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Evaluation and comparison of staining effect of *Punica granatum* flower extract on testis and ovary of Wistar rats: First results

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The evaluation and comparison of the staining effect of pomegranate (*Punica granatum*) flower extract (dye solution) on histological sections of rat testis and ovary provides a simple, quick technique for the visualisation of both cells. In the developed procedure, this work shows the staining effect of *P. granatum* flower extract used at different pH of dye bath and temperatures on the histological sections of rat testis and ovary. A 20% stock solution of *P. granatum* flower extract was prepared by dissolving 20 g of the dye in 100 mL ethanol at room temperature for several hours. After 24 h, the deep-red coloured solution was filtered (0.45 µm filter) to remove any undissolved dye. This stock solution was stored at room temperature and each day a 5 mL sample was taken and adjusted to different pH with dilute ammonia (NH_3) using pH meter. Best staining effects conditions were obtained by testing the different pH of dye (1-2, 4-5) and temperature. The testis and ovary are stained in different colors; lamina propria section and spermatogonia cells of testis were more purple than the same sections of ovary at room temperature and pH of 1-2 dye bath. Therefore, the developed method has been applied successfully for the visualization of rat testis and not the ovarian tissue.

Key words: Histological section, ovarian, pomegranate, *Punica granatum,* testis**.**

INTRODUCTION

Natural dyes are environmental friendly. They exist in a lot of plants (Hu et al., 2017). For example, pomegranate is an important fruit crop of tropical and subtropical regions (Chandra and Babu, 2010). The flowering habit of pomegranate is influenced by the climatic condition of the geographical region where it is grown (Babu, 2010). Plants and their extracts are used for medicinal purpose both for the prevention and treatment of human diseases (Zeliha et al., 2016). Pomegranate extract is widely used for the treatment of some diseases in health sciences. For example, olive anthracnose is caused by different species of *Colletotrichum* spp. and may be regarded as the most damaging disease of olive fruits worldwide. A pomegranate peel extract (PGE) is very effective in controlling this disease (Pangello et al., 2017; Davulcu et al., 2014). Pomegranate peel extract decreases the damage induced by testis torsion (Beigi et al., 2017).

Natural compounds are important resources of many anticancer drugs. Pomegranate is a kind of antioxidant-rich fruit. Its peel and seed have potential anticancer

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activities. The extract from *Punica granatum* (pomegranate) peel induces apoptosis and impairs metastasis in prostate cancer cells. Prostate cancer is a big threat to males due to its poor prognosis and high mortality rate (Deng et al., 2017). In contrast to pomegranate or its peel, pomegranate flower is less used in health studies. For example, *P. granatum* flower has played an important role in the treatment of diabetes in herbal medicine (Xu et al., 2017). PG could be used as a dietary supplement in the treatment of chronic diseases characterized by atherogenous lipoprotein profile, aggravated antioxidant status and impaired glucose metabolism and also in their prevention (Bagri et al., 2009).

Constituents of the flowers of *P. granatum* were determined by spectroscopic analysis (Wang et al., 2006). Six known compounds were identified by comparing their spectral data with values reported in the literature as ellagic acid, 3, 3',4'- tri-O-methylellagic acid, ethyl brevifolin carboxylate, urolic acid, maslinic acid, and daucosterol (Wang et al., 2010; Mahmoud et al., 1994). According to their sources, dyes are divided into two classes as natural and synthetic (Nagar et al., 2005). Dyes from liquids in which they are completely or at least partially soluble are applied to different materials like textiles, leather, paper, hair, food, cells at different temperatures and time (Zollinger, 2004; Guler and Benli, 2017). Mostly in histological studies the most important and used dye is hematoxylin. It is used to demonstrate general tissue structures (Avwioro, 2002). This is the first study where pomegranate flower extract is used on testis and ovarian tissues. The aim is to elucidate the differences that occur when we dye testis and ovarian tissues under the same conditions, to reveal the positive and negative results of dyes and to direct future studies.

MATERIALS AND METHODS

Flowers of *P. granatum* were obtained from local markets of Kayseri. In this study, two Wistar albino male and female rats weighing 150-250 g were obtained from Erciyes University Experimental and Clinical Research Center (DECAM) and used according to the decision of the ethics of the committee 16/144 dated 16.11.2016.

Preparation of the extract by cold waiting process in ethanol

Dried flowers of *P. granatum* were ground into a dark red-black powder using manual grinding machines (Waring, commercial). The dry powder of the plant weighed 20 g (Schimadzu bl 3200). The powder was kept in 100 mL ethanol for 24 h at room temperature. Then the extract (20 % w/v) was filtered two times by blue filter paper. The filtrate was stored in the refrigerator at 4°C and then was used for staining.

Preparation of sections

Testis and ovarian tissues were carefully dissected, trimmed of all fat, and blotted dry to remove any blood. They were fixed in 10% formal saline. The fixed tissues were transferred through a graded series of ethanols. On day 1, they were placed in 70% alcohol for 7 h, then transferred to 90% alcohol and left in the latter overnight. On day two, the tissues were passed through three changes of absolute alcohol for 1 h each, then cleared in xylene. The tissues were infiltrated with three changes of molten parafin wax at 1 h intervals in an oven at 58°C. The tissues were embedded in wax and blocked embedding. The specimens were oriented so that sections would be cut perpendicular to the long axis of the testis and ovary. These sections were designated vertical sections. Serial sections of 5 µm thick were obtained from a block using a rotary microtome, they were attached to slides and dried at 65°C for 45 min.

Staining method

A 300 mL *P. granatum* flower extract (20% w/v, stain solution) was distributed to the four 80 mL beakers in portions of 75 mL. Two of the beakers were left empty, and pH (1-2) of the solutions was measured until pH of 4-5 was obtained. The NH_3 solution was added to the other two beakers. They were mixed with glass stirrer. Prepared testis and ovary were submerged into the four beakers. At the first stage, the first and third beakers were left at room temperature for an hour. An hour later, the slides were removed from the stain solutions and washed with distilled water, and left to air-dry. At the second stage, the second and fourth beakers were placed in the oven at 100°C for one hour. An hour later, the slides were removed from the dye solutions and washed with distilled water, and left to air-dry. Photographs of the testis and ovarian tissues on the eight slides were taken at 10×50 magnification by light microscopy (Olympus BX-51, Japan).

RESULTS

Testis and ovarian tissues were stained in pomegranate flower extract. Acidity and dyeing temperature were changed in the extract. Dyeing results of the tissues are given in Figure 1. We examined and compared the dyeing results of the tissues. More different regions, cells and erythrocytes were stained with green, brown, light red colors in ovarian tissue at 100°C dyeing temperature. This is seen in Figure 1a, tissue surface was stained with light purple-green at room temperature in Figure 1b.

Testis tissue was stained in different colors at different temperatures and dye solution pH of 4-5. Epithel cells in testis tissue were stained in brown color at 100°C as seen in Figure 2a. But, the same cells have very light green color at room temperature as seen in Figure 2b. When the pH of dye solution was changed, tissue surfaces were stained in different colors as seen in Figure 3a, b and c. As seen in Figure 3b, best dyeing color of spermatogonia and sertoli cells appeared at pH 1-2 or in more acidic dye solution as purple. Because cell nucleus contains histon proteins which are base, strong acidic dye molecules interact with base protein molecules making staining to occur. Comparing the pictures of testis and ovarian tissues in Figures 5a and 4a, testis tissue is stained as purple color more than ovarian tissue at pH 1-2 in strong acidic dye solution. Ovarian and testis tissues have light green-yellow color at pH 4-5 in weak acidic dye solution. This is seen in Figures 4 c and 5c.

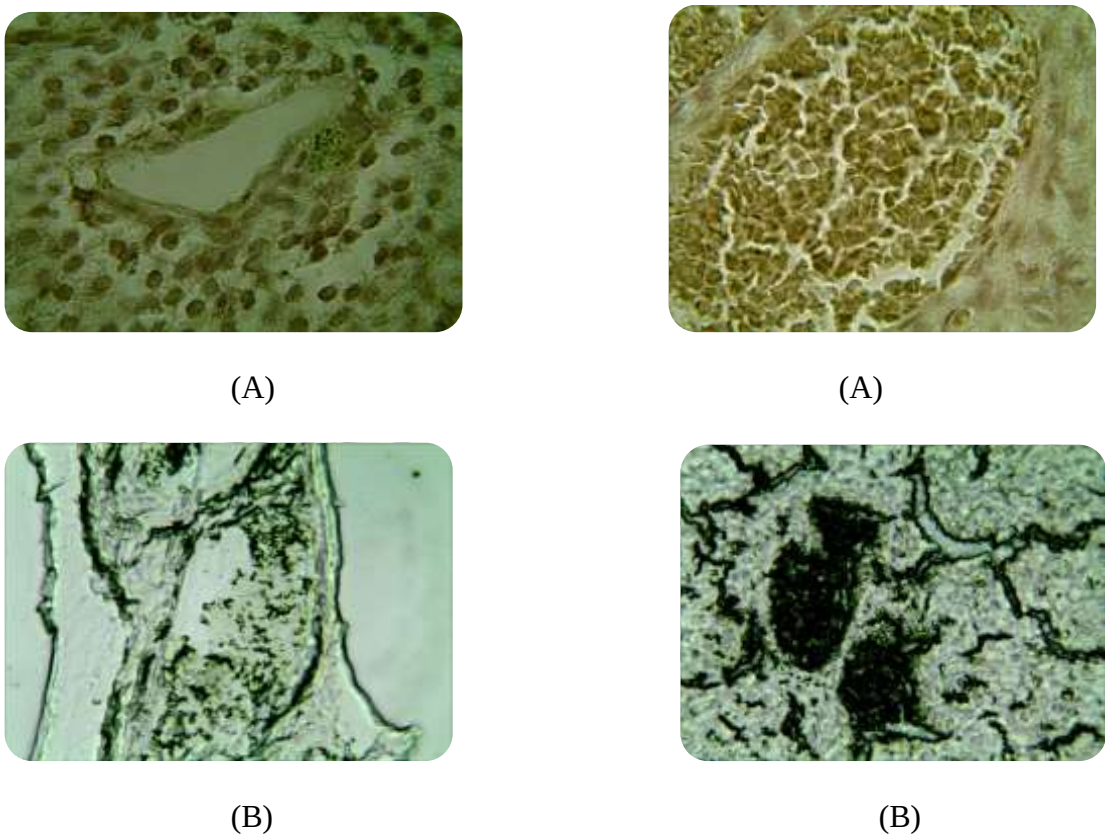


Figure 1. Photographs of the ovarian tissue at pH value 4-5 for one hour in pomegranate flower extract ×50 A) at 100°C, B) at room temperature.

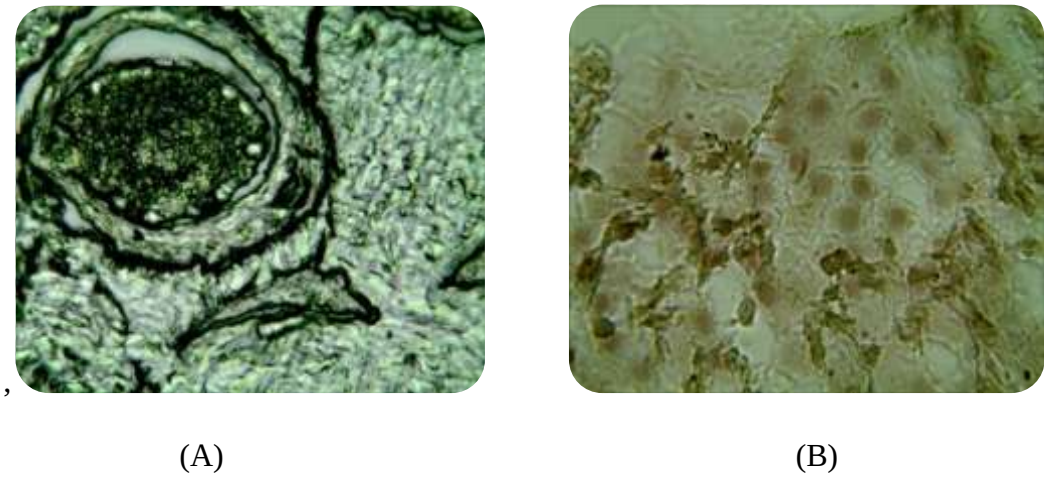
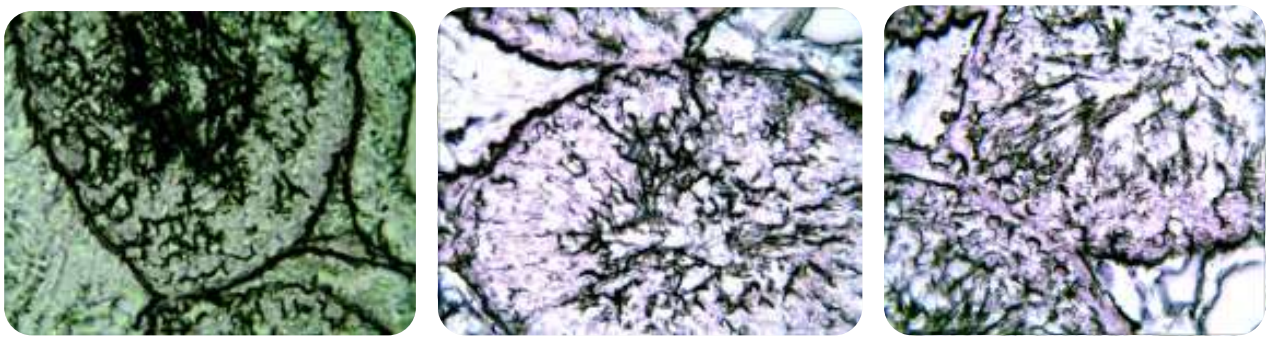


Figure 2. Photographs of the testis tissue at pH 4-5 for one hour in pomegranate flower extract ×50 A) 100°C, B) at room temperature.

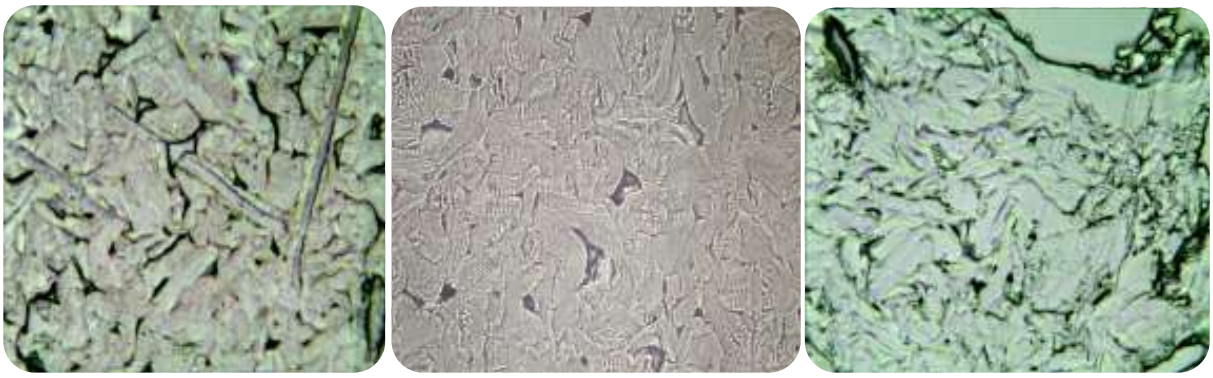
DISCUSSION

Our study focused on the use of non-allergenic, non-toxic and ecofriendly natural pigment for histology. Bassey et al. (2012) also used ethanol solvent from a family of hibiscus only to stain testis tissue. The pH value of the extract is in the range of 4-5. They applied the extract to the tissue in varying amounts and durations at constant



(A) (B) (C)

Figure 3. Photographs of testis tissue at different pH, at room temperature in pomegranate flower extract ×50 A) pH 4-5, B) pH 1-2.



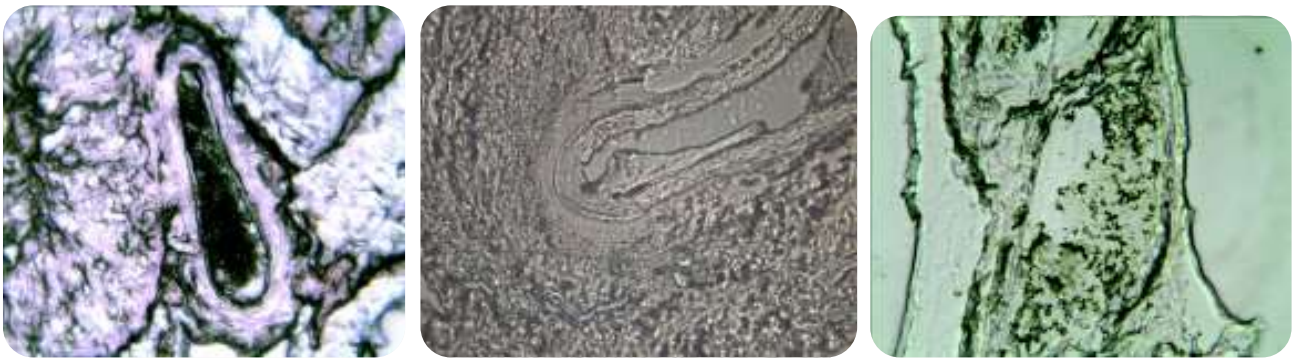
(A) (B) (C)

Figure 4. Photographs of ovarian tissue at room temperature in pomegranate flower extract ×50 (A) pH 1-2, (B) Without extract, (C) pH 4-5.

room temperature. In a short period of time, they achieved successful dyeing in dense concentration. In this study, we tried to stain both testis and ovarian tissues at two different temperatures and pH values. When the pH value of the paint extract was 4-5, there was a lighter color in the tissue at room temperature, and not pink. However, when the pH value of the dye extract is 1-2, the spermatogenic and interstitial cells were well defined as a purple-pink color. But this did not change the color of the ovarian tissue. This is thought to be due to the fact that the distribution and amount of molecules in the basic or acidic cells in both tissues are different. At 100°C dyes, even darker, brown dyes were observed in the section of the testis. This is the positive effect of temperature increase on dyeing yield.

The ovum showed diffuse cytoplasmic staining at different temperatures with different colors. The nucleus was unstained in tissues. Ovarian tissue was not affected by change in pH. This is seen in Figure 4a, c. Ovarian tissue was not dyed in Figure 4b. When compared with others, follicular and epithelial cells in testis tissue were stained (Figures 2s and 3). The most acidic dye solution gave positive dyeing result in testis tissue (Figures 5a and 3b). From this, testis tissue has more basic molecules or cells than ovarian tissue.

Our findings suggest that pomegranate flower or *P. granatum* flower can be mostly used as a stain for testis histological examination and histopathological diagnoses than the examination of ovarian tissue. But, hematoxylin is an acidic, plant-derived nucleus dye. In particular, the testis tissue was stained by pomegranate flower extract in strongly acidic medium having colour like hematoxylin. After working to increase the intensity of color, this extract will be nominated for hematoxylin equivalence. Because eosin is a synthetic dye, we do not use it to compare the color that the pomegranate flower extract



(A) **(B)** **(C)**

Figure 5. Photographs of testis tissue at room temperature in pomegranate flower extract ×50 (A) pH 1-2, (B) Without extract, (C) pH 4-5.

gives to testis and ovarian tissues. This work is the first study of the testis and ovary staining, and we hope to obtain better staining results with subsequent studies, to compare the results with hematoxylin control stains and show the results more photomicrographically.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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