Research Article

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Evaluation of natural staining on Wistar rat EMI region using different metal salts

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Abstract

Background: *The natural staining of cells has recently become a growing research topic. Roselle (Hibiscus sabdariffa) chemical structure has two main components that contain more chromophore groups. Objectives: The aim of this study is to dye cells and elements in two important parts of the Wistar rat uterus with roselle extract containing dense flavonoids and metal salts.*

Methods: *In this study, dried and clean Hibiscus sabdariffa obtained from the local market was used for histological staining of uterine layers (EMI region) in Wistar rat tissue. Different cell structures and their different pathological evaluations are made in the EMI region. In order to more reach different cells, solutions of one percent anhydrous SnCl2 (tin chloride), 1% FeSO4.5H2O (ferrous sulfate), and 1% KAlSO4.12H2O (alum) metal salts in distilled water were used in this study.*

Results: *In staining with roselle extracts containing alum and iron sulfate, tissue red-colored endometrial cell staining, and blue-purple stained inflammatory cells were observed in staining with roselle extract containing 1% SnCl2.*

Conclusions: *Cytoplasmic and nuclear staining intensity, bluing, and clarity were better when SnCl2 was used as the bluing or bruising agent. A purple chelates and coordinate covalent bonding between Flavonoids in the roselle structure and the protein structures in the cell was formed by tin metal in SnCl2 solution. In fact, that there are blue-purple-stained regions in different shades in the cell structure suggests that flavonoids stain different uterine cells thanks to SnCl2. This situation may help to evaluate different and complex pathological findings in the uterus.*

Keywords: Tin Chloride, Emi Region, Wistar Rat, Hibiscus Sabdariffa, Roselle

Introduction

A histopathology lab is where the specimen gets processed and stained to view under a microscope for interpretation. Exposure to the chemicals used in these processes causes various health hazards to the laboratory technicians, pathologists, and scientists working in the laboratory [1]. Hence, there is increasing awareness among people of natural products. Due to their non-toxic, eco-friendly properties, low pollution, and lower side effects, natural dyes are used in many day-to-day products. Although the African continent possesses plentiful plant resources, only a small amount has been exploited so far. *Hibiscus Sabdariffa* is a plant cultivated in many countries globally [2]. In Sudan, it is called Karkade; the *Hibiscus sabdariffa* extract is usually used as a drink; hot tea-like in winter and cold in summer. The water extract of *Hibiscus sabdariffa* containing flavonoids is red in color and acidic in taste. The plant has several well-known industrial, medical, and nutritional uses [3-5]. *Hibiscus sabdariffa*, also known as roselle, has been used in histopathology and cytological examinations. Studies have demonstrated that specific cell components like bar bodies would help diagnose and evaluate as a dye for sperm and, testis morphology [6-9]. The chemistry of the roselle is essential for useful staining. The two significant roselle components are delphinidin 3-sambubioside (*Hibiscus sabdariffa*) and cyanidin 3-sambubioside [10, 11]. The dyes occur in a ratio of approximately 2:1 delphinidin: cyanidin, two minor components, delphinidin 3-glucoside, and cyanidin 3-glucoside, are present [11,

12]. The uterus wall in tissue consists of three parts or three layers; endometrium, myometrium, and perimetrium [13]. Flavonoid structure in Hibiscus sabdariffa extract has occurred coordinate covalent bonding or complex formation with protein structure in tissue by mordants like shown in Figure 1.

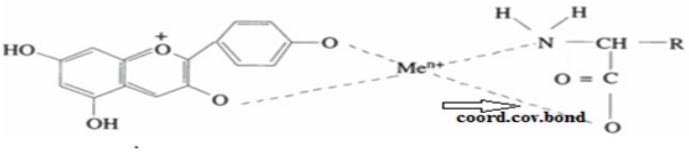

Figure 1: Showing coordinate covalent bonding by metal salts between protein monomer and flavonoid [14]

The Female Wistar rat uterus is a reproductive organ. Some aromatic chemicals like Bisphenol A (BPA) are some of the most widespread endocrine-disrupting chemicals used in the manufacturing of plastics. Many pieces of evidence report many harmful effects on health [15]. The plants we use for uterine staining contain dyes containing phenolic and aromatic groups. However, compared to synthetic substances, they do not cause toxic effects on sensitive uterine tissue. In fact, in studies conducted in cancer lines, its therapeutic effects, not harmful, are mentioned. For Example, Oxidative stress-based cytotoxicity of chemical components of roselle like delphinidin and cyanidin in colon cancer cells is mentioned [16-18]. These chemical components as known flavonoids in the roselle (*Hibiscus sabdariffa*) plant structure do not act by themselves. But they make complexes in different colors by different mordants [19]. So that, In this study, different uterine cells in layers were stained in different colors with different mordant substances.

Materials and Methods

Hibiscus sabdariffa was obtained from the local markets in Kayseri. All the chemicals were used in analytical pure like alum ($KAlSO_4.12H_2O$), ferrous sulfate (FeSO4.5H2O), tin chloride anhydrous (SnCl2), isopropanol, chloroform, xylene, and distilled water.

Preparation of Hibiscus sabdariffa L. extract

The dried *Hibiscus sabdariffa* was ground to a dark red-black powder using a manual grinding machines (Waring, commercial). Fifty gr dry powder was put into 100mL chloroform, boiled for five minutes, and cooled down to room temperature. Then 50ml distilled water was added, and the extract (50 % w/v) was filtered by Whatman No 1 filter paper. This process was continued until 300mL extract was obtained. The filtrate was directly used for staining or put into the refrigerator at 4°C to prevent extract degradation.

Preperation of animals

Two rats (male/female) were used with a weight ranging between 150-250 g. The rats were raised in wire cages and the appropriate food and drink were placed, with proper temperature, light and humidity controlled. The experiments on rats were carried out according to the ethics of scientific research, as the approval of the Ethinical Scientific Committee for Animal Rights was obtained under No. (16/144) [20].

Preparation of sections

Tissue fats of rats were eliminated by cutting and blood removed. Defatted tissues were immersed in the formalin solution. For dehydrating process, they were immersed in the from 70 % to 90% graded series of ethyl alcohol solutions, then cleared with xylene. The Paraffinization process was applied to the tissues to occur paraffin block. Tissue sections were obtained from paraffin by using a microtome device and then sections were taken onto the slides and dried at 65°C for 45 min. So that tissue preparates carrying tissue section were been ready for staining process [21].

Staining protocol

1. The slides were left in an oven at 60ºC for two hours.
2. Paraffin wax was removed from all sections of uterus tissue with xylene.
3. The slides were rehydrated through 100, 90, 80, 70, 50 and 30% isopropanol.
4. The slides were rinsed in distilled water.
5. The 300 mL roselle extract obtained was divided into 50 mL pieces.
6. Three endometrium slides and three myometrium slides were used.
7. Firstly, three endometrium slides were used for staining according to the below groups
   a. The first group was stained in 50mL roselle extract containing 1% alum solution for 30 min, then rinsed in distilled water for 5–10 sec.
   b. The second group was stained in 50mL roselle extract containing 1% ferrous sulfate and 1% alum solution for 30 min, then rinsed in distilled water for 5–10 sec.
   c. The third group was stained in 50mL roselle extract containing 1% tin chloride and 1% alum solution for 30 min, then rinsed in distilled water for 5–10 sec.
8. Secondly, three myometrium slides were used for staining according to the above groups
9. All slides were dehydrated slides with 100% isopropanol, cleared with xylene, and mounted with DPX. Light microscopy (Olympus BX-51, Japan) images of six slides were taken separately at 200× magnification [22].

Results

The image results obtained from the light microscope of the stained endometrium and myometrium layers (EMI) were examined. With the 1% alum solution on the EMI region, nuclei and cytoplasm were darker red (Figures 2a, 3a). With the 1% ferrous sulfate solution, nuclei and cytoplasm were lighter red (Figures 2b, 3b). With the 1% tin chloride solution, nuclei and cytoplasm were dark blues (Figures 2c, 3c). In general, although nuclei could be distinguished, the contrast was suboptimal. However, staining with 1% Tin chloride was provided improved contrast and a blue hue (Figures 2b and 3b).

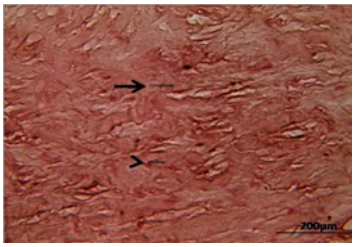

Figure 2a

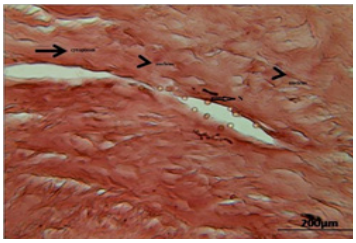

Figure 2a

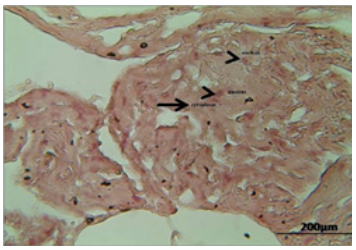

Figure 2b

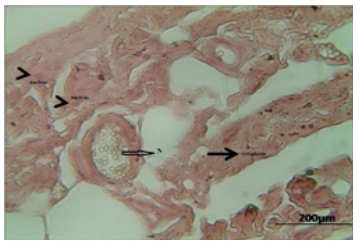

Figure 3b

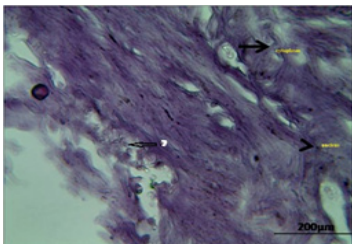

Figure 2c

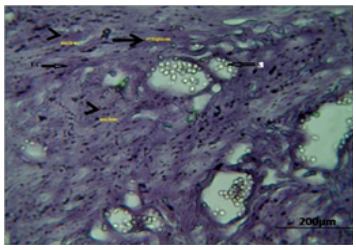

Figure 3c

Figure 2: Photographs of staining myometrium in *Hibiscus sabdariffa* extract with a: 1% alum solution, b: 1% ferrous sulfate + alum solution, c: 1% tin chloride + alum solution (N: neutrophils) (at magnification 200 ×).

Figure 3: Photographs of staining endometrial in *Hibiscus sabdariffa* extract with a: 1% alum solution, b: 1% ferrous sulfate +alum solution, c: 1% tin chloride + alum solution (N: neutrophils, EC: Endometrial cells) (at magnification 200 ×).

Discussion

I previously examined the tissue dyeing studies conducted with *Hibiscus sabdariffa* in solvent and mordant substances while preparing the extract. In the study by Bassey et al., powder, dried *Hibiscus sabdariffa* was extracted with 70% ethanol, and it was observed that rat sperm cells were dyed red without using mordant. In another study conducted by Omorodion and Achukwu, the filtrate obtained after roselle, kept at room temperature for 24 hours in 70% ethanol, was divided into parts, and potassium alum, iron alum, acid, and alcohol were added to each part. Staining results on tissues were generally obtained as red in the nucleus or cytoplasm. Generally, in studies conducted with roselle, extracts in water or ethanol were used as dye solutions, iron or potassium alum mordants were preferred, and it was stated that dyeing without mordant was practical [23, 24]. In a recent study, the cell nucleus being darker blue thanks to substances such as ammonium hydroxide and tap water was been used in the hematoxylin dyeing process. It has even been reported that sodium hydroxide is a more effective bluing agent than ammonium hydroxide [25].

In this study, the same staining procedures were applied to uterine sections and similar results were obtained. In staining using only alum, although the nucleus is not contrasted and clear, the cytoplasm was stained dark red (Figures 2a, 3a). In a staining study using ferrous sulfate, the nucleus is stained clearer and darker red than the cytoplasm (Figures 2b, 3b). These results are consistent with previous studies applied to different tissues. However, in the staining containing 1% Tin chloride solution, the cell nucleus and cytoplasm in the sections were stained in violet-dark blue color (Figures 2c, 3c). There are various cells in the uterine tissue endometrium. Neutrophils also play a role in the indicator of inflammation in the cell. It is important to identify many diseases in the uterus such as endometrial neoplasia by staining neutrophils, secretory cells, and endometrial cells in the diagnosis and treatment of cancer [26-29]. In previous studies, staining not done on the uterine or other tissues with the dye solution was formed by adding tin chloride to the roselle's chloroform-water extract, and good staining results of neutrophils (N), endometrial cells (EC) were obtained. The endometrial-myometrial interface (EMI) is an important region of the human or rat uterus. The endometrium sits directly on the myometrium so that the staining result or color intensity of myometrium was similar to the endometrium [30]. This indicates that the study is original in terms of extract, mordant substance, and tissue.

Conclusion

Chloroform used in the extract's preparation was preferred because it makes a more reddish solution compared to the extract of roselle in the water. Tin chloride solution added to the dye solution acted as an agent that turns the red dye color of roselle into blue. When the tin chloride solution was used, the uterine tissue color changed from red to blue. One percent Tin chloride solution can be used as a new and alternative mordant substance in tissue staining studies with plants. Thus, different and vivid color support can be provided in the pathological evaluation of the EMI region in rat uterine tissue.

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Declaration of Conflicting Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement

The experiments on rats were carried out according to the ethics of scientific research, as the approval of the Ethinical Scientific Committee for Animal Rights was obtained under No. (16/144).

References

1. Ananthalakshmi Ramamoorthy, S. R., Jeddy, N., Thangavelu, R., & Janardhanan, S. (2016). Natural Alternatives for Chemicals Used in Histopathology Lab-A Literature Review. Journal of clinical and diagnostic research: JCDR, 10(11), EE01.
2. Mohamed, B. B., Sulaiman, A. A., & Dahab, A. A. (2012). Roselle (*Hibiscus sabdariffa* L.) in Sudan, cultivation and their uses. Bull. Environ. Pharmacol. Life Sci, 1(6), 48-54.
3. Mohamed, M. A., Elkhalifa, A. E. O., & Agab, M. A. (2012). Biochemical investigation on *Hibiscus sabdariffa* L.(karkade) seed-based sudanese fermented food locally known as furundu. Electronic Journal of Environmental, Agricultural and Food Chemistry, 11(3), 265-78.
4. Cid-Ortega, S., & Guerrero-Beltrán, J. A. (2015). Roselle calyces (*Hibiscus sabdariffa*), an alternative to the food and beverages industries: a review. Journal of Food Science and Technology, 52(11), 6859-6869.
5. Odigie, I. P., Ettarh, R. R., & Adigun, S. A. (2003). Chronic administration of aqueous extract of *Hibiscus sabdariffa* attenuates hypertension and reverses cardiac hypertrophy in 2K-1C hypertensive rats. Journal of ethnopharmacology, 86(2-3), 181-185.
6. Bassey, R. B., Osinubi, A. A., & Oremosu, A. A. (2012). Staining effect of *Hibiscus sabdariffa* extract on sperm cell morphology of Sprague–Dawley rats. Journal of Histotechnology, 35(3), 110-113.
7. Omorodion, N. T., & Achukwu, P. (2017). Investigation of *Hibiscus sabdariffa*(Roselle) as histological stain and in assessment of bar bodies. Am. J. Biomed. Sci, 9(1), 15-19.
8. Egbujo, E. C., Adisa, O. J., & Yahaya, A. B. (2008). A Study of the Staining Effect of Roselle (*Hibiscus sabdariffa*) on the Histologic Section of the Testis.
9. Bassey, R. B., Bakare, A. A., Peter, A. I., Oremosu, A. A., & Osinubi, A. A. (2012). Factors influencing extract of *Hibiscus sabdariffa* staining of rat testes. Biotechnic & Histochemistry, 87(6), 403-407.
10. Wong, P. K., Yusof, S., Ghazali, H. M., & Man, Y. C. (2002). Physico-chemical characteristics of roselle (*Hibiscus sabdariffa* L.). Nutrition & Food Science.
11. Cardon, D. (2007). Natural dyes. Sources, tradition, technology and science, 268.
12. Sinela, A., Rawat, N., Mertz, C., Achir, N., Fulcrand, H., & Dornier, M. (2017). Anthocyanins degradation during storage of *Hibiscus sabdariffa* extract and evolution of its degradation products. Food Chemistry, 214, 234-241.
13. Ross MH and Pawlina W. (2014). Histology: a text and atlas. 6th ed. Philadelphia: Lippincott Williams & Wilkins.
14. Adem, Ö. N. A. L. (1996). Extraction of dyestuff from onion (Allium cepa L.) and its application in the dyeing of wool, feathered-leather and cotton. Turkish Journal of Chemistry, 20(3), 194-203.
15. Dhagga N, Gupta P, and Seema S. (2017). Hıstologıcal Effects of Bısphenol- A on Reproductıve Organs of Female Wıstar Rats. Int J Adv Res, 5(11), 152-156.
16. Cvorovic, J., Tramer, F., Granzotto, M., Candussio, L., Decorti, G., & Passamonti, S. (2010). Oxidative stress-based cytotoxicity of delphinidin and cyanidin in colon cancer cells. Archives of biochemistry and biophysics, 501(1), 151-157.
17. Thomasset, S., Teller, N., Cai, H., Marko, D., Berry, D. P., Steward, W. P., & Gescher, A. J. (2009). Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs?. Cancer chemotherapy and pharmacology, 64(1), 201-211.
18. Zhang, Q., Yang, W., Liu, J., Liu, H., Lv, Z., Zhang, C., ... & Jiao, Z. (2020). Identification of Six Flavonoids as Novel Cellular Antioxidants and Their Structure-Activity Relationship. Oxidative medicine and cellular longevity, 2020.
19. Alshamar, H. A., & Dapson, R. W. (2021). Use of roselle extracted from *Hibiscus sabdariffa* for histological staining: a critical review and rational stain formulation. Biotechnic & Histochemistry, 96(2), 94-101.
20. Kuşçulu, N. G., & Aslan, H. G. (2019). Evaluation of an extract of the Punica granatum flower as a biological stain of rat tissues: a preliminary study. Molecular biology reports, 46(1), 581-585.
21. Kuşçulu, N. G. (2018). Evaluation and comparison of staining effect of Punica granatum flower extract on testis and ovary of Wistar rats: First results. African Journal of Biotechnology, 17(32), 989-993.
22. Luna, L. G. (1968). Manual of histologic staining methods of the Armed Forces Institute of Pathology.
23. Surendra L, Nambiar KS, Haragannavar VC, et al. (2018). Staining Efficacy of Rose Extract in Comparison with Eosin Stain: A Histological Study on Oral Tissues. World J of Den, 9(6), 500-504.
24. Benard, S. A. (2008). Iron-Roselle: a progressive nuclear stain substitute for hematoxylin. Journal of Histotechnology, 31(2), 57-59.
25. Mohandas, R., Ramani, P., Sherlin, H. J., Gheena, S., Ramasubramanian, A., Don, K. R., ... & Santhanam, A. (2020). 0.5% sodium hydroxide as a bluing agent-A comparative study. Drug Invention Today, 14(6).
26. van den Brand, A. D., Rubinstein, E., de Jong, P. C., van den Berg, M., & Van Duursen, M. B. M. (2019). Primary endometrial 3D co-cultures: A comparison between human and rat endometrium. The Journal of steroid biochemistry and molecular biology, 194, 105458.
27. Rogers, P. A. W., Macpherson, A. M., & Beaton, L. (1992). Reduction in endometrial neutrophils in proximity to implanting rat blastocysts. Reproduction, 96(1), 283-288.
28. Jarboe, E. A., & Mutter, G. L. (2010, November). Endometrial intraepithelial neoplasia. In Seminars in diagnostic pathology (Vol. 27, No. 4, pp. 215-225). WB Saunders.
29. Carthew, P., Edwards, R. E., Nolan, B. M., Martin, E. A., Heydon, R. T., White, I. N., & Tucker, M. J. (2000). Tamoxifen induces endometrial and vaginal cancer in rats in the absence of endometrial hyperplasia. Carcinogenesis, 21(4), 793-797.
30. Uduwela, A. S., Perera, M. A. K., Aiqing, L., & Fraser, I. S. (2000). Endometrial-myometrial interface: relationship to adenomyosis and changes in pregnancy. Obstetrical & gynecological survey, 55(6), 390-400.