ISSN 1984-5693
Vol.16, 2024,
http://dx.doi.org/dx.doi.org/10.22407/1984-5693.2024.v16.p.e.20241603

Artigo Científico

Vaccinium macrocarpon AITON (ERICACEAE): ANALYZES OF COMMERCIAL SAMPLES WITH A PHARMACOGNOSTICAL APPROACH

Vaccinium macrocarpon Aiton (Ericaceae): análises de amostras comerciais com uma abordagem farmacognóstica

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Submetido em: 06.04.2024; Aceito em: 21.05.2024; Publicado em: 23.05.2024.

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Abstract: The cranberry (*Vaccinium macrocarpon* Aiton - Ericaceae*)* is a known food product in Brazil and recently is being quite consumed because of its effects against bacteria in the urinary tract. Information on the anatomical description of this fruit and its chemical markers is rare, making studies regarding the authenticity of this species difficult. Samples of dried fruit were obtained in Campinas city (SP) and in Rio de Janeiro (RJ). Capsules of cranberry were obtained in Nova Iguaçu city (RJ). It also was performed histochemical analysis of dried fruits. Phytochemical tests were accomplished after preparation of hydroalcoholic extracts, to detect the condensed tannins, the chemical markers that are responsible by the therapeutic effect. Cranberry capsules were analyzed, the slides were observed and the results compared to the dried fruit. Histochemical tests of cranberry with the observed fruit provided preliminary and fast results about the presence of lipids in the cuticle, phenolic compounds in the pericarp and lignin in the xylem. Colorimetric assay with the hydroalcoholic extracts reacted positively to condensed tannins. After analysis comparisons with specialized bibliography were made. A morphological and micromorphological description was established, which can be used as a basis for analyzing various cranberry products and also rapid colorimetric tests that can assist in the preliminary detection of specific metabolites, requiring their association with instrumental methods for more accurate results.

Keywords: cranberry; *Vaccinium macrocarpon;* authenticity; microscopy; Pharmacognosy.

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Resumo: Cranberry (*Vaccinium macrocarpon* Aiton - Ericaceae) é um produto alimentício conhecido no Brasil e recentemente vem sendo bastante consumido, devido aos seus efeitos contra bactérias do trato urinário. Informações sobre a descrição anatômica deste fruto e seus marcadores químicos são raras, dificultando estudos quanto à autenticidade desta espécie. Amostras de frutos secos foram obtidas na cidade de Campinas (SP) e no Rio de Janeiro (RJ). Cápsulas de cranberry foram obtidas na cidade de Nova Iguaçu (RJ). Também foi realizada análise histoquímica dos frutos secos. Testes fitoquímicos foram realizados após a preparação dos extratos hidroalcoólicos, para detecção dos taninos condensados, marcadores químicos responsáveis pelo efeito terapêutico. Foram analisadas cápsulas de cranberry, observadas as lâminas e comparados os resultados com os frutos secos. Os testes histoquímicos do cranberry com o fruto seco forneceram resultados preliminares e rápidos sobre a presença de lipídios na cutícula, compostos fenólicos no pericarpo e lignina no xilema. O ensaio colorimétrico com os extratos hidroalcoólicos reagiu positivamente aos taninos condensados. Após análise foram feitas comparações com bibliografia especializada. Foi estabelecida uma descrição morfológica e micromorfológica, que pode ser utilizada como base para análise de diversos produtos de cranberry e também testes colorimétricos rápidos que podem auxiliar na detecção preliminar de metabólitos específicos, sendo necessária sua associação com métodos instrumentais para resultados mais precisos.

Palavras-chave: cranberry; *Vaccinium macrocarpon;* autenticidade; microscopia; Farmacognosia.

INTRODUCTION

Vaccinium macrocarpon Aiton (Ericaceae), usually called “cranberry”, is a species whose fruit has become known in Brazil, especially in cooking, where there are various commercial products, and it can be consumed fresh or dried (HALE *et al*., 1986). The action against urinary tract bacteria was demonstrated (TURNER, 2006) and assigned to condensed tannins named proanthocyanidins A, polymers of flavan-3-ol and flavan-3,4-diol, are the responsible metabolites for this therapeutic property (MENTZ & PETROVICK, 2003; NAVARRO *et al*., 2014).

In Ericaceae, the fruits can arise from a superior or inferior ovary, with 4 to 10 carpels, with a whole placenta or with two lobes and many ovules. Seed small, fusiform, oval or laterally compressed, straight endosperm and embryo. Some genera can be recognized by fruits characters, but in the indehiscent fruits, sometimes is difficult this recognition. The *Vaccinium* genera is characterized by the globose fruit, with 4mm diameter, hard cell walls, like stones, thickened and few seeds (BARROSO *et al*., 1999).

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However, when *V. macrocarpon* products suffer adulteration or counterfeiting, their properties are impaired and do not cause the therapeutic effect.

As cranberry is considered a relatively expensive fruit (HALE *et al*., 1986), and also its derivative products, and it is an imported ingredient not commonly found in Brazil, but in cold regions like North America, Europe, British Columbia, Central America and regions of the African continent, Asia and Oceania (KHANEJA *et al*., 2015), there is a great possibility that such adulteration in Brazilian products may occur.

Due to the lack of information on diagnostic characters, this study aimed to subsidize analysis of food microscopy and herbal products by the description of morphological and anatomical standards of commercial samples of *V. macrocarpon*, as well as to detect specific metabolites, like the condensed tannins, through colorimetric tests, in order to identify possible adulterants in cranberry products.

MATERIAL AND METHODS

Commercial samples of dried fruits completely developed were obtained in two different regions: in a market of Campinas (SP) and Rio de Janeiro city (RJ), Brazil. These samples were analyzed to establish distinct quality parameters. The cranberry capsules were acquired in Nova Iguaçu city (RJ), in a "natural products store", to compare its results to the obtained through the dried fruit and evaluate the usefulness of the characters described in the exams for authenticity of raw materials.

Histochemical tests and anatomical analysis

Histochemical tests were performed after freehand cuts through Ranvier microtome, dripping the specific reagents on the material and assembling slides (KRAUS & ARDUIN, 1997; FIGUEIREDO *et al*., 2007), to quickly detect the presence or absence of metabolites in cells and tissues. These reagents were Sudan III to detect lipids, lugol to detect starch, 3% ferric chloride in water to detect phenolic compounds, floroglucine to lignin and 3% aqueous solution of ferric chloride and 5% tannic acid to mucilages. As a comparison standard, data from the American Herbal Pharmacopoeia were used, in addition to other specialized bibliographies, in the search for additional information (NAVARRO *et al*., 2014).

In order to identify the morphological structures of *V. macrocarpon* through anatomical characterization, the method[12] was followed to perform paraffin inclusion in dried fruit. First, the fruit was dehydrated in increasing ethanolic series.

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The samples were immersed in hydro-alcoholic solutions (50%, 70%, 80%, 90% and 100%) for 1h each, next in ethanol:xylol solutions (ratios 3:1, 1:1, 1:3) for 24h each and in xylol 100%, for more 24h. Then, paraffin infiltration was performed at 100°C, slowly, every 24h, changing xylol for paraffin until total substitution. After paraffin inclusion, the fruits were sectioned on rotary microtome YADI YD – 315, and transversal sections were obtained with 16-20 μm thickness.

To dewaxing, samples were immersed in xylol 100% for 20 minutes, followed by xylol:ethanolic decreasing series (ratio 3:1, 1:1, 1:3 for 10 minutes each), and in ethanolic:distilled water decreasing solution series (ratio 100%, 90%, 80%, 70%, 50% for 10 minutes each) and finally distilled water for 5 minutes; then, semi-permanent and permanent slides were assembled and the transversal sections were colored with toluidine blue 0,05%.

After, glicerin 50% in deionized water (to semi-permanent slides) was used as mounting medium (JOHANSEN, 1940; KRAUS & ARDUIN, 1997). Next, slides were observed under Alltion binnocular photomicroscope, where the photomicrographs were obtained. Analysis of some samples were carried out using the Phenon Pro-X Scanning Electron Microscope (SEM), located at the IFRJ microscopy laboratory, in Nilópolis *campus*, to investigate the innermost structures of the fruit. The dried fruit samples were prepared by immersing them in hydroalcoholic solutions (70%, 80%, 90%) for 10 minutes each, and immersed in 100% alcohol for 1 hour. Then, the samples were immersed in solutions of ethyl alcohol: hexamethyldisilazane (HMDS) (ratios 3: 1, 1: 1, 1: 3) for 10 minutes and then in 100% HMDS for 20 minutes, replacing the critical point by drying. Then, the sample was submitted to the SEM freezing module (-25°C for 40min), replacing the metallization (HARZIN-CHONG & MANEFIELD, 2012). After evaporation of the solvent, the fruits were observed through the Phenon Pro-X SEM, with beam power of 10 and 15kV.

For analysis in the Inspect S50 SEM (FEI Company Brand, Hillsobro, OR, USA), located in the National Institute of Technology (INT) in Rio de Janeiro, Brazil, the samples were dehydrated in an acetonic series (30%, 50%, 60%, 70%, 80% and 90% for 20 minutes each and next 100% for 24 hours). Evaporation of acetone was performed by Leica EM – CPD 030 Critical Point equipment and then, samples were coated with gold by Emitech K550X metalizer . After these procedures, the fruits were analyzed through SEM Model Inspect S50 (FEI Company Brand, Hillsobro, OR, USA), at 15kV (SOUZA, 1989; NASCIMENTO *et al*., 2016).

Phytochemical tests

The colorimetric assay was performed to detect specific secondary metabolites responsible for the therapeutic effect. The procedure used is as described by MATOS (1997), after obtaining hydro-alcoholic extracts (100g of each dried fruit sample and 100mL of alcohol). The specific solution used was ferric chloride 3%, to detect condensed tannins.

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Commercial product analysis

After these procedures to establish a standard from dried fruit, the same methods were performed to the cranberry commercial product. First, the internal content of 14 cranberry capsules, used as food supplement was weighed and diluted in a 50% glycerin solution in water. Next, semi-permanent slides were mounted using glycerin 50% in distilled water after colored with toluidine blue 0,05% were also analyzed under Alltion photo microscope; and colorimetric assay with ferric chloride 3% was performed in test tube, to assess their authenticity, comparing the results with the obtained with the dried fruit.

RESULTS AND DISCUSSION

Histochemical tests and anatomical description

Histochemical tests provided rapid detection of metabolites present in the pericarp. The results are shown in the Table 1.

By the anatomical analysis of the dried fruit, the following characters were detected: the fruit is a tetracarpelar berry, with more than one seed per locule and axial placentation (Figure 1A). There is a cuticle in its outermost part, whose thickness measures between 1.3 and 2 μm (Figure 1B – C), that react positively to Sudan III test. The exocarp is thin, red in color, which is subdivided into two or more layers: the first, more external, is made up of epidermal tabular cells, well pigmented, with straight walls and content evidenced by droplets dispersed in the cytoplasms (Figure 1C - E). After, there are a second hypodermical layer with different shape and size than the first layer. In general, these cells are larger in length and width. They still have an elongated shape, but tend towards an isodiametric shape. Afterwards, until to three additional layers can occur, with the same characteristics as the second. However, these cell layers are often discontinuous. There is a more compact arrangement than all the other cells that form the mesocarp. These cells can be strongly pigmented too, as in the more superficial extract. Next, a succulent mesocarp is composed of globose cells with thin walls (Figure 1F) and internal content that stores phenolic substances that react positively to the ferric chloride test, which also occurs in the exocarp. The endocarp has a unique layer of elongated epidermal cells of predominantly fusiform aspect, with straight walls (Figure 1G); in these regions, some rare stomata can be found (Figure 1H), these stomata, can be observed only in in frontal view.

Collateral vascular bundles can occur throughout the mesocarp, branching toward the exocarp and the endocarp, where the vessels elements and fibers react positively to the floroglucine test that detect lignin in the cell walls of these cells. The small seeds (about 2mm length), present in variable number (Figure 1A), don´t form lumps and have and ornate forehead, whose wall are highlighted by their high relief, with a shape that resembles that of the brachysclereids (Figure 1I).

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Presented histochemical results were in accord to the literature (KRAUS & ARDUIN, 1997; FIGUEIREDO *et al*., 2007), specially about the brown color generated by the reaction with 3% ferric chloride, that evidences the presence of phenolic compounds in the cells of *V. macrocarpon* (BROWN *et al*, 2012; NAVARRO *et al.,* 2014; OSZMINIAŃSKI *et al*., 2017; WANG *et al.*, 2017), which can represent condensed tannins.

Colorimetric test with the hidroalcoholic extract showed a positive result for condensed tannins. Condensed tannins generate a green precipitate and hydrolyzed tannins generate a blue precipitate (MATOS, 1997).

The chemical reaction of the ferric chloride with the extract occurs between the hydroxyls in the ortho position in B ring and Fe^{3+} (FIGUEIREDO *et al*., 2007; MARCHINI, 2015). This result corroborates the possible presence of proanthocyanidins, the chemical markers of this species. However, more precise isolation and identification techniques will ensure a safer result. In this paper, the obtained results corroborated with Kaneja *et al.* (2015), also observed some thin layers of cells in the epicarp and globose cells in the mesocarp, as well as Upton *et al*. (2011), that described reddish cells in the epicarp, and the vascular bundles in the mesocarp. The seeds are described as with elongated thick cells that do not form lumps. Is rare, in the literature, the description about stomata in endocarps was, but Bergman in 1920 and after, Turner (2006) also observed this structure. Although all the characters described are compatible with the characteristics of Ericaceae species, dried fruits of other species, like *Vitis vinifera*, *Morus* spp., *Hibiscus sabdariffa* can be easily mixed in the raw material because they have similar characteristics of the fruits. Therefore, stomata appear to be characteristics in pericarps of Ericaceae species (BERGMAN, 1920). Thus, the stomata in the endocarp are the best diagnostic characters in the cranberries fruits.

Table 1. Histochemical assays for preliminary detection of typical *Vaccinium macrocarpon* metabolites.

Reagents	Expected metabolites	Presence or absence in the pericarp cells	Generated color
Sudan III	Lipids	Presence in the cuticle and in the walls of exocarp cells	Orange
Lugol	Starch	Absence	-
3% Ferric chloride	Phenolic compounds	Presence in the pericarp	Brown
Floroglucine	Lignin	Presence in xylem (including fibers)	Red
3% Ferric chloride + 5% tannic acid	Mucilage	Absence	-

(-) Nonspecific staining

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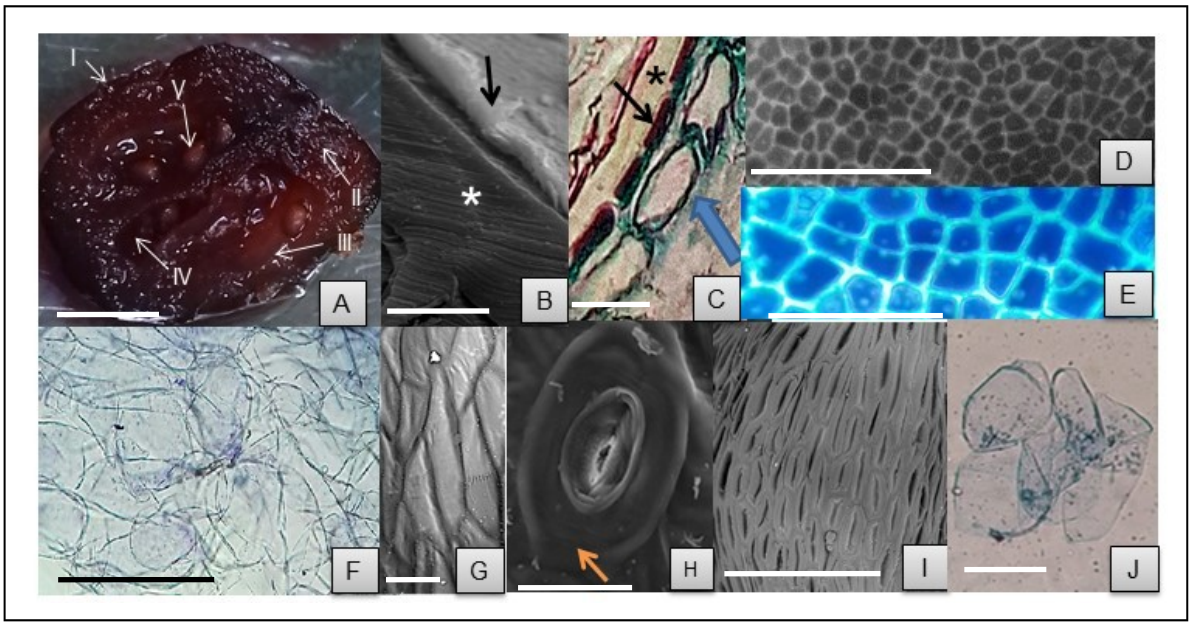

Figure 1. A: External morphology of *Vaccinium macrocarpon* fruit, showing the regions that make up the pericarp. I - Exocarp; II - Mesocarp; III - Endocarp; IV - One of the locules where the pericarp was found. V - Seeds (average fruit dimensions: length = 40.48 mm, width = 5.5 mm). (Bar: 2 cm). B-J: Histological and anatomical aspects. B: cuticle of the fruit in detail (arrow) on the outer periclinal wall (*) (Bar: 5µm). C: Transversal section of the exocarp region (I), showing the thick cuticle (*), the outer (black arrow) and the inner (blue arrow) layer of the exocarp (Bar: 20µm). D-E: Outer layer of the exocarp (I), in frontal view (Increases: 40x and 100x, respectively). Phase contrast in E. (Bars: 200 µm). F: Globose parenchymatic cells that forms the mesocarp (II) in detail (Bar: 200 µm). G: Paradermic view in SEM of cells that form the endocarp (III and IV), in detail. (Bars: 50µm). H: Stomata in detail, seen through the SEM, in the endocarp (Bar: 25 µm). I:Sclereified cells that form the forehead of the seeds (V) (Bar: 300 µm). J – Group of globose parenchimatic cells from the mesocarp, in detail, found in food supplement capsules (Bar: 20µm).

Phytochemical analysis and commercial product analysis

The hydroalcoholic extract reacted positively to the presence of condensed tannins in the colorimetric assay, presenting a green precipitate that characterizes it (MATOS, 1997).

The previous results obtained in this study intended to establish different quality parameters so that they could be compared to commercial products made of cranberry. The cranberry gelatin capsules were weighed, and their pink internal content powder were 6,71g.

ISSN 1984-5693
Vol.16, 2024,
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The microscopic analysis of the powder showed a similar result to the dried fruit. Globose parenchimatic cells were found in the capsules, similar to those observed in the fruit mesocarp (Figure 1J). The colorimetric assay performed with the powder capsules showed a positive result for condensed tannins, evidenced by the formation of the green precipitate (MATOS *loc. cit.*).

The purpose of the analysis of the commercial samples was to compare the results with the information obtained with the dried fruit. After diluting the capsules contend powder in glycerin solution and observe slides under the microscope, it was found the same mesocarp cells aspect of *V. macrocarpon*. However, confirm its authenticity, it was necessary to perform colorimetric assays to verify the presence of condensed tannins, because other species of berries have similar mesocarps. Nevertheless, the chemical analysis can present better and more accurate results, if associated to instrumental assays like High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR).

CONCLUSIONS

The obtained results allowed us to conclude that:

V. macrocarpon presented anatomical characteristics that can to auxiliate in its diagnosis, like the layers that forms the exocarp and the presence of stomata in the endocarp, allowing the detection of possible adulterations in its products. The obtained results suggest that all the samples were authentic.

- The histochemical tests allowed to detect where the different metabolites groups were localized in the pericarp.
- The colorimetric assays were quick to detect the condensed tannins. The combination of these tests with microscopic analysis can provide important additional information in recognizing the species. However, only the colorimetrical test is insufficient to confirm the presence of chemical markers, like the proanthocyanidin A. Chemical analysis can present better and more accurate results if associated with instrumental tests such as High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR).

ACKNOWLEDGMENTS

The authors are grateful to IFRJ for the financial support and infrastructure; to D.Sc. Maurício Magalhães de Paiva, M.Sc. Alan Menezes do Nascimento and Centro de Caracterização em Nanotecnologia para Materiais e Catálise (CENANO) from the National Institute of Tecnology (INT) for the partnership in the SEM analysis.

ISSN 1984-5693
Vol.16, 2024,
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