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Evaluation of annatto (*Bixa orellana* L.) methanolic extract in association with methylene blue for double staining in vegetable histology

Avaliação do extrato metanólico de urucum (*Bixa orellana* L.) em associação com azul de metileno para dupla coloração em histologia vegetal

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The annatto is a yellow-orange dye extracted from the pulp of *Bixa orellana* L. seeds, a tree native of tropical forests. The composition of this dye consists basically of carotenoids, such as bixin, norbixin and nobixate. Bixin, a highest amount compound, has a liposoluble characteristic that facilitates its interaction with cell membranes. The present study evaluated the dye association viability in vegetable histology of *Bixa orellana* L., *Morinda citrifolia* L. and *Petiveria alliacea* L. leaves. The annatto dye was obtained by extraction in methanol (1:6 w/v) by maceration of *Bixa orellana* seeds. The analyzes were carried out in cross sections of the vegetal samples collected in the "Berta Lange Morretes" Medical Garden of Federal University of Maranhão (UFMA). After clarification with sodium hypochlorite, the staining was performed in an average immersion time of 10 seconds, for both the urucum + methylene blue association in the 2:1 (A1) and 1:1 (A2) concentrations as well as for the blue methylene 1% and annatto dye 2%. All sections were mounted between blade and coverslip with distilled water, and visualized under an optical microscope. The A1 allowed a better morphological visualization of the anatomical structures due to the acidic characteristic of bixin which reacts with basic pH components of plant, and the alkaline characteristic of methylene blue that reacts with structures of acidic nature. This association represents a staining technique that can contribute alternatively as plant histological dyes.

Keywords: Plant anatomy, Annatto, *Bixa orellana* L.

O urucum é um corante amarelo-alaranjado extraído da polpa das sementes de *Bixa orellana* L., uma árvore nativa das florestas tropicais. A composição deste corante consiste basicamente em carotenóides, como bixina, norbixina e nobixato. A bixina, composto de maior quantidade, tem característica lipossolúvel que facilita sua interação com as membranas celulares. O presente trabalho avaliou a viabilidade da associação de corantes em histologia vegetal nas folhas de *Bixa orellana* L., *Morinda citrifolia* L. e *Petiveria alliacea* L. O corante de urucum foi obtido por extração em metanol (1:6 p/v) através da maceração de sementes de *Bixa orellana* L. As análises foram feitas em secções transversais das amostras vegetais coletadas no Horto Medicinal "Berta Lange Morretes" da Universidade Federal do Maranhão (UFMA). Após a clarificação com hipoclorito de sódio, a coloração foi realizada em um tempo médio de imersão de 10 segundos, tanto para a associação urucum + azul de metileno nas concentrações 2:1 (A1) e 1:1 (A2) como para o azul de metileno 1% e corante de urucum 2%. Todos os cortes foram montados entre lâmina e lamínula com água destilada e visualizados em microscópio óptico. A A1 possibilitou melhor visualização morfológica das estruturas anatômicas devido à característica ácida da bixina, que reage com componentes de pH básico do vegetal, e a característica alcalina do azul de metileno que reage com estruturas de natureza ácida. Essa associação representa uma técnica de coloração que pode contribuir de forma alternativa como corantes histológicos vegetais.

Palavras-chave: Anatomia vegetal, Urucum, *Bixa orellana* L.

1. INTRODUCTION

The *Bixa orellana L.* is a woody plant of the family Bixaceae that is original of Tropical America and native to the Amazon and Atlantic Forest. In Brazil it is known as "urucum", "urucu", "açafroa", among others. The fruits or "cachopas" are capsules of two parts, which contains of 30 to 45 seeds, being able to be ovoid, cordiformis, spherical and in some cases flattened. The "cachopas" can reach up to 5 cm in width, being glabrous or hairy and its coloration varies from green to intense red [1, 2].

The Brazil leads the world production of annatto seeds with a harvest estimate in 2016 of just over 10.000 tons, representing 39% of world production followed by Peru with 22%, Ivory Coast and Ghana with 18% and Kenya with 7% [3]. The world importance of annatto is given by possibility of extracting a dye from pericarp of the plant seeds, which in turn receives the international name of annatto [4]. The annatto presents as a mixture of yellow-orange pigments because it consists of carotenoids, such as bixin, norbixin and nobixate [5], and is widely used by food, pharmaceutical and textile industries [6].

The main pigment of annatto is the bixin, corresponding to more than 80% of carotenoids found in these seeds. This compound corresponds on average to 2.5% of the dehydrated seeds weight and it is an apocarotenoid originated by carotenes cleavage [7]. Removal of methyl ester group from bixin gives norbixin, a dicarboxylic acid [8]. A structural difference confer to bixin a liposoluble properties due to methyl ester group presence in the molecule, while norbixin presents greater water solubility due to carboxyl group presence, which is the site of interactions with water molecules [9].

The extraction methods of *Bixa orellana* L. pigments, for chemical composition determination or investigation of activities in living organisms, can be carried out with solvents such as propylene glycol, oil, pure water and alkaline solutions. Depending on the solvent used, the extraction can be more or less selective in according to conveniences of each job [5].

The leaf blade of different plant species consists of tissues with their own characteristic functions, which influence the affinity with various dyes used. As an example, parenchyma cells usually have thin walls composed of cellulose, hemicellulose and pectic substances. The cell wall of the collenchyma has cellulose, a large amount of pectic substances and water. The sclerenchyma has a secondary cell wall thickened, lignified or non-lignified. The fibers are long cells with thick secondary cell walls, usually lignified [10]. The walls of the epidermal cells may contain lignin and more often cutin [11].

Thus, some synthetic dyes have high affinity for certain substances present in plant structures, such as zinc chloride that selectively dye the cellulose in blue. The lignin is specifically stained with hydrochloric acid (saturated phloroglucinol solution in hydrochloric acid 20%) in reddish-purple and with iodinated zinc chloride in yellow [12].

In plant anatomy works, the coloring methods used are not accompanied by the corresponding bibliographical references, making it impossible to obtain the necessary information for accomplishment of these as well as for obtained results interpretation. In addition, various methods described for distinguishing the tissues do not correctly indicate the type and concentration of dye used, as well as preparation of the solutions involved [13].

Considering that staining is a crucial process in microscopic analysis of plant species and in a laboratory routine, alternative techniques of staining are needed. Therefore, it becomes necessary to study and search for new dyes with quality comparable to those usually employed. Another important factor in obtaining new colorants is their low cost of production, their good yield and their low toxicity.

Annatto's dye has some characteristics that justify its wide use, among which are its coloring power, facility of extraction and its wide availability in nature [8], even though toxicological data on its use are limited [4]. Its use in plant histological sections has not yet been explored as well as its association with other dyes.

In the search for new options for plant histological staining, the present work aimed to associate methylene blue with annatto dye, in order to contribute to studies that search the production of an effective and low-cost vegetable histological dye.

2. MATERIALS AND METHODS

The aerial parts of *Bixa orellana* L., *Morinda citrifolia* L. and *Petiveria alliacea* L. were collected in the "Berta Lange Morretes" Medical Garden of Federal University of Maranhão (UFMA) in March, 2013. Specie of the samples was catalogued at Atticus Seabra Herbarium of Federal University of Maranhão (UFMA) under number 1815, 3212 and 1162, respectively.

The Annatto dye was obtained by extracting 50 g of fresh and whole seeds in 300 ml of methanol (1:6 weight/volume) by maceration for two days. Then, the obtained extract was concentrated in a rotary evaporator obtaining a final volume of 200 mL and a concentration of 2% of pigments in relation to seeds total weight, calculated from the dry weight.

After obtaining annatto dye (COR-U), it was prepared the associations of this with blue methylene 1% synthetic dye (REAGEN®). A pilot test was performed using only annatto dye, and two associations were made: A1 (annatto dye + methylene blue 1% in a ratio of 2:1 v/v) and A2 (worked with the same and respective components, but in a ratio of 1:1 v/v). The cuts colorations obtained from associations of dyes were compared with cuts stained only with methylene blue 1% (COR-AZ).

After cleaning botanical material, samples of *Bixa orellana* L., *Morinda citrifolia* L. and *Petiveria alliacea* L. leaves were cross-sectioned by free hand using a cutting blade, followed by sodium hypochlorite clarification and washing in distilled water. Subsequently, the staining procedure was started by classifying each sample into three subgroups, depending on dye used type. In the first group, the cuts were immersed for 10 seconds in A1, with later removal of excess with distilled water. Already in the second and third groups, the same process was performed, but A2 or COR-AZ was used respectively as a coloring alternative.

All cuts were mounted between blade and coverslip with distilled water. The sections were visualized and the observations were recorded using optical microscopy (OLYMPUS®) with photographic camera (SAMSUNG®) coupled. The results were compared with standard staining technique used in plant histology according to methodology proposed by Oliveira et al. (2008) [12], checking the color quality obtained with the naked eye. The analysis was based on contrast quality obtained with A1 and A2 compared to usual color COR-AZ and COR-U alone.

3. RESULTS AND DISCUSSION

With the coloration obtained only with annatto dye (Figure 1), it was no possible to obtain a different contrast between histological tissues of the plant. The coloration obtained with use of A1 (Figure 2) was satisfactory, because using a higher ratio of annatto dye it was possible to obtain a better contrast. It is possible that bixin, because it is the compound in greater abundance in dye, is responsible for contrast quality of this coloration. The A2 use (Figure 3) did not provide a quality coloration, since there was a limited dye penetration into cuts probably due to bixin being present in low ratio.

Comparing A2 with COR-AZ (Figures 2 and 4) it is possible to detect much more expressive contrasts with A2. The structures identification, such as annular and angular collenchyma, is much easier, since the reaction of COR-U dye with the thick wall of tissue is more evident. In addition, tonality and contrast are more present in relation to conventional technique.

The magnitude obtained is due to acidic characteristic of bixin [14], which reacts with cytoplasmic basic components present in the tissues, such as cortical parenchyma, collenchyma and phloem, which have a cell wall and organic substances that are favorable to reaction. Methylene blue (basic dye) is responsible for acidic elements staining, such as xylem, epidermis and fibers, since they have a secondary wall with lignin that due to presence of aldehydic and phenolic groups has acid reaction [12].

It was still possible to obtain very diverse colors among the vegetal components. The cortical parenchyma presented a brown coloration, and collenchyma and phloem presented a greenish coloration (Figure 2). This proves that, in addition to cell walls composition, the tissues have different substances in their composition, such as starch, phenolic substances and crystals, which allows different reaction types [15].

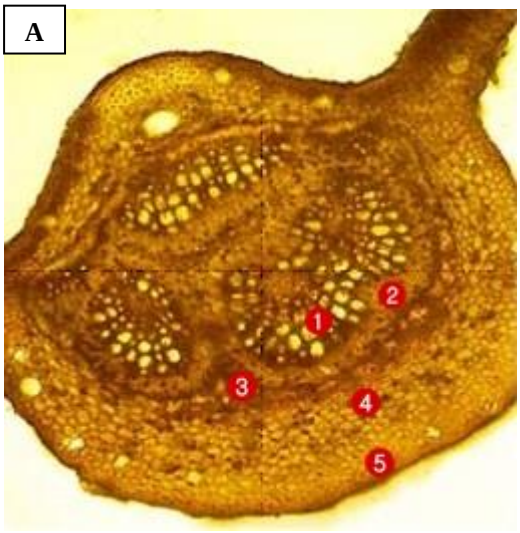

Figure 1: Leaf cross sections of Bixa orellana L. stained with annatto dye.
Legend: 1 = xylem. 2 = phloem. 3 = sclerenchyma fibers. 4 = cortical parenchyma. 5 = collenchyma.

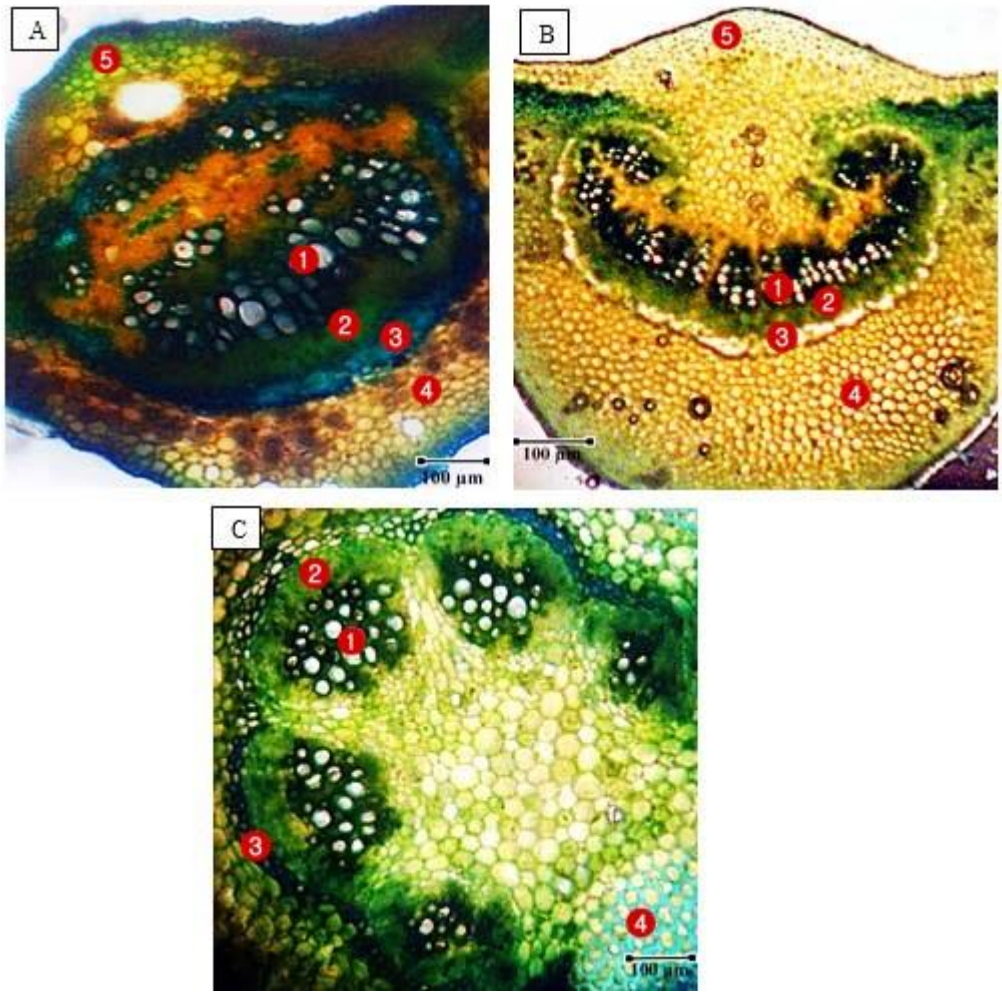

Figure 2: Leaf cross sections stained with annatto dye + methylene blue 1% in the ratio of 2:1 (v/v). (A) Bixa orellana L. (B) Morinda citrifolia L. e (C) Petiveria alliacea L. Legend: 1 = xylem. 2 = phloem. 3 = sclerenchyma fibers. 4 = cortical parenchyma. 5 = collenchyma.

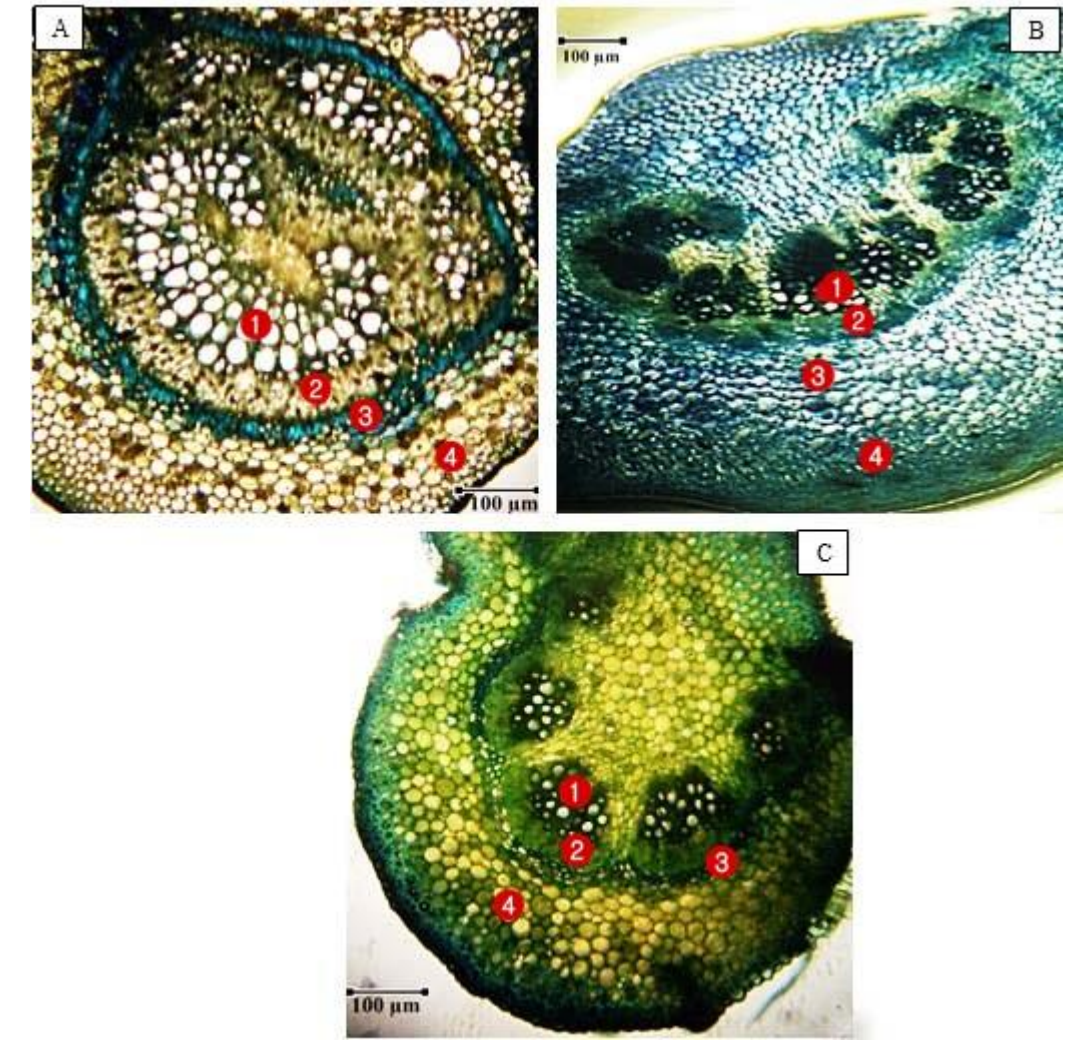

Figure 3: Leaf cross sections stained with annatto dye + methylene blue 1% in ratio 1:1 (v/v). (A) Bixa orellana L. (B) Morinda citrifolia L. e (C) Petiveria alliacea L. Legend: 1 = xylem. 2 = phloem. 3 = sclerenchyma fibers. 4 = cortical parenchyma.

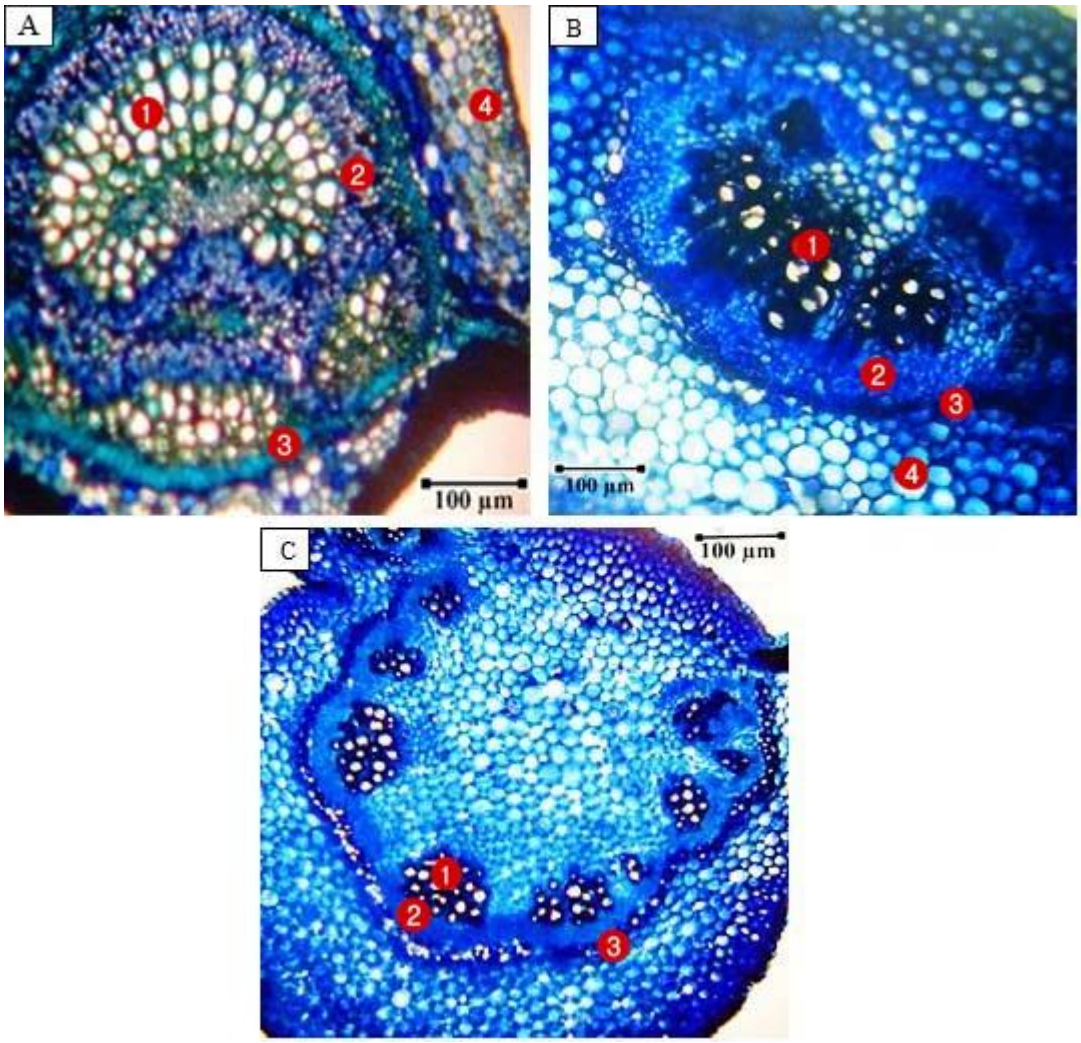

Figure 4: Leaf cross sections stained with methylene blue 1%. (A) Bixa orellana L. (B) Morinda citrifolia L. e (C) Petiveria alliacea L. Legend: 1 = xylem. 2 = phloem. 3 = sclerenchyma fibers. 4 = cortical parenchyma.

4. CONCLUSION

The associations of annatto extract and methylene blue 1% (2:1; v/v) represents an innovative and effective technique for the histological staining of vegetable cuts. In addition, it is an alternative method and of production low cost. It was possible to observe larger contrast between the plant morphological structures facilitating the vegetal characterization, unlike the isolated use of these dyes.

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