Original Article

Toward developing natural histologic stains using anthocyanins: A novel approach

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Abstract

Introduction: In the present-day scenario, wherein histotechnological laboratory personnel come into contact with numerous hazardous chemicals every day, laboratories are emphasizing on development of safer and environment-friendly alternatives globally which are easily available and feasible. In this context, we have attempted to utilize anthocyanins, a family of pigments naturally occurring in fruits and flowers and which are already used as natural food colorants, for assessing their utility as histological stains.

Materials and methods: Juices of thin consistency from well-ripened pomegranates were obtained by using a juicer with blender functions. Formalin-fixed paraffin-embedded 4 μm thin sections were immersed in this solution for 2 h, during which the solution was periodically monitored. Several modifications were attempted such as the addition of *Citrus limon* (lemon) extract or acetic acid, change in orientation of the slides and refrigeration of the solution during the staining procedure.

Results: A peculiar pattern of reasonably diagnostic staining was observed in which the basal and suprabasal cells, basement membrane, inflammatory cells and collagen fibers stained prominently with a magenta color. The addition of lemon, horizontal orientation of slides and refrigeration each separately led to an improvement in staining characteristics. The addition of 4% acetic acid and refrigeration of the solution each led to an improvement in longevity of the staining solution.

Conclusion: Anthocyanins could potentially be used as viable histological stains having advantages of availability, feasibility, color stability and nontoxicity, although numerous modifications to improve longevity of staining solution and staining characteristics are warranted by further research for which the present study could serve as a pretext.

Keywords: Anthocyanins, coloring agents, flavonols, histologic technique, pomegranate, tissue stains

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INTRODUCTION

Synthetic dyes, although more efficient, have been reported to be hazardous to human health, thereby resulting in the cutback in their usage.[1] Furthermore, the scientific community has experienced repeated shortages or changes

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in the quality of standard stains such as hematoxylin.[2] This disruption in supply becomes even more pronounced in times such as pandemics wherein much of the economy is focussed on procuring essential equipment for medical personnel. Thus, the emphasis has been laid in laboratories worldwide to develop and utilize nontoxic chemicals that are friendly to the environment, with a similar need arising in histological staining procedures.[3]

One such naturally occurring pigments are anthocyanins that belong to the same group of chemical dyes as hematein which is the principle chromophore in hematoxylin.[2] Anthocyanins comprise a multitudinal group of more than 600 water-soluble phenolic pigments that are responsible for blue, purple and red color of various fruits, flowers and vegetables (e.g.,: beetroot, pomegranate, grapes, roses and red cabbage).[4-6] These pigments are generally tasteless and odorless with substantial color stability[6] and have thus been widely promoted as safe-to-consume coloring agents in foods and beverages.[5,7] Furthermore, various researchers have successfully demonstrated that anthocyanins obtained from *Hibiscus sabdariffa* (Hibiscus), *R. indica* (Rose) and *Bougainvillea glabra* (*Bougainvillea*) and beetroot[8-10] can be effectively used for histological staining.

In this context, aiming to develop natural, eco-friendly histological stains, we attempted to utilize *Punica granatum L.* (pomegranates) as a source of anthocyanin pigments to test the hypothesis of whether these pigments could be used for staining of histopathological sections. The selection was carried out based on the fact that pomegranates are nutrient-dense fruits rich in anthocyanins and flavonols which have properties of a natural dye.[11] The fruits are easily available globally and thrive even in various drastic environmental conditions such as harsh winters, droughts and desert areas.[12] In addition, the refractive index (RI) of *P. granatum* extract as determined by biochemical analysis was found to be 1.58,[13] which is close to that of dried proteins having an RI of 1.53 and also to RI of commonly used mounting media.[14] These factors would facilitate achieving the objective requirements of a quintessential histological stain including ease of availability, economic feasibility, reasonable color stability, nontoxicity, sustainability and compatibility with tissues and mounting media.[15]

MATERIALS AND METHODS

Overall, the methodology utilized can be structured as follows:

i. Procurement of fruits and obtaining their extract
ii. Obtaining histological sections followed by their deparaffinization and rehydration through graded alcohols to water
iii. Addition of 10 ml *Citrus limon* (lemon) extract or 4% acetic acid (if applicable, for respective modifications)
iv. Measuring pH of the anthocyanin solution
v. Staining with anthocyanin solution for 2 h
vi. Dehydration, clearing and mounting with Dibutylphthalate Polystyrene Xylene (DPX)
vii. Examination under a light microscope and obtaining digital photographs
viii. The methodology has been discussed in detail in the subsequent test.

Selection and procurement of fruits

Four well-ripened pomegranates were acquired from local vendors in the city of Mumbai for each session of staining. Care was taken to ensure that the pomegranates were adequately red in color, fresh and were not damaged physically which could possibly reduce the quantity of pigments obtained from the fruit leading to a reduction in the quality of subsequent staining.

Extraction of juice

Refrigerated fresh fruits were sufficiently washed and cut into halves, following which the edible portion of the fruit containing juice and seeds was separated from the peel manually [Figure 1a].[13] This portion was then blended in a food processor with blender functions (Morphy Richards Essentials 100FP, India). An added advantage to using the device was that all the impurities and seeds were automatically separated and only a relatively noncontaminated juice of thin consistency was obtained. In case of presence of impurities or a thicker consistency of juice, as observed with processors that are unable to produce such a solution, a suitable size of Whatman filter paper of Grade no. 1 could be used to obtain a solution of the desired consistency.[16]

Four pomegranates provided around 200 ml of pure watery extract that was dark red in color due to rich content of anthocyanins present in the fruit [Figure 1b]. The fresh extract obtained was transferred to an airtight container and stored in the refrigerator until the time of staining procedure, at the time of which it was transferred to two clean glass Coplin jars of 100 ml capacity each.

Staining procedure

Formalin-fixed paraffin-embedded blocks of oral tissues obtained from patients that had undergone crown lengthening procedure were selected for the purpose of this study and procured from the archives of the Institutional Department. The particular selection was done to ensure

Figure 1: Illustrates certain steps before beginning the staining procedure. (a) Edible part of the pomegranates utilized to prepare the staining solution. (b) Staining solution in Coplin jar. Note the red color of anthocyanin solution due to acidic pH. (c) Measurement of pH by a digital pH meter prior to commencement of staining procedure. (d) Staining solution filled in a container into which slides could be oriented horizontally during the staining procedure

that none of the valuable pathological tissue was misspent as the histopathologic staining potential of the pigments was yet unconfirmed. Sections of 4 μm thickness were taken on glass slides from the selected specimen blocks by means of a semi-automated microtome (Leica Biosystems, Germany). It was ensured that minimal time was expended between obtaining the extract and staining procedure. This would ensure that the solution utilized was maintained as fresh as possible at the time of staining procedure.

The sections were deparaffinized in xylene and rehydrated to water in increasing grades of alcohol (50%, 75%, absolute alcohol) for 2 min in each grade. At this point, pH of the anthocyanin solution was measured by means of a digital pH meter [Figure 1c] immediately following which the slides were introduced into the Coplin Jar filled with 100 ml of anthocyanin solution. The timing and pH were promptly noted down at the three points of time-at beginning the procedure, 1 h after commencement and finally before removing the slides from the jar at the end of 2 h. The procedure was standardized based on previous studies pertaining to the use of anthocyanin solutions obtained from various flowers that were used for histological staining.[9,10] However, the timing suggested by previous researchers was found to produce inadequate results, and thus, overall duration of 2 h was selected for the purpose of staining by authors in the present research that provided optimum results. Prolonged immersion in anthocyanin solution was also found to be detrimental to the staining quality which has been discussed later.

After removing the slides from the staining solution, they were dehydrated in increasing grades of alcohol (50%, 75%, absolute alcohol), cleared in xylene and finally mounted with a resinous mounting media-DPX (Merck specialties private LTD, Mumbai). The stained sections were observed using a biological trinocular research microscope (BA210 LED Trino"Motic," Wetzlar, Germany) in a bright field under ×4, ×10 and ×40 magnifications of an objective lens. Photographs of the stained specimens were obtained by means of 1.3 MP Digital Microscope Camera (Moticam 1SP) and compatible software (Motic Images Plus v2.0, Motic China Group Co., Ltd., Xiamen, China). Since the objective of research was primarily to visualize the staining pattern produced by anthocyanin extracts obtained from *P. granatum*, the staining was performed without any counterstain and not compared to any other standard stain. Only the results obtained following staining solely with anthocyanin extracts obtained from *P. granatum* have been illustrated and discussed by the authors.

Attempted modifications

A number of factors that could influence the staining of histological sections were modified and observed for changes in nature of staining. These modifications included (i) addition of 10 ml extract from *Citrus limon* to the staining solution, (ii) addition of 10 ml of 4% acetic acid to the staining solution, (iii) altering the temperature for the duration of the procedure wherein the staining solution at 4°C in a refrigerator for the entire duration of staining

procedure and (iv) orienting the slides in a horizontal container filled with staining solution [Figure 1d] instead of vertical orientation in a Coplin jar. The abovementioned modifications were tested singly without combining any of the two to ensure that only the changes in nature of staining most probably related to that particular factor could be identified.

RESULTS

Characteristics of staining

A generalized pink to reddish staining of the histological section was observed which was reasonably diagnostic, as illustrated in Figure 2. The basement membrane, nucleoli and bundles of collagen fibers comparatively stained more intensely. The basement membrane was most prominently stained exhibiting a magenta pink color with a slight reduction in intensity, but similar staining was observed in the cytoplasm of cells of the basal layer of stratified squamous epithelium. Intensity was further reduced in the suprabasal layers and was faintly light pink in the cells of layers of the stratum spinosum further away from the basal lamina. Extremely mild intensity of staining was noted in upper spinous layers. Staining gradually reduced in intensity toward the surface layers of the stratified squamous epithelium; however, the surface keratin layer showed a distinct pattern of light brown staining. However, one astonishing finding was that nucleoli of the cells stained darker and could be observed prominently through the epithelial layer. In fact, nuclear staining was more discernible toward the superficial layers because of a lack of cytoplasmic staining. This is in accordance with results from previous studies that concluded that anthocyanin extracts can be utilized as viable substitutes for hematoxylin for nuclear staining.[9,10]

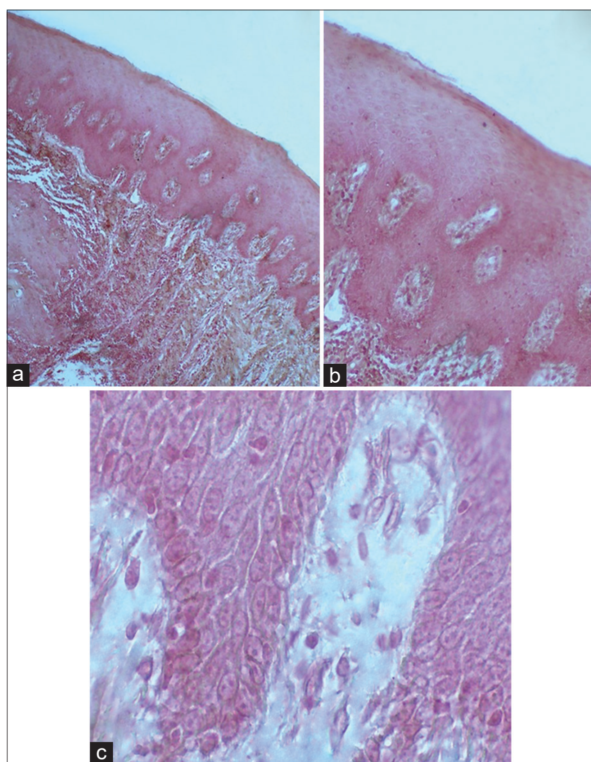

Figure 2: Illustrates staining achieved by anthocyanin solution under. (a) Scanner view (×4). (b)×10 magnification. c) 40x magnification

Overall, the connective tissue stroma [Figure 3] appeared as a number of prominently stained elements on a background of faint light brown ground substance. The nuclei of inflammatory cells exhibited a prominently stained magenta/dark pink color. Similar intensity and color of staining were observed in collagen fiber bundles as well. The endothelial and reticular lining of blood vessels stained relatively darker brown with the intravasated vascular elements exhibiting an extremely faint light brown color.

Observations in unaltered staining solution

The final pH of pure staining solution was invariably 3. 6 at the time of commencement of the procedure and did not undergo any measurable alteration when recorded during and at the end of the staining procedure. This pH was within the recommended optimum range for anthocyanin solutions, i.e., 2.5–4.[10] Staining intensity improved with an increase in the amount of time the slides were kept immersed in the staining solution drastically during the first 2 h, following which there was a very slight or no improvement. Thus, this was determined as the most efficient duration for obtaining an acceptable intensity of staining. Increasing the duration did not show adequate improvement in results when the investment of time factor was considered, rather, reduction in staining intensity could be observed in sections that were kept in solution for prolonged periods of time. Deposition of an aggregate or precipitate was observed at the bottom of the Coplin jar after the staining solution was stored in it for more than 4–5 h [Figure 4].

This could possibly include deposition of anthocyanin pigments present within the solution due to gravity explaining

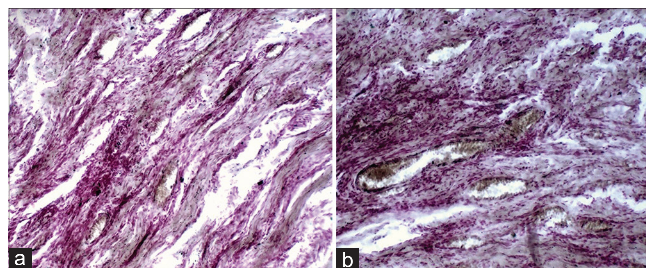

Figure 3: (a) illustrates staining of connective tissue achieved by anthocyanin solution under scanner view. (b) Illustrates staining of connective tissue achieved by anthocyanin solution under ×10 magnification

the reduction in staining intensity of histological sections due to prolonged staining times in vertical orientation. Furthermore, this would explain better staining in slides oriented horizontally during staining procedure due to even deposition of the pigments due to gravity on the specimen. Thus, a freshly prepared solution was required for an efficient and intense staining. Furthermore, on storage of the solution at room temperature for more 2 days, frothing was visible on the surface of staining solution and also it attracted certain household insects. Therefore, it is strongly recommended to discard the solution on the same day after completion of the staining procedure, if used at room temperature.

The results of various modifications attempted individually are illustrated in Table 1.

DISCUSSION

The extract of pomegranates comprises abundant amounts of anthocyanins, catechins and flavonols which are its core chromophores.[12] Anthocyanins have a red hue in acidic pH, shifting toward purple at neutral pH and bluish at higher pH. Cyanidin and delphinidin are the most prominent flavonoids that have been demonstrated to be present in pomegranates.[6] The pigments are predominantly in the form of 2-phenyl-flavylium cations responsible for red color of the solution which are stable at lower pH values,[7,17] as observed in our staining solution of pH 3.6. The ability of a dye to stain specific tissue structures is determined to a large extent by pH of the stain wherein acidic structures would be stained by basic dyes, while basic structures would be stained by acidic dyes.[18] Owing to the strong affinity of anthocyanin extract obtained from pomegranates for the cytoplasm, it can be inferred that it is acidic in nature, thereby explaining its prominent magenta staining on the basement membrane, inflammatory cells and collagen fibers. *P. granatum* contains flavonoids, which are typically acidic polyphenolic compounds having an ability to release hydrogen from their hydroxyl group. Hence, this gives the solution extracted from *P. granatum* the ability to stain the basic components of the tissues. However, the light pink staining of the cytoplasm could be due to the blockage of reactive sites in the cytoplasm due to the presence of acetic acid in the *P. granatum* extracts which is in accordance with a similar observation made earlier by Egbujo *et al.*[19] Another possible reason for faint cytoplasmic staining in the superficial layers of the epithelium could be that squamous epithelial tissue is more resistant to penetration of anthocyanin solution than relatively more porous structures such as lymph nodes or brain tissue used in previous studies.[20] Polymerization of anthocyanins and tannins, both of which are present in pomegranates,[13] at acidic pH also contributes to its color stability.[17,21] This ionic nature of anthocyanins could be responsible for reagent-tissue interactions such as Coulombic attractions with proteoglycans present in the

Figure 4: Illustrates deposition of an aggregate at the bottom of the jar after storing the staining solution and leaving it undisturbed for more than 4 h

Table 1: Effects of modifications of staining solution

Modification	Final pH	Effect on: Staining characteristic	Effect on: Shelf life of solution	Rationale for effect of modification
Pure unmodified *Punica granatum* extract	3.6	Basement membrane nucleoli, collagen fiber bundles-prominent magenta color Cytoplasm-light pinkIntercellular substance, blood elements-light brown	24-48 h	N/A
Addition of 10 ml extract of *Citrus limon* (lemon)	3.1	Increased intensity	Reduced (<24 h)	Reduced pH lead to increased color stability
Addition of 10 ml 4% acetic acid	3.4	No effect	Increased (48-72 h)	Acylation of anthocyanins make the pigments more stable
Orienting the slides horizontally during staining	3.6	Slight increased intensity with more even distribution of pigments throughout sections in different places on the slides	No effect	Gravitational deposition of the pigments on the slides
Placing the slides in refrigerator at 4°C throughout the duration of staining	3.6	Slightly increased intensity	Increased (>72 h)	Anthocyanins are more stable at lower temperatures

basement membrane and nuclear staining in some cells. In addition, hydrogen bonding which is favored by collagen substrate, and hydrophobic effect increasing the system entropy leading to general dispersion of the pigments could explain the peculiar staining pattern observed.[22]

Another mechanism that possibly contributes toward the affinity of anthocyanins is self-aggregation of the pigments.[23] The cation aggregates tend to build up on surfaces with negative charge density, for example, granular content of inflammatory cells, which could be the reason for their observed prominent staining.[22] Besides aggregation, co-pigmentation of flavylium cations with compounds present in pomegranate juice such as tannins and phenolic acids enhances the color intensity as well as stability.[22] This is achieved by protecting the anthocyanins from production of colorless compounds by nucleophilic attack of water molecules that would reduce the staining intensity.[23] Anthocyanins have also been found to possess fluorescing properties.[24] Therefore, further studies in this regard could augment their diagnostic role as histological stains by using fluorescent microscopy.

Acylation or malonylation of anthocyanins enhance their color stability, and thus, acylated pigments are preferred as food colorants.[7,17] Thus, the addition of *Citrus limon* extract achieved superior staining properties and greater stability owing to its various compounds contributing to this phenomenon combined with further acidification of the solution.[25] Similarly, the addition of 4% acetic acid prolonged the shelf life of solution due to improved stability of the staining solution by acylation of the anthocyanin pigments present in *P. granatum* extract.

In case of modification in orientation of the slides during staining procedure, the superior staining results obtained by placing the slides horizontally in a container filled with staining solution could be purely because of passive deposition of pigments evenly throughout the specimen. A fraction of anthocyanins possibly settled down at the bottom of the Coplin jar due to gravity, thus making less pigments available for the histological section to take up. This deposition could also explain formation of an aggregate that was observed toward the bottom of the jar within 4–5 h after it was filled with staining solution. In addition, it would account for most of the stain being taken up within the first 2 h and subsequent negligible improvement in staining with extended periods of immersing the slides in solution.

The anthocyanin pigments are less stable and demonstrate deterioration of color at higher solution temperatures.[17] Storage of anthocyanin solutions at 4°C has been recommended to minimize the degradation in color quality due to sunlight and higher temperatures.[10] The greater stability of anthocyanins at lower temperatures account for improved staining results when the staining procedure was carried out in a refrigerated environment. It would explain the reason behind the extension of shelf life and reusability of the staining solution for longer periods of time when refrigerated.[26,27]

The present research being a novel approach to utilize fruit juice for staining in itself poses a number of limitations. First, the need to prepare a fresh solution every few days even if the staining solution was stored in the refrigerator makes the procedure relatively more tedious. Furthermore, a certain amount of contamination was also observed inevitably in the stained specimens, which points toward the requirement of more refined techniques for removal of contaminants. Although various modifications that led to the improvement in the longevity of staining solution or staining properties have been discussed in the present research, further research is warranted to develop modifications of the staining solution that would enhance the staining characteristics or durability. Therefore, the staining solution would have to go numerous modifications until it could be standardized as a histological stain for optimum results.

To summarize, *P. granatum* can serve as a viable source for anthocyanin pigments which can effectively stain certain components of histological sections to provide a reasonably diagnostic picture. Utilizing these pigments for staining of histological specimens provides the advantages of availability, economic feasibility, nontoxicity and sustainability. As for the drawbacks associated with a natural staining solution, researchers could further look into improving and developing better staining solutions. More understanding of the mechanisms of peculiar staining patterns by anthocyanins in histology is required for which the present research could serve as a pretext.

CONCLUSION

Undoubtedly, extract from *P. granatum* does not possess as efficient staining properties and the standard dyes available commercially. However, it does have the following advantages wherein it is more feasible, eco-friendly, easily available, safely disposable, nonallergic, nontoxic and noncarcinogenic. The present research findings demonstrate that naturally occurring pigments such as anthocyanins can be effectively utilized as histopathological stains and how certain modifications affect the staining

properties. The results obtained by staining the histological specimens by anthocyanin pigments opens an arena for further similar research to explore, identify and employ other natural sources of pigments in histopathology. By using this research as a base, researchers can attempt to better understand natural stains, utilize greater variety of naturally occurring pigments for staining or implement modifications to achieve more optimum staining properties using anthocyanins. Development of such stains would definitely attend to the present need of modern-day laboratories for using safer and more environment-friendly chemicals.

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Conflicts of interest

There are no conflicts of interest.

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