LUMEN ET VIRTUS

Pharmacognostic study of *Vernonia ferruginea*, *Vernonia polyanthes* and *Vernonia westiniana* species

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ABSTRACT

Introduction: Medicinal plants have always been the subject of discussion, with knowledge of their use dating back to ancient times in the history of civilization. Vernonias are species known as "assa-peixe", and Vernonia polianthes is popularly used to treat various pathologies. The aim of this study was to carry out pharmacognostic methods to identify species of Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana. Identifying a species becomes difficult when the plants are acquired in the form of powders, dry extracts or in liquid form such as tinctures and extracts. In this way, pharmacognostic tests were carried out, making it easier to identify the species. Methodology: Based on the physical and chemical methods described in the Brazilian Pharmacopoeia VI edition and on the website of the Brazilian Society of Pharmacognosy, some tests were carried out to identify the secondary metabolites present in the species: Vernonia ferrugínea, Vernonia polyanthes and Vernonia westiniana. Results and Discussion: In the results obtained, chemical reactions and physical methods were used to reliably identify the species studied. When determining the ash content, it is possible to differentiate them, which is more evident through chemical methods, which through reactions, determine the presence of flavonoid glycosides, alkaloids, terpenes and saponin compounds. Another practical method that provided an identification of the compound 3,7dimethyl-quercetin, isolated by high-performance liquid chromatography (HPLC), was comparative thin layer chromatography (SDLC), which verified its presence in Vernonia polyanthes and was not found in Vernonia westiniana. From the same isolated substance it was possible to quantify it in methanolic extracts and in the infusion, with higher concentrations being found in Vernonia ferruginea extracts and lower in Vernonia polyanthes infusion. Conclusion: From this pharmacognostic study, it was possible to establish a method for identifying Vernonias species, however, for better security it is necessary to use more technologically modern methods.

Keywords: Medicinal Plant, Pharmacognostic Control, Identification, Phytotherapics.

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INTRODUCTION

The use of medicinal plants is as old as the history of civilization itself. Entire peoples often associated the healing power of medicinal plants with magic and religious rituals. Cordeiro et al., 19961, reported in their manuscript that, even today, Western folk medicine performs rituals in which they mix knowledge of the healing properties of medicinal plants with rituals and prayers to expel diseases1.

Brazil has the largest medicinal flora in the world, but there was a period when medicinal plants were discredited. This was due to the advance of the pharmaceutical industry in the world, corroborating a new concept of medicine, often palliative2.

Today, the use of medicinal plants is becoming increasingly widespread, not only in Brazil, but also in other countries, especially in Europe. A few years ago, only the countryside population, who grew both edible and medicinal plants, used them to cure a wide variety of ailments through knowledge acquired from their grandparents or great-grandparents3.

Although the Brazilian flora is recognized as one of the most important, in numerical, economic, ornamental, ecological and medicinal terms in the world, there are few works on the subject4.

Some species are easily found, distributed throughout the country, but there is a major problem in the lack of quality plant material, which is often not reliably identified5. In pharmacies, products derived from medicinal plants are purchased in the form of tinctures or powders, which are hardly ever tested for quality and/or botanical identification6.

One species widely used in folk medicine is Vernonia polyanthes, mainly for the treatment of bronchitis, respiratory system disorders and kidney problems7,8. It is also indicated for rebellious coughs, flu, skin conditions, muscle pain and rheumatism9. As it has morphological similarities with two other species, Vernonia ferruginea and Vernonia westiniana, it is extremely important to carry out pharmacognostic studies to determine quality assurance, such as: comparative thin layer chromatography, total ash, acid-insoluble ash, to confirm the species Vernonia polyanthes, which is often misclassified by laypeople9.

2 METHODOLOGY

The species, Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana, were collected near São José do Rio Preto - SP, identified at the Biosciences Institute - UNESP, in partnership with the botany department of the educational institution. After collection and identification, weeding was carried out and the leaves and flowering tops were separated and dried in a circulating air oven at 37º C for 48 hours.

After drying, the species were pulverized and stored in plastic bags to be used in pharmacognostic tests. For this work, a bibliographic survey was also carried out, using articles available in databases: lilacs, web of science, pubmed and scielo, with the aim of learning about the secondary metabolites present in the respective species, facilitating their pharmacognostic identification. The tests were carried out based on the methodologies described in the Brazilian Pharmacopoeia VI edition10, and on the website of the Brazilian Society of Pharmacognosy (SBF), in the teaching link11.

PHYSICAL METHODS

The determination of moisture in the powders of the species Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana was based on the method described in the Brazilian Pharmacopoeia10. The characterization of the method is based on the loss due to desiccation in an oven and aims to determine the amount of volatile substances of any nature eliminated under the conditions specified in the monograph10. The quantitative determination of total ash was also carried out according to the method described in the Brazilian Pharmacopoeia10. A quantity of the powder of each of the species was measured, placed in a porcelain crucible, previously desiccated and the mass recorded. The respective samples were incinerated at a temperature of 450°C, then, after cooling in a desiccator, they were weighed and the percentage of ash of the respective powders of the species used was calculated.

In the test to determine the moisture content, a 50mL beaker was used for each of the species, in powder form, and left in an oven at 105º C for 6 hours, after which time they were placed in a desiccator to cool. Next, the beakers were taken and the masses individually weighed using tongs. 1g of each species was placed in the beakers separately and taken to an oven at 105º C for 1 hour, determining the respective masses to check the moisture content10.

After determining the total ash, the crucibles containing the ash of the respective species were each added certain amounts of 7% hydrochloric acid and heated for 5 minutes. After this time, they were removed from the heat and left on the bench, covered with watch glass, until they cooled down, after which the respective residues from each crucible were transferred to filter paper and dried on a hot plate. Once dry, they were incinerated in crucibles in the muffle furnace until constant weight to determine the concentration of acid-insoluble ash10.

The determination of total flavonoid content was carried out according to the method described in the article by Silva (2016)12, in which 0.5ml aliquots, in triplicate, of each sample of the hydroalcoholic extracts of Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana, were added to an equal volume of 5% aluminum chloride methanolic solution ($AlCl_3$). It was left to stand for 15 minutes and read on a UV spectrophotometer (Bel Photomic) at a wavelength of 420nm. The

total flavonoid content was determined using a calibration curve with quercetin, purchased on the market, at concentrations of 0, 25, 50, 75, 100 mg/ml, considering a variation of +/- 5%12. The total flavonoid content was calculated from the straight line equation obtained from the curve of the standard graph, and the results were expressed in mg of quercetin per 100g of each of the Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana species.

Comparative thin layer chromatography (CCCD) was used to identify the presence of 3,7 dimethoxyquercetin, isolated in the methanolic extract of Vernonia polyanthes and also found in Vernonia ferruginea, in infusions of Vernonia polyanthes and Vernonia westiniana. The method consists of obtaining infusions of the species mentioned, applying them to silica gel plates separately, with the isolated substance (3,7 dimethoxy-quercetin) used as a marker.

After applying the extracts and quercetin to the plate, it was placed in a glass vat containing BAW eluent (butanol, acetic acid and water) in respective concentrations of 65:25:15, impregnating the vat to elute the extracts applied. After reaching the top line, the plate was removed from the eluent, left to dry for a few minutes and revealed with a saturated solution of ceric sulphate and resublimated iodine in a glass vat.

For the quantification of the substance 3,7 dimethyl quercetin, isolated in the methanolic extract of Vernonia polyanthes in another study, a Waters Millipore chromatograph was used, equipped with a Waters model 501 binary pump system, UV detector (Waters model 486), Phenomenex C-18 reverse phase column (250 x 4.60 mm, 5 µ) and mobile phase composed of methanol and water. The samples used were obtained from the extracts of Vernonia ferruginea and Vernonia polyanthes, the latter of which was also infused for the quantification of quercetin by constructing a calibration curve using five different concentrations of the standard.

The external standard method was used for the quantitative analysis of 3,7 dimethyl quercetin, present in the methanolic extracts of the polyanthes and ferruginea species. Obtained in a standard solution of the substance isolated in the methanolic extract of Vernonia polyanthes, in the following concentrations: 2.0; 0.26; 0.035; 0.0047; 0.00063mg/mL. The sample was prepared from the MeOH extract by taking 100mg of the extract and diluting it in 1mL of methanol. The infusion was prepared at 10% (w/v) of the dried plant in boiling distilled water and left to cool.

CHEMICAL METHODS

The chemical methods used to identify the secondary metabolites present in the species studied were based on the Brazilian Pharmacopoeia and the website of the Brazilian Society of Pharmacognosy.

To determine cardiotonic glycosides, the following reactions were carried out: Liebermann-Burchard reaction to identify the steroidal nucleus; Kedde reaction to identify the pentagonal lactone

ring; and Keller-Kilianireaction to identify 2-deoxysugars. For flavonoid compounds, the Shinoda or Cyanidin reactions, reaction with aluminum chloride, reaction with ferric chloride, reaction with sodium hydroxide were carried out, as well as for anthraquinones, possible Borntraeger reactions were analyzed, being a microsublimation process.

For saponin glycosides, a physical process consisting of stirring the solution to detect foam formation was used. In the chemical process, general reactions, the Rossol reaction, the Micthell reaction, the Rosenthalen reaction and the reaction with sulfo-vanillic reagent were carried out. In the specific reactions, the Liebermann-Burchard reaction was carried out, as well as reactions using chloroform solution. The general reaction with trichloroacetic acid and the specific Salkowski reaction were also carried out.

Analysis of alkaloids, the metabolite responsible for different antimicrobial activities, was carried out on the pulverized plant and after extraction with dilute sulphuric acid and chloroform, it was detected with neutral lead acetate. For the determination of tannins, general reactions are observed: reaction with 2% ferric chloride, reaction with neutral lead acetate. Specific reactions with lead acetate and glacial acetic acid and reaction with ferric chloride.

RESULTS AND DISCUSSION

The pharmacognostic tests were proposed in order to identify species of Vernonia ferrugínea, Vernonia polyanthes and Vernonia westiniana, as they are morphologically similar and, in pharmacies, they are purchased in the form of dry powder and microprocessed, or even as a dry extract This makes it difficult to identify the species, so pharmacognostic tests are of great value for this determination

The species Vernonia ferrugínea, despite having a large amount of flavonoids, is very similar to Vernonia polyanthes, so for most of the pharmacognostic tests, the species Vernonia polyanthes and Vernonia westiniana were used, as they have similar morphological characteristics

In the tests to determine water loss, as described in materials and methods, using the leaves and stems of Vernonia polyantes and in the same way for Vernonia west iniana, we obtained values for loss due to desiccation of 8.8% and 10%, respectively. The moisture content can directly influence the ash content and other tests

In the quality control of medicinal plants, a simple, easily reproducible test that offers good security in the identification of species is the total ash index and acid-insoluble ash. According to the analyses described in the materials and methods section, the results obtained showed a clear difference in the ash content of the two species (Table 1), while the ash content of Vernonia polianthes reached indices between 0.32g of total ash and 0.10g of acid-insoluble ash, the indices

obtained with Vernonia westiniana reached values of 0.39g and 0.09g, respectively, and in Vernonia polyanthes values of 0.17g and 0.11g were obtained, respectively.

Thus, the ash content is a simple and quick way of differentiating between the species: Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana With this result, it can be concluded that the total ash and acid-insoluble ash index test can clearly differentiate the Vernonia species studied.

Table 1 - Total and acid-insoluble ash from Vernonia ferriginea, Vernonia polyanthes and Vernonia westiniana.

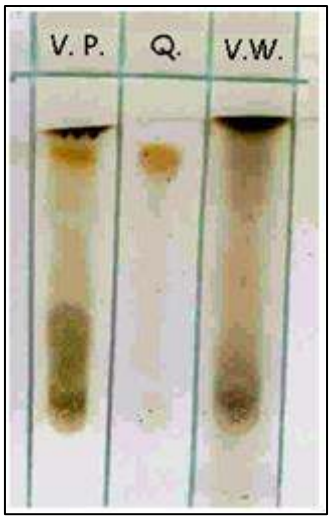
Species	Total ashes	Acid-insoluble ashes
Vernonia polyanthes	0,32g (10,7%)	0,10g (3,33%)
Vernonia westuniana	0,39g (13,0%)	0,09g (3,0%)
Vernonia ferruginea	0,17g (7,12%)	0,11g (3,52%)

The corresponding hydrogenic potential (pH) obtained for both the infusion and the methanolic extract of Vernonia polyanthes and Vernonia westiniana was 5.78 and 7.27 respectively. As there were no significant differences in the infusion with the methanolic extract, it can be said that Vernonia polyanthes has a higher acidity index than Vernonia westiniana.

The use of medicinal plants is directly related to the manipulation of magistral formulas in pharmacies, the vast majority of which are acquired in powder form (dried and ground), making it difficult to identify the plant macroscopically. A quick and practical method for pharmacies to identify vetal drugs is the CCCD method for the methanolic extract and infusion of Vernonia polyanthes, using a commercial standard of 3,7 dimethyl quercetin, which can be easily found on the market.

The methodology proved to be effective for the proposed test, as the presence of the substance in the extract and infusion of Vernonia polyanthes was clearly visible using a specific developer for flavonoids (Figure 1), and the method can also be guaranteed, as 3,7 dimethyl quercetin is not present in any other similar species of the genus studied so far.

Figure 1 - CCCD of the methanolic extract of Vernonia polyanthes and Vernonia westiniana with the 3,7 dimethyl quercetin standard.



V.P. - Methanolic extract of Vernonia polyanthes.
Q. - Standard of 3,7 dimethyl quercetin in methanol.
V.W. - Methanolic extract of Vernonia westiniana.

After isolating the metabolite 3,7-dimethyl quercetin from the methanolic extract of Vernonia polyanthes, it was used as an external standard to quantify its presence in methanolic extracts of Vernonia polyanthes and Vernonia ferruginea species and Vernonia polyanthes infusion .

The calibration curve made it possible to verify the linearity of the detector (UV-vis) within the concentration ranges evaluated, where a correlation index value of 0.99959 was achieved. This means that the method used in the analysis of the substance studied follows a linear correlation in the concentration ranges considered.

According to the data obtained from the calibration curve, linear regression was used to determine the quantification of the substance 3,7 dimethyl quercetin, present in the MeOH extracts of Vernonia polyanthes and Vernonia ferruginea, obtaining a concentration of 0.13mg/mL and 0.54mg/mL in each extract, respectively, and a concentration of 0.063mg/mL in the Vernonia polyanthes infusion.

Determining the amount of flavonoids present in extracts is a good indicator of pharmacological effects, as they are considered antioxidants and anti-inflammatories and are found in most plant species. Quantifying this metabolite is therefore important for the safe and effective use of the species studied, in order to demonstrate therapeutic activity.

The results obtained in the quantification of flavonoids for the species: Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana, based on spectrophotometric methods, in the quantification of quercetin, provided a percentage (m/m) of the flavonoid in each medicinal species studied. Thus, according to the results obtained in the tests, it was determined that Vernonia ferriginea had a percentage of 1.21%, Vernonia polyanthes had a percentage of 0.75% and Vernonia westiniana had a percentage of 0.05% of the flavonoid determined from quercetin, which means that this species does not have significant amounts of flavonoids, as shown in Table 2.

Table 2 - Percentages of total flavonoid concentrations, based on the values obtained with quercetin as a marker.

Medicinal plant	Flavonoid content obtained
Vernonia ferruginea	1,21% m/m
Vernonia polyanthes	0,75% m/m
Vernonia westiniana	0,05% m/m

The determination of cardiotonic glycosides, according to the Liebermann-Burchard reaction, showed positive results for the species Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana, with coloration in a ring with a slightly reddish edge, indicating the presence of cardenolides and bufadienolides. This reaction is characteristic of steroid and triterpenoid compounds, because the reagent promotes dehydration and dehydrogenation of the fundamental nucleus, which results in derivatives with conjugated double bonds and are therefore colored. For this reason, this characteristic is common in cardenolide and bufadienolide compounds.

The determination of flavonoid glycosides, based on the Cyanidin test, as it is characteristic of the largest number of substances in this class, with a positive result for the species: Vernonia ferruginea and Vernonia polyanthes, because according to the quantification of total flavonoids, the species Vernonia westiniana, did not present significant quantities in its constitution, corroborated the finding, according to the test result, of the occurrence of red coloration in the sample. The study with ferric chloride and sodium hydroxide showed negative results, and an inconclusive result in the case of the reaction with aluminum chloride, as no change in the color of the reaction was observed.

Tests to determine the presence of anthraquinone derivatives are usually orange in color. In the tests carried out using the Borntraeger reaction, which should show a reddish-pink color indicating the presence of these anthraquinone derivatives, no change in color was observed. Therefore, the result for the presence of anthraquinones was negative in all the species studied. In the microsublimation process, after heating on a plate under a metal ring, crystals should be present for positive results, so the result obtained was considered negative, confirming the result of the previous reaction, with no crystals being identified in any of the species.

Another test that offers reliable identification is the determination of saponin glycosides. The physicochemical method was used and, after uninterrupted shaking of the test tubes containing the diluted samples for 5 seconds, the formation of foam was observed in all the tubes, corroborating the indication of the presence of saponin compounds. Even after leaving the extracts to stand for 30 minutes, the formation of foam remained stable in two species, Vernonia ferruginea, Vernonia polyanthes, which were considered positive for the Rossol, Mitchell, Rosenthalen reaction and showed a slight reddish brown reaction. As for the Vernonia westiniana species, the test was negative as the foam formed did not remain stable after 30 minutes.

In the sulfo-vanillic and trichloroacetic acid reactions, the results were negative for the species, as they didn't show any specific coloration. In the Salkowski tests, the species Vernonia

ferrugínea and Vernonia polyanthes showed a triterpenoid nucleus and Liebermann-Burchard showed a steroid nucleus due to the coloration of the samples, which confirms the presence of saponin compounds in the extracts.

The presence of alkaloids in the samples also shows many of the therapeutic actions of the species studied, and the result obtained in the reaction with neutral lead acetate was positive, as it showed the formation of a white precipitate in the samples of: Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana, which indicated the presence of alkaloids, in the formation of insoluble complexes.

The tannins present in the Vernonia species were detected using the reaction with ferric chloride, which showed a characteristic green color for condensed tannins or catechuic for the samples of Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana, indicating the presence of hydrolysable tannins. The test with neutral lead acetate showed a whitish precipitate, confirming the presence of hydrolysable tannins in all three species, as did the reaction with lead acetate and glacial acetic acid, confirming the presence of hydrolysable tannins.

CONCLUSION

In this way, we can conclude that the tests carried out to identify the species Vernonia ferruginea, Vernonia polyanthes and Vernonia westiniana are favorable for the metabolite compounds found in the species, which corroborate quality assurance, contributing to greater safety for pharmacies that use powders to prepare formulations.

However, it is known that these tests are not totally conclusive and that there needs to be more accurate control, with isolation and identification of the compounds, using technologically modern methods with precise and safe results. Therefore, in order to make quality control more reliable, a phytochemical study of the species should be carried out.

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