the threshold concentration of bitterness of an extract and the dilute solution of quinine chloride. Thus, there are astringent, oily, mucilaginous, bitter and pungent tastes. The bitterness index was higher in the first two collections (table 5), it shows that the drug contains bitter substances, especially considering the dilution used in the test. To Wasicky et al.,²³ bitterness index has solid bases theoretical and experimental, which competes with the best biological methods. However, Mello et al.,²⁴ affirmed that this test may introduce errors due to individual variability, since it doesn't statistically reproducing real information. Thus, to perform this test it is necessary a previous training of experimenter, which combined with statistical analysis, allow that the biological variability be minimized, becoming a reliable method.

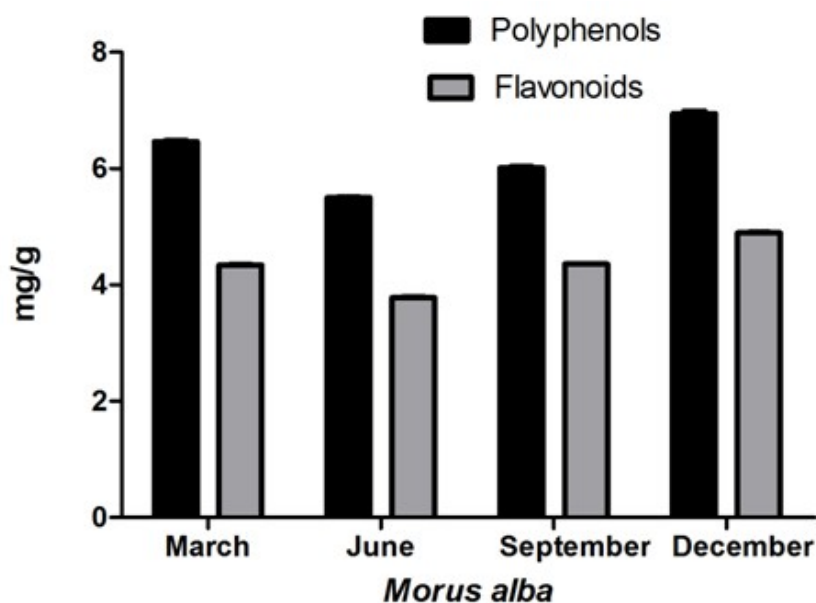
There were differences in about 11 % in yield's assessment of 70 % ethanol extracts. The highest yields were on June and September. The pH of the aqueous extract was neutral (pH 7.0) on March and slightly acid (pH 6.0) in other months, this suggests presence of neutral and acid substances, respectively, in *M. alba* leaves.

Table 5 — Bitterness index, yield, pH of *Morus alba* leaves.

Month	Bitterness index (units/g)	Yield (%)	pH
March	1.037	18.0	7
June	1.037	29.61	6
September	0.84	26.47	6
December	0.84	24.35	6

In relation the organoleptic properties, the four collect showed small variations, they were odorless and the aqueous extract showed a slight bitter flavor. The fresh leaves showed a light green color in the collection of March, mid-green on June and September and dark green on December. Total polyphenols content found in ethanol extracts of *M. alba* on March, June, September and December were 6.467 ± 0.012 ; 5.503 ± 0.007 ; 6.016 ± 0.020 ; 6.943 ± 0.046 milligrams of gallic acid per gram of dried plant (figure 2). The regression curve obtained was $Y = 0.2879x - 0.0685$ ($R = 0.9912$). The highest content of polyphenols was found in collection on December (6.943 ± 0.046 mg/g). The content of flavonoids found was 4.345 ± 0.008 ; 3.782 ± 0.016 ; 4.362 ± 0.005 ; 4.893 ± 0.013 milligrams of rutin per gram of dried plant. The regression curve obtained was $Y = 0.1821x - 0.1545$ ($R = 0.9935$).

Figure 2 — Contents of polyphenols and flavonoids in ethanol extracts produced in four seasons.



Plants can accumulate phenolic compounds in their metabolism under various stress conditions.^{25,26} Second Paliyath et al.,²⁷ most plants suffer physiological and biochemical damage by exposure to different temperatures. Sývacý et.al.,²⁸ found significant differences in polyphenol content of *M. nigra* and *M. alba* stem in the four seasons.

Through statistical analysis used in this study, was checked significant differences in the polyphenol and flavonoids content in four collections, only on March versus September there were not significant differences in flavonoids content. This fact can be explained by changes in climate, collection time, soil formation, plant age, temperature and total rainfall. The information obtained in this study has been relevant to characterization and physico chemical quality control of *M. alba* leaves.

Conclusion

Due to widespread use of *M. alba* leaves in traditional medicine, it is necessary to obtain quality standard for the use of this plant. The methods employed were adequate to assess the quality control of vegetable drugs in study, since it hasn't description in Pharmacopoeias. The dosages of polyphenols and flavonoids in the four collections showed higher content in summer.

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