Molecular Biology Reports (2019) 46:581–585
https://doi.org/10.1007/s11033-018-4510-3

ORIGINAL ARTICLE

Evaluation of an extract of the *Punica granatum* flower as a biological stain of rat tissues: a preliminary study

Nilgün Güler Kuşçulu[1] · Halime Güzin Aslan[2]

Received: 4 June 2018 / Accepted: 19 November 2018 / Published online: 5 December 2018
© Springer Nature B.V. 2018

Abstract
Natural dyes and especially hematoxylin, which is herbal, are widely used in staining tissues. The aim of this study is to evaluate the staining status of different tissues taken from rats with pomegranate flower extract. For this reason, 2 Wistar albino rats, one male and one female, were used as stain biomaterial. A histological follow up procedure was applied to the lung, kidney, liver, and heart tissue samples taken from the rats and the unstained preparates of these tissues were prepared. As the source of the dye, the dry flowers of *Punica granatum* (*PG*) obtained from local markets of Kayseri were used. Each tissue sample underwent the same staining procedure with the same temperature, duration, and dye solution. Before and after the staining procedure, ×40 images of the tissue preparates were taken using a light microscope. Generally, different tones of staining were observed in the nuclei and cytoplasms of all cells and epithelium cells. Staining in parts specific to each tissue occurred. For example, there were light stains on the glomerular cells and the Bowman capsule in the kidney tissue Differences in staining can only be explained by molecular diversity differences in tissue. However, in order to improve the initial staining results obtained in this study, it is possible that working with different temperatures, pH values, mordant substances, and dye that the dye molecules in the extract will provide more vivid colors with different molecules in the tissues.

Keywords *Punica granatum* · Wistar albino · Glomerular cells · Bowman capsule · Hematoxylin

Introduction

Natural dyes are obtained from plants, animals, and minerals Plant sources have been commonly used for dyeing different materials. Plants consist of many organic compounds and some of them contain color molecules called dye. Dye molecules basically have aromatic and phenolic structures. The dye molecules are extracted from plants by polar solvents. These extracts have been used in food, textiles, pharmacology, and histology fields to dye different molecules. It is known that several histological techniques, which are used to provide nuclear stains, consist of natural phenolic compounds, which are structurally related to anthocyanins. These include carminic acid (used in carmine dyeing) and hematoxylin as dye resources. Anthocyanins in red cabbage, dahlias, *Hibiscus sabdariffa*, and *Curcuma longa* have also been used for histological dyeing. So have many parts of the pomegranate, including its flowers, which are mostly used for medicine. As a dyeing agent, the peelings of the pomegranate are used in dyeing textiles [1–11]. However, no studies in literature could be found regarding the use of the peel or flowers of pomegranate for dyeing tissues or cells. For this reason, we tried to dye and evaluate various rat tissues we selected with pomegranate flower extract. For this, information on the anatomic and chemical structure of each tissue will be used. When we look at the structure of lung tissue, we can see that alveoli, which constitute one of the structural elements of the respiratory system, are located in the lung within the main and distal bronchia. Pulmonary alveoli are functional units where gas exchange occurs within the lungs [12]. Type I and Type II alveolar cells exist in the walls of the alveoli. Type II alveolar cells excrete phospholipids, which are known as lung surfactants and decrease the surface tension of alveoli [13, 14].

✉ Nilgün Güler Kuşçulu
nguler@erciyes.edu.tr

✉ Halime Güzin Aslan
guzina@erciyes.edu.tr

[1] Department of Chemistry Technology, Mustafa Çıkrıkçıoğlu Vocational School, Kayseri University, 38250 Kayseri, Turkey

[2] Department of Chemistry, Faculty of Science, Erciyes University, Kayseri, Turkey

The liver, which is the largest internal organ in the human body, is located in the upper right section of the abdomen, right underneath the diaphragm. The liver is the only parenchymatous organ that receives dual blood flow. While the portal vein provides 75% of the blood intake of the liver, the hepatic artery provides roughly 25% [15]. The venous drainage of the liver is provided by the hepatic veins. When the liver is evaluated morphologically, it can be seen to be separated into the left and right lobes by the falsiform ligament. The Riedel lobe and the left lobe of the liver reaching out to underneath the left diaphragm are common variations. The Riedel lobe starts from the right lobe of the liver and reaches out to the inferior [16]. Since the liver is a very large organ, in anatomic evaluations, anatomies of the segmental, vascular, hepatic, arterial, hepatic venous, and portal venous parts are separately taken into consideration [17].

The aim of this study is to apply the same procedure to four separate (lung, liver, kidney, and heart) tissues obtained from rats using *Punica granatum* flower (PGF) extract and evaluate the results of staining using the anatomic characteristics of the tissues.

Materials and methods

Tissue supply

In this study, 2 Wistar albino rats, one female and one male, weighing 150–250 g and raised at the Erciyes University Experimental and Clinical Research Center (DEKAM) were used. This study was conducted with the ethics committee number 16/144 dated 16 November 2016. The rats were kept in cages, at 21 °C with normal daily organization, 12 h of light/dark, and fed and watered appropriately. The rats were put to sleep with ketamine and xylasine and their tissues taken, so their lives were ended. All of the procedures were performed according to ethical guidelines.

Preparation of the tissue preparates

The tissue samples taken from the rats were fixed in a 4% formaldehyde solution. After fixing, routine histological tissue follow up was performed. The tissue samples were first dehydrated though increasing series of alcohol, made transparent with xylol, and blocked by burial in paraffin. The 5 µm cross sections taken from the tissue paraffin blocks for dyeing were then applied the staining procedure to see the effect of the *Punica granatum* flower. The intensity of staining was evaluated as negative (−), weak (+), medium (++), and very intense (+++). As a negative control, the cross sections were immersed in distilled water instead of *Punica granatum* extract.

Deparaffinization of tissue preparations

Paraffin was removed from the preparate by the classical method. Preparates were left in an oven at 60 °C for one night. The next day they were removed from the oven and allowed to cool to room temperature for 3–5 min.

The preparation of the pomegranate flower extract solution

20 g of the pomegranate flowers, which were obtained dry and clean from the local markets of the Talas region of the city of Kayseri, were immersed in 100 ml of distilled water. The mixture was boiled for 15 min. After covering with para film, the mixture was left to wait one night at room temperature. The mixture was twice filtered using Whatman blue band filters. The filtered mixture that was obtained was used as a dyeing solution for tissues.

Dyeing of the tissues with pomegranate flower extract

90 ml of pomegranate flower extract solution was added into the staining jar. One each of the undyed preparates of lung, liver, kidney, and heart tissues were immersed in the extract solution. The beaker was left in a 60 °C oven (NUVE 500) for 1 h. The preparate slides were taken from the solution and washed with a large amount of distilled water. The preparations were allowed to stand in 100%, 96%, 80%, and 70% ethyl alcohol for 5 min (for dehydration). They were closed after they were immersed in xylene for 3 min. The preparate slides were examined under 40× magnification with a light microscope. The staining procedure was applied to the same tissues of two different rats under the same conditions, but since there is no significant difference in the staining results, the results of staining of the tissues of a single sex are given in this study.

Dyeing of the tissues with hematoxylin and eosin (H&E) for positive control

The following staining procedure was applied to the heart, lung, liver, and kidney tissues in the same way.

The sections were deparaffinized with two changes of xylene, for 10 min each. They were then Re-hydrated in two changes of absolute alcohol, for 5 min each. The sections were left in 95% alcohol for 2 min and in 70% alcohol for 2 min and then washed briefly in distilled water. They were then stained in a Harris hematoxylin solution for 8 min. Afterwards, they were washed in running tap water for 5 min and then differentiated in 1% acid alcohol for 30 s. They

were again washed in running tap water for 1 min. Next, they were plunged into 0.2% ammonia water or saturated lithium carbonate solution for 30 s to 1 min. They were again washed in running tap water for 5 min, rinsed in 95% alcohol with 10 dips, and counterstained in eosin-phloxine solution for 30 s to 1 min. Finally, the sections were dehydrated through two changes of 5 min each in absolute 95% alcohol,cleared in two 5 min changes of xylene, and mounted with xylene based mounting medium.

Results

The cross sections of the lung, kidney, liver, and heart tissues, which underwent pomegranate flower extract staining, were examined. In the lung tissue sample, the epithelium cell cytoplasm in the bronchiole structure and the straight muscle cell cytoplasms surrounding those were found to be stained at a weak (+) level. However, no significant staining was seen in the epithelium cell and straight muscle cell nuclei. While the cytoplasms of the cells forming the alveolar structures were stained at a weak level, a medium (++) level of staining was seen in the nuclei of certain cells. Additionally, the straight muscle cells forming the blood vein wall in the tissue had the same staining as the straight muscles in the bronchiole wall (Fig. 1a). No significant staining was observed in the ligature structure and elements forming the tissue stroma. No staining was seen in the lung cross sections that were not stained with the *Punica granatum* flower extract (Fig. 1b).

In the kidney tissue cross sections, weak (+) staining was seen in the nuclei and cytoplasms of the proximal and distal tubule epithelium cells. Weak (+) staining was also observed in the glomerular cells forming the renal corpuscle and the structure of the Bowman capsule (Fig. 2a). No significant staining was observed in the ligature structure and elements forming the tissue stroma. No staining was seen in the kidney cross sections that were not stained with the *Punica granatum* flower extract (Fig. 2b)

In the liver tissue cross sections, the hepatocyte cell nuclei and cytoplasms were observed to be stained in a weak (+) manner (Fig. 3a). No significant staining was observed

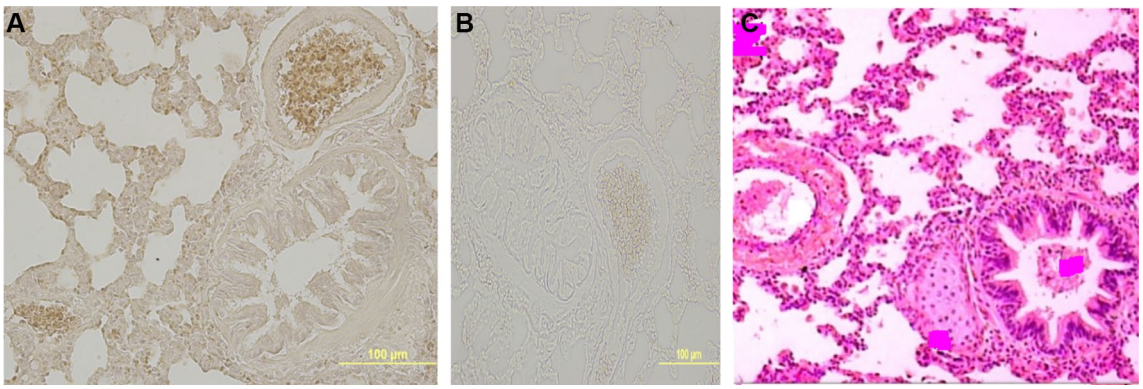


Fig. 1 **a** The lung tissue sample which underwent *Punica granatum* flower (PGF) extract application. **b** Negative control without dye. **c** Positive control with H&E

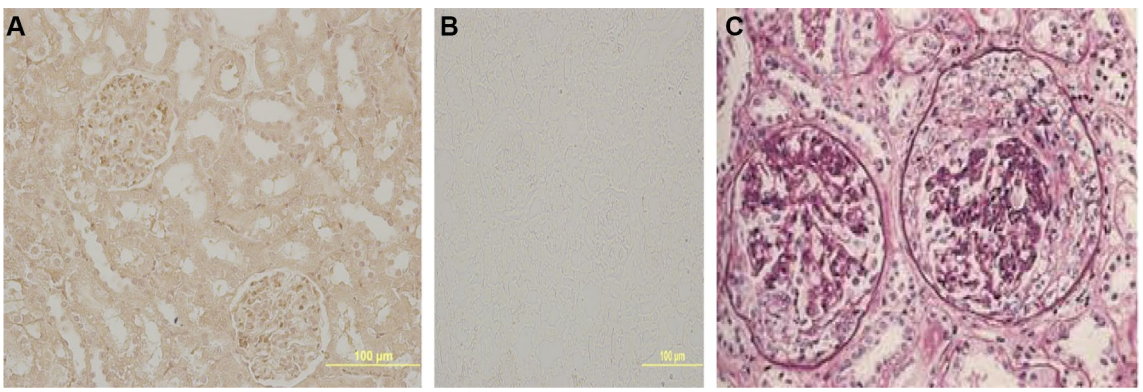


Fig. 2 **a** The kidney tissue sample which underwent *Punica granatum* flower extract application. **b** Negative control without dye. **c** Positive control with H&E

in the ligature structure and elements forming the tissue stroma. No staining was seen in the liver cross sections that were not stained with the *Punica granatum* flower extract (Fig. 3b)

In the heart tissue cross sections, a weak (+) staining was seen in the muscle cell nuclei and cytoplasms. While a medium (++) level of staining was seen in the ligature cell nuclei in the tissue stroma, no staining was seen in their cytoplasms (Fig. 4a). No staining was seen in the heart cross sections that were not stained with the *Punica granatum* flower extract (Fig. 4b)

In this study, heart, lung, liver, and kidney tissues were stained with H&E for positive control. Hematoxylin and eosin are used together because they stain different places in the cell. The nucleated regions in the tissues with hematoxylin are stained in blue, while the cytosols with eosin are stained in pink. Since PGF extract was used alone for tissue staining, a sharp color separation between the cytoplasm and the nucleus in the tissues was weaker than the positive control. With PGF, the cytoplasm in the tissues is stained in the same color, while the nucleus are darker. This difference is better understood when looking at the negative control images that do not contain any dye.

Discussion

We can think of the dyes performed in his study as histochemical procedures. Histochemical procedures are applied based on the basic characteristics of tissues or cells. All dyeing methods should answer these questions: (1) For which tissue component is the dyeing done? (2) Why do certain components take dye while others do not? (3) Can dyeing be performed for all tissue components? [18–21]. When we sought the answers to these questions in our study, stains were performed on lung, liver, kidney, and heart tissue components, with the same herbal extract, to evaluate the dyeing and interaction of the same dye on different tissues. The striated muscle structure of the heart muscle and the textural integrity of the heart muscle cells (the appropriate physical structure) will facilitate the adsorption between the dye molecules and the cellular molecules. Therefore, more

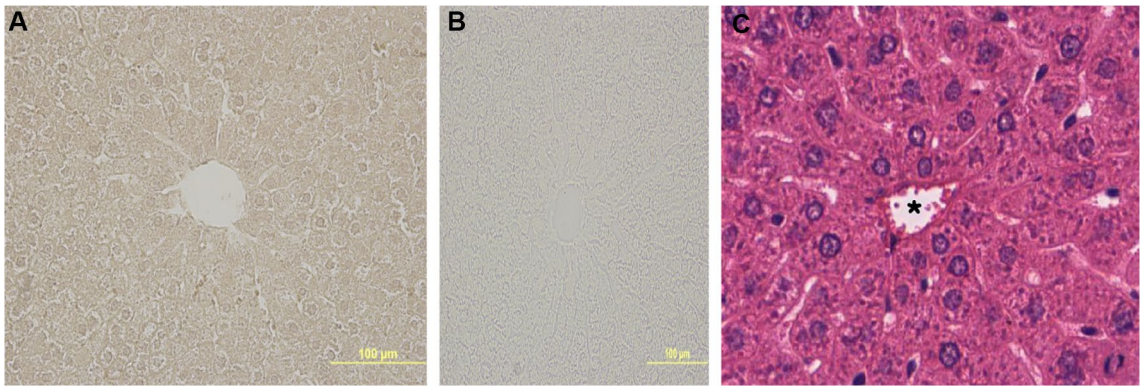


Fig. 3 **a** The liver tissue sample which underwent *Punica granatum* flower extract application. **b** Negative control without dye. **c** Positive control with H&E

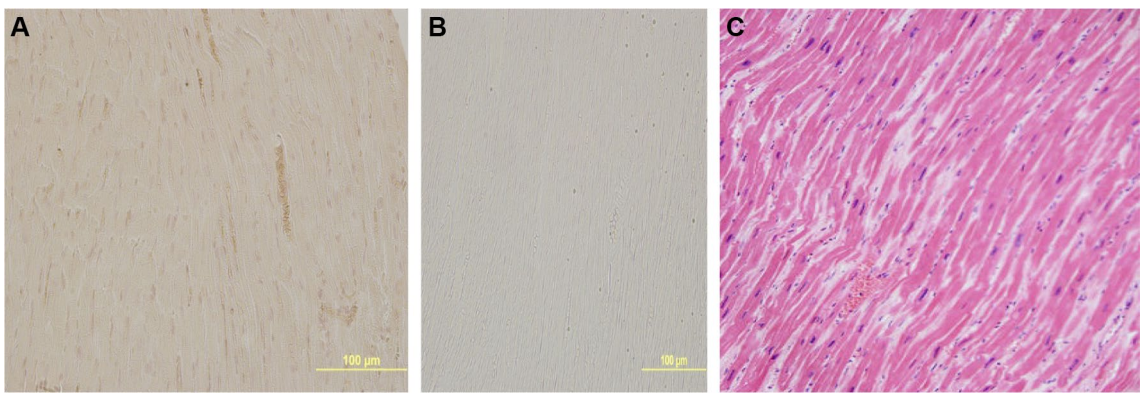


Fig. 4 **a** The heart tissue sample which underwent *Punica granatum* flower extract application. **b** Negative control without dye. **c** Positive control with H&E

efficient staining of the heart muscle cells is appropriate for this extract. In this study, the binding of dye molecules and the heart muscle molecules on the cell surface by adsorption is called the staining. It is not possible to say chemical bonding, for this it is necessary to perform characterization and spectroscopic studies. However, the lack of gaps between the heart muscle cells and their proper structure suggest the possibility of chemical binding between adhesion molecules (CAM's) and dye molecules.

While there was staining in only the cytoplasm of the straight muscle and muscle cells of the lung, there was staining in both the cytoplasm and the nuclei of the muscle cells of the heart. The different translation (cytoplasm) and transcription (nucleus) time and locations in the cells or the different protein rates in cell cytoplasm and nuclei may have caused this. The pomegranate flower extract is strongly acidic. For this reason, it will interact with the alkaline structures in the cells. Since DNA and RNA are acidic molecules, more intense dyeing will be seen in the cell nucleus, where the protein density is higher, in nuclear staining. The differences in the anatomical structures of the tissues can cause different staining results even if the same dye extract is used. For example, type I and type II alveolar cells exist in the walls of the lung alveoli. Type II alveolar cells excrete phospholipids, which are known as lung surfactants and decrease the surface tension of alveoli. Phospholipids form complexes with dye molecules and prevent the staining the dye would do in alveoli walls or the nucleus. This would cause faint and intense stained sections to form in the same lung tissue.

While no significant staining could be seen in the ligaments forming the stroma of lung, liver, and kidney tissues, a medium level staining could be seen in the nuclei of the ligaments forming the stroma of the heart tissue. The heart muscle cells do not have division abilities like other tissues. Since this would cause a difference in the number of tissue elements and molecules, dye molecules would exhibit ionic interactions with more molecules in a fixed and wide surface. Thus, more staining would be seen in heart muscle cells. As a result, in our study, which was performed by holding the dyeing conditions and the density of the dye solution content at a minimum, the heart tissue was more effectively stained with the water solution of the pomegranate flower extract. When temperature, pH, and dyeing duration, which comprise the dyeing conditions, are changed and mordant substances are added to the study, it will be possible to obtain darker stain results and in different colors, resulting in those similar to H&E staining in unstained tissue sections.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Suebkhampet A, Sotthibandhu P (2012) Effect of using aqueous crude extract from butterfly peaflowers (*ClitoriaTernatea* L.) as a dye on animal blood smear staining. Suranaree J Sci Technol 19(1):15–19
2. Lillie RD, Pizzolato P, Donaldson PT (1975) Hematoxylinsubstitues: a survey of mordant dyes tested and consideration of the relation of their structure and performance as nuclear. Stain Technol 5:25–41
3. Bassey RB, Osinubi AA, Oremosu AA (2012) Staining effect of *Hibiscus sabdariffa* extract on sperm cell morphology of Sprague-Dawley rats. J Histotechnol 35(3):110–113
4. Avwioro OG, Onwuka SK, Moody JO, Agbedahunsi JM, Oduola T, Ekpo OE, Oladele AA (2007) Curcuma longa extract as a histological dye for collagen fibres and red blood cells. J Anat 210:600–603
5. Chigurupati N, Saiki L, Gayser CJ, Dash AK (2002) Evaluation of red cabbage dye as a potential natural color for pharmaceutical use. Int J Pharm 241:293–299
6. Ephraim P, Lansky A, Robert A, Newman A (2007) *Punicagranatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J Ethnopharmacol 109:177–206
7. Batta AK, Rangaswami S (1973) Crystalline chemical components of some vegetable drugs. Phytochemistry 12:214
8. Huang TH, Harada M, Yang Q, Li GQ, Yamahara J, Roufogalis BD, Li Y (2005) Pomegranate flower extract diminishes cardiac fibrosis in Zucker diabetic fatty rats: modulation of cardiac endothelin-1 and nuclear factor-kappaB pathways. J Cardiovasc Pharmacol 46:856–862
9. Hernandez F, Melgarejo P, Tomas-Barberan FA, Artes F (1999) Evolution of juice anthocyanins during ripening of new selected pomegranate (*Punicagranatum*) clones. Eur Food Res Technol 210:39–42
10. Davulcu A, Benli H, Şen Y, Bahtiyari M (2014) Dyeing of cotton with thyme pomegranate peel. Cellulose 21:4671–4680
11. Singh R, Srivastava S (2015) Exploration of flower based natural dyes—a review. Res J Recent Sci 4:6–8
12. Kılıç C, Anatomisi A (2011) J Clin Anal Med. https://doi.org/10.4328/JCAM.964
13. Sinnatamby CS (2006) Last's anatomy; regional and applied, 11th edn. Churchill Livingstone, New York, pp 219–224
14. Johnson D, Shah P (2005) Thorax. In: Standring S (ed) Gray's anatomy, 39th edn. Churchill Livingstone, Edinburgh, pp 951–1079
15. Gryspeerdt S, Van Hoe L, Marchal G, Baert AL (1997) Evaluation of hepatic perfusion disorders with double-phase spiral CT. Radiographics 17:337–348
16. Yano K, Ohtsubo M, Mizota T, Kato H, Hayashida Y, Morita S et al (2000) Riedel's lobe of the liver evaluated by multiple imaging modalities. Intern Med 39:136–138
17. Başak M, Akan D (2015) Karaciğerin ve Safra Yollarının Radyolojik Anatomisi. Turk Radiol Semin 3:336–348
18. Zeidel ML, Hoening MP, Palevsky PM (2014) A new CJASN series: renal physiology for the clinican. Clin J Am Soc Nephrol 9:1271
19. Guyton AC, Hall JE (2011) Textbook of medical physiology, 11th edn. Saunders Elsevier, Philadelphia
20. Widmaier EP, Raff H, Strang KT (2010) Vander's human physiology: the mechanisms of body function. Chapter 12. 12th edn. McGraw-Hill, New York
21. Doran F (2005) Histokimya. Aegean Pathol J 2:62–70