Original Article

Using Black Carrot Extracts as an Alternative Biological Dye for Tissue Staining

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ABSTRACT

Background: Tissue staining is pivotal in histology and histopathology, shouldering a noteworthy role in identifying and classifying tissues and diseases. Due to their non-production of toxic effluents, the utilization of plant-based dyes aligns harmoniously with environmental sustainability and the well-being of laboratory personnel and the general public. Furthermore, this approach is highly cost-effective, further enhancing its appeal.

Objectives: This research study explored the feasibility of staining various tissues in mice, such as the liver, kidney, intestine, and cartilage, utilizing a dye extracted from black carrots.

Methods: An ethanol extract of 200 g of fresh black carrots (*Daucus carota* L.) was prepared using 95% ethanol saturated with two different solvents in 200 mL of distilled water. Subsequently, the prepared sections of mice tissue were immersed in the extracted dye solution for 20 minutes, followed by assessment using a light microscope. Hematoxylin-eosin staining was used as a control.

Results: The dye extracted from the black carrot using alum and acetic acid successfully stained the cartilage, kidney, intestine, and liver tissues, giving them a bluish-gray coloration. Phytochemical screening further confirmed the presence of anthocyanins in the black carrot extract.

Conclusion: The dye derived from black carrots exhibits natural tissue staining capabilities, making it an alternative to hematoxylin-eosin in histology and histopathology laboratories.

Keywords: Black carrot, Histology, Natural dye, Staining, Tissue

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Introduction

Staining techniques have been used to enhance accurate descriptions of the microscopic structure of tissues, which is necessary for histopathological diagnosis (Alturkistani et al., 2016; Adisa et al., 2017). Immediately after sectioning, tissue sections appear dull and unremarkable under the microscope, making morphological distinction exceedingly difficult (Richardson & Lichtman, 2015). There are two distinct dye types: Synthetic dyes produced through chemical processes and natural dyes derived from natural sources (Benkhaya et al., 2017). In histology and histopathology, natural dyes are commonly used. Hematoxylin campechianum is the most widely used natural dye derived from a tree native to Mexico (Mahapatra et al., 2020). However, despite its widespread use in histology (Baghkheirati et al., 2023; Khodayari et al., 2023; Mohamed Amine et al., 2023), it shows certain limitations, including high cost and issues related to supplementation. Therefore, it is advantageous to explore other natural alternatives to overcome these drawbacks (Alshamar & Dapson, 2021; Kusculu & Eser, 2022).

Using non-allergenic, non-toxic, and eco-friendly natural dyes has gained significant consideration due to the growing environmental awareness, aiming to avoid the hazards associated with certain synthetic dyes (Chaudhary et al., 2020). Dyes containing azo bonds, nitro, or amino groups, are carcinogenic which induce liver and urinary bladder tumors in experimental animals. The reduction of azo dyes results in the formation of aromatic amines, many known mutagens, and carcinogens (Ajileye et al., 2015). In contrast, natural colors derived from minerals, insects/animals, and plants offer a compelling alternative in terms of safety with no health hazards. They are easily disposable, biodegradable, and can be transformed into compost for agricultural purposes once removed (Iqbal & Ansari, 2021). Moreover, natural dyes have been used since ancient times to stain materials such as wool, skin, silk, carpets, and cotton, irrespective of their origins (plants, animals, or minerals) (Yusuf et al., 2017). Notably, a wide range of natural dyes have been derived from different parts of plants, however, out of approximately 2000 available options only around 150 commercially utilized (Tochhawng et al., 2019).

The desirable properties of natural dyes have been demonstrated in various scientific publications. For instance, tissue staining with black mulberry fruits (*Morus nigra*) has proven effective in identifying and differentiating parasites (Tousson & Al-Behbehani, 2010) and nervous tissues (Tousson & Al-Behbehani, 2011). In a study conducted by researchers at the Pathology Department of Unilorin Teaching Hospital in Nigeria in 2017, an aqueous extract solution of *Hibiscus sabdariffa* was used as a substitute nuclear stain for hematoxylin, resulting in successful demonstration of skin morphology and connective tissue (Agbede et al., 2017).

Scientifically known as *Daucus carota L.* ssp. *sativus* var. *atrorubens* Alef. black carrots are also called "Kaali Gajar" in India. The plant is consumed in Turkey, Afghanistan, Pakistan, and India (Nabi et al., 2023). These purple-black carrots are rich in anthocyanins, particularly red anthocyanins, with antioxidant properties. Anthocyanins, depicted in Figure 1, are natural red and purple pigments widely present in nature. Red anthocyanin pigments are highly stable and vibrant, finding application in various industries, including food and beverages (Zamora-Ros et al., 2011).

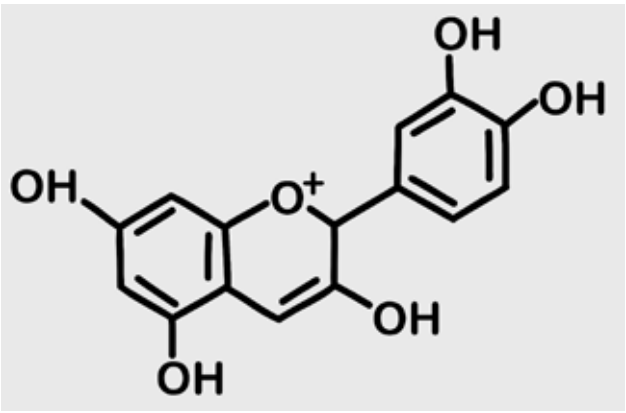

Figure 1. Chemical structure of anthocyanin

Several studies have investigated black carrot anthocyanins' beneficial properties and coloring capabilities as a natural food colorant (Espinosa-Acosta et al., 2018; Chinchón-Payá et al., 2020). Furthermore, besides their use in biological staining, black carrot extracts have shown potential as medicinal compounds, possessing anticancer, anti-inflammatory, and cholesterol- and glucose-reducing properties (Akhtar et al., 2017). Based on these findings, the possibility of utilizing extracts derived from black carrots for tissue staining has been explored. This natural dye is environmentally friendly, cost-effective, and long-lasting which make it as a viable alternative to conventional synthetic dyes for histological and histopathological diagnostic purposes.

Materials and Methods

Extraction of dye solution from black carrots

The fresh black carrots were procured from the online market in Mashhad City, Iran. They were cleansed with water and divided into smaller segments. The carrot pieces weighing 200 g were then subjected to boiling in 200 mL of distilled water, to which 20 g of sugar was added to augment color stability. The process of boiling was executed for 2 minutes. Once the temperature reached 45°C, a blend of 100 mL ethanol alcohol (95%) and 0.1% (v/v) HCl (1 M) was added to the boiled carrot mixture, following two different compositions as specified in Table 1.

The mixture was kept for 24 hours to facilitate settling. The mixture was left undisturbed for 24 hours to aid in separating its components. To maintain the clarity of the extract, the mixture was filtered multiple times using Whatman No. 1 filter paper, with the filtration process repeated twice. Subsequently, the filtered mixture was centrifuged at 5000 rpm for 10 minutes. Finally, the resulting solution was evaporated in a water bath until the final volume reached 100 mL. The extracted solution was stored in a dark place at 4°C until further use. The pH of the extract was evaluated using a pH meter (Sana sl-901), calibrated with pH 4.0 and pH 7.0 solutions (Buitrago-Osorio et al., 2022).

Preparation of sections and staining

Two male mice weighing 25 and 30 g were used for the experiment. Four tissue samples in two replicates, from kidney, liver, cartilage, and intestinal tissues, were col-

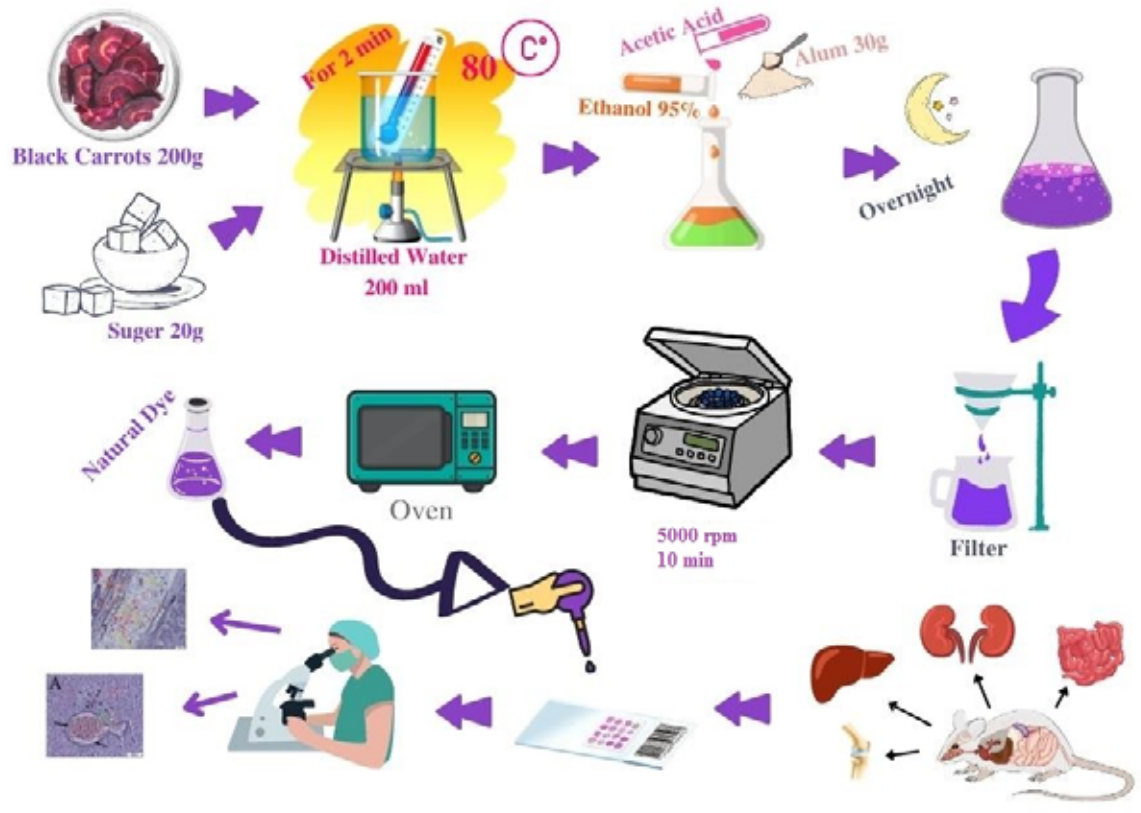

Figure 2. The steps of the experiment

lected. The samples were subsequently fixed in a 10% buffer formalin solution for 24 hours. The extracted tissues underwent dehydration in a series of ethanol solutions (70%, 80%, 90%, and 100%). They were purified in xylene before being embedded in paraffin (Merck, Germany). The paraffin-embedded tissues were sectioned using a rotary microtome (Leica RM 2145; Germany) into 5-mm cross-sections. The solvent xylene was used to remove all paraffin from the tissue sections and rehydrated in an ethanol series. The sections were stained with two black carrot solutions for 20 minutes at 37˚C and the results were compared with hematoxylin-eosin (H&E) stained sections (as control). After histological staining, images were captured using a light microscope (models BX51 and 60; Olympus, Tokyo, Japan) with a digital camera (model DP12; Olympus). Two qualified observers evaluated all photomicrographs to avoid any potential bias (Figure 2).

Results

The maceration process was used to extract black carrots for this investigation (Nuryanti et al., 2012). The yield of ethanol extracts from 200 g of black carrot samples is presented in Table 2. The calculation was based on the weight of the samples after the extraction process.

Staining with solvent A

Examination of cartilage sections stained with solvent A (pH=5.4) revealed clear visibility of the perichondrium, chondroblasts in the perichondrium, lacunae of chondrocytes, and chondrocyte nuclei. The dye extracted from black carrots demonstrated good contrast in identifying cellular and tissue structures within the cartilage tissue (Figure 3a).

In renal tissue sections, distinct renal structures were observed, including the kidney capsule, renal glomeruli, Bowman's capsule, and urinary space. When exposed to the black carrot dye, the nuclei of distal tubular cells exhibited stronger contrast than the proximal tubules, making the distal tubules easier to identify (Figure 3c).

Liver tissue slices stained with black carrot extract displayed visible hepatocyte nuclei, hepatic cell cords, sinusoidal gaps, portal vein, bile ducts, liver capsule, and hepatic arteries. Applying dye derived from black carrots facilitated the necessary contrast and structural distinction in the liver tissue (Figures 4e and 4g).

When small intestinal tissue sections were stained with black carrots, the lamina propria, muscle layers, goblet cells, and brush border margins of the intestinal villi surface were all examined (Figure 5i). The nuclei of cells absorb the hematoxylin dye and appear dark violet or blue. In contrast, the cytoplasm of specific cells such as cartilage, kidney (Figures 3b, 3c and 3d), liver (Figures 4f, 4f and 4h), and small intestinal tissues (Figure 5j) absorb the eosin dye and stain pink.

Staining with solvent B

Examination of tissue sections stained with solvent B revealed that increasing the pH (pH=6.8) during the stages of solution processing significantly reduced the color intensity and color retention on the prepared tissue sections. Additionally, the necessary contrast for identifying tissue and cellular components was reduced considerably. As a result, detecting cell membranes and the location of intestinal glands, renal glomerular tissue, and hepatic cords has become challenging.

The results indicated that solvent B exhibited lower staining potency than solvent A, as the sections stained with solvent B displayed reduced staining intensity or even complete color loss from the tissues (Figure 6).

Table 1. The composition of the two distinct solvents utilized for extracting natural plants

Solvent	Alum (g)	Acetic Acid (mL)	Ethanol Alcohol 95% (mL)
A	30	30	100
B	-	-	100

Table 2. The Percentage yield of the extract from black carrot

Plant	Weight of Sample Used (g)	Weight of Samples After Extraction (g)	Yield (%)
Black carrot	200	71	64.5

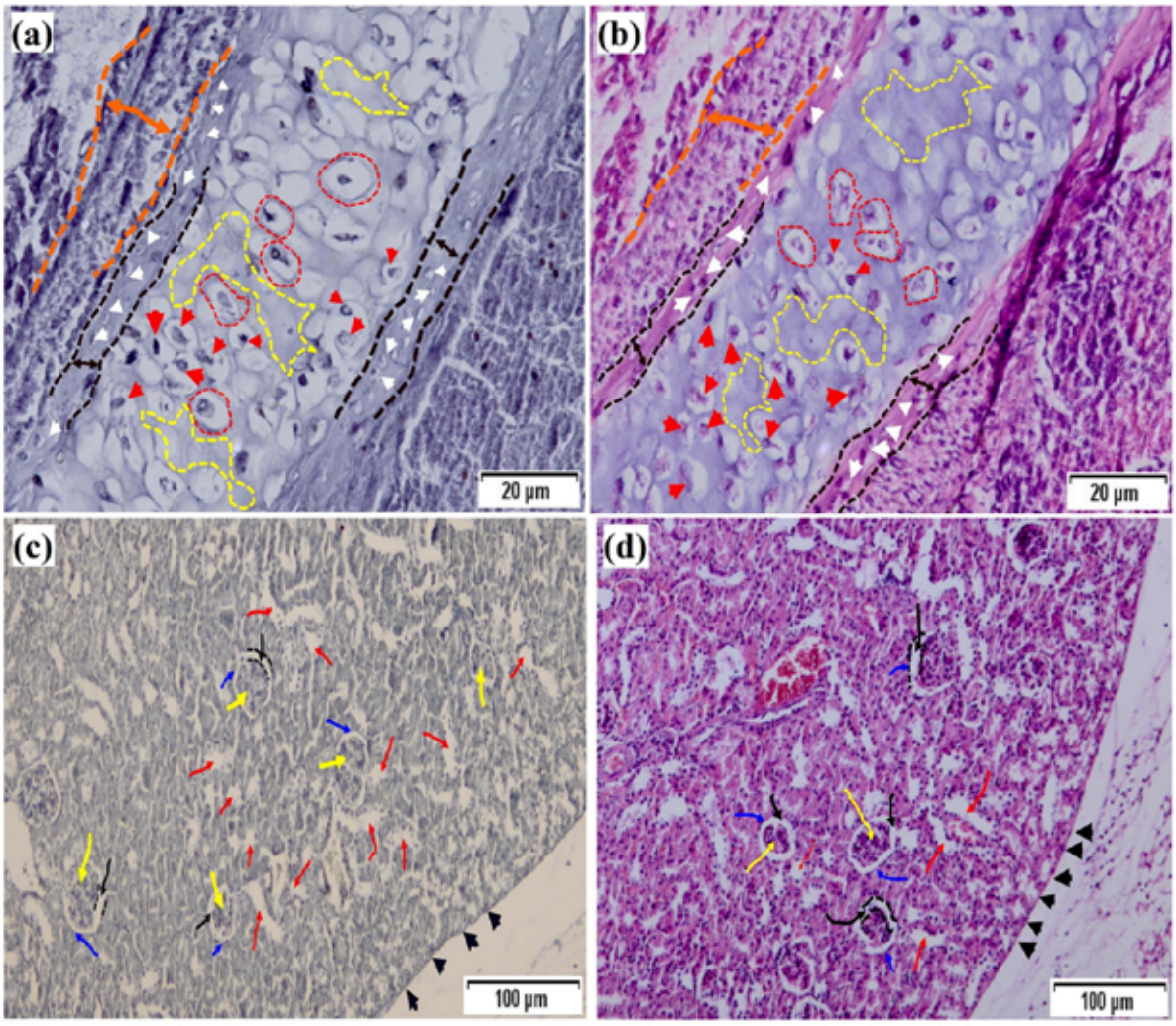

Figure 3. Photomicrographs of cartilage tissue (a, b) and kidney tissue (c, d), stained with solvent A (a, c) and haematoxylin-eosin (b, d) (x100 magnification)

In the photos (a) and (b), chondrocyte nuclei (red arrowheads), chondroblasts (white arrowheads), matrix (circled by yellow broken lines), chondrocytes in lacunae (encircled by red broken lines), connective tissue (orange broken lines), and perichondrium (black broken lines) are seen. In the photos of (c) and (d), the renal capsule (black arrowheads), distal convoluted tubule (red arrows), Bowman's capsule (blue arrows), glomerulus (yellow arrows), and urinary space (black arrows) are seen.

Discussion

Combining hematoxylin and eosin, a synthetic dye, highlights general tissue structures like muscle fibers and connective tissue. Hematoxylin is a type of basic dye that stains acidic components within cells, while eosin is an acidic dye that stains the essential cytoplasmic components of cells. A counter stain, typically a nuclear stain, contrasts the principal stain, making the stained structures more visible. However, some acidic counter stains may lighten or remove the nuclear stains (Mahapatra et al., 2020). Using plant dyes as quality indicators has been a long-standing tradition. Nevertheless, obtaining a complete and consistent plant color poses challenges during processing and storage (Mohammad Azmin et al., 2022). Anthocyanins, a specific subgroup of flavonoids, play a pivotal role in imparting color to various fruits, vegetables, and plants, encompassing hues that span from orange and red to purple and blue (Nistor et al., 2021; Agcam et al., 2017; Blando et al., 2021). Black carrots have gained recognition for their abundant presence of anthocyanins, predominantly acylated compounds (Algarra et al., 2014). Anthocyanins exhibit a fragile nature and are prone to degradation. Various factors, including pH, copigmentation, storage temperature, enzyme presence, light, oxygen, anthocyanin structure, and anthocyanin concentration, influence anthocyanin stability (Ghareaghajlou et al., 2021; Enaru et al., 2021;

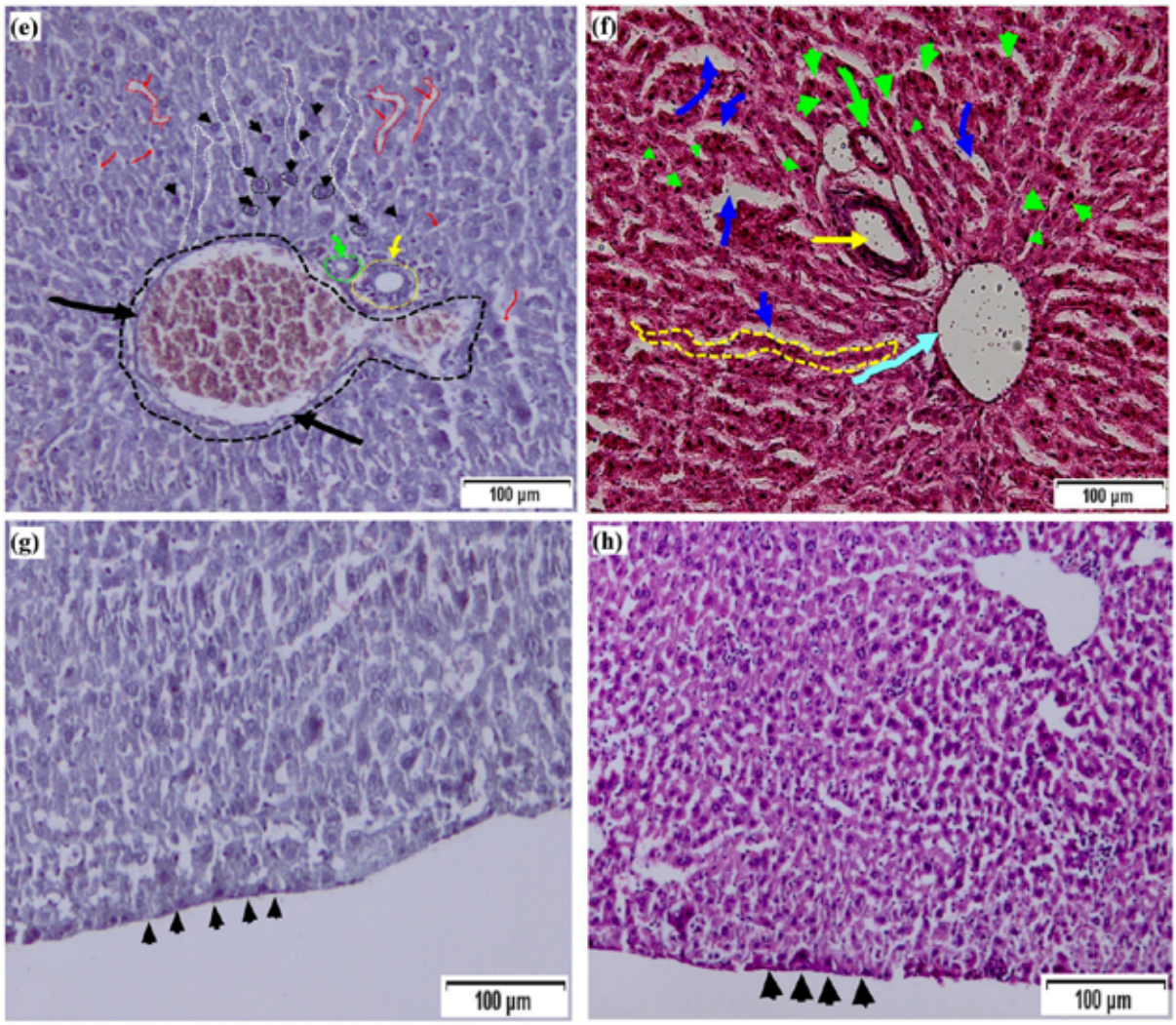

Figure 4. e and g) Liver stained with black carrot extract; f and h) Liver stained with and haematoxylin-eosin (x100 Magnification); e) and f) Portal vein (light cyan and black arrow), sinusoidal spaces (blue and red arrows), hepatic cell cords (white and yellow broken lines), hepatocytes nuclei (black and green arrowheads), bile duct (yellow arrow) and portal artery (green arrow); g) and h) Capsule (Glisson's capsule) (black arrowheads)

Gençdağ et al., 2022). The successful coloring of tissues during the staining process relies on the ability of the dye to form solid connections or attachments with the tissue. Otherwise, the stain would be quickly removed from the tissue when it is washed with another solution. Ionic bonding, which occurs when oppositely charged ions are attracted to each other, is the primary type of bonding that plays a crucial role in histological staining (Kiernan, 2018). Acidic solutions, such as HCl, enhance the stability of anthocyanins. However, it is essential to note that HCl can potentially damage plant cell membranes and dissolve the anthocyanin pigments within the cells (Fei et al., 2021). Copigmentation is a phenomenon where pigments form molecular or intricate associations with other colorless chemical molecules or metallic ions, such as aluminum^{3+}, leading to a shift or intensification of color. During copigmentation, complex interactions between copigments and anthocyanins are formed, resulting in increased stability. It occurs because the interactions reduce the frequency of contact between anthocyanins and water molecules, thereby minimizing anthocyanin degradation (Mu & Li, 2019). This investigation used acetic acid and alum as copigments in the solvent (referred to as solvent A). Acetic acid was chosen due to its ability to create an acidic environment, which promotes the excellent stability of anthocyanins and enhances their staining properties (Buwaeyusoh & Jantarat, 2022). The structure of anthocyanins, specifically the cyanidin molecule, undergoes protonation at low pH levels, forming a positively charged ion or cation. As the pH increases, the cyanidin molecule becomes deprotonated, forming a negatively charged ion or anion. This

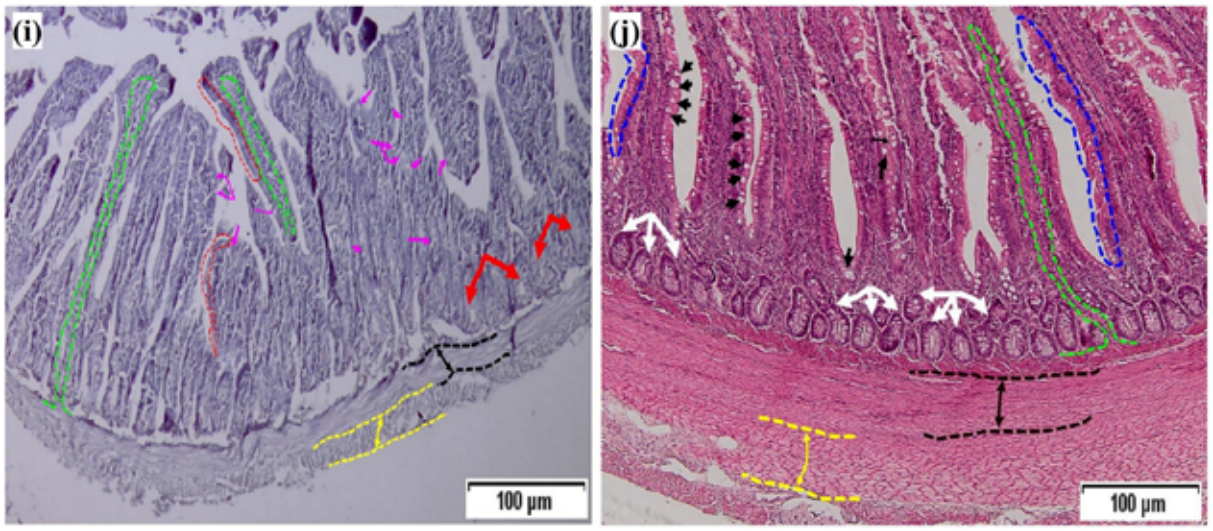

Figure 5. i) Small intestinal tissue, stained with black carrot extract; j) Small Intestinal tissue, stained with haematoxylin-eosin (j) (x100 magnification)

Note: The intestinal glands: Red and white arrow; Goblet cells: Black arrowheads and magenta arrow; Lamina propria: Green broken lines; Inner circular muscle: Black broken lines; Outer longitudinal muscle: Yellow broken lines; Epithelium: Blue and red broken lines

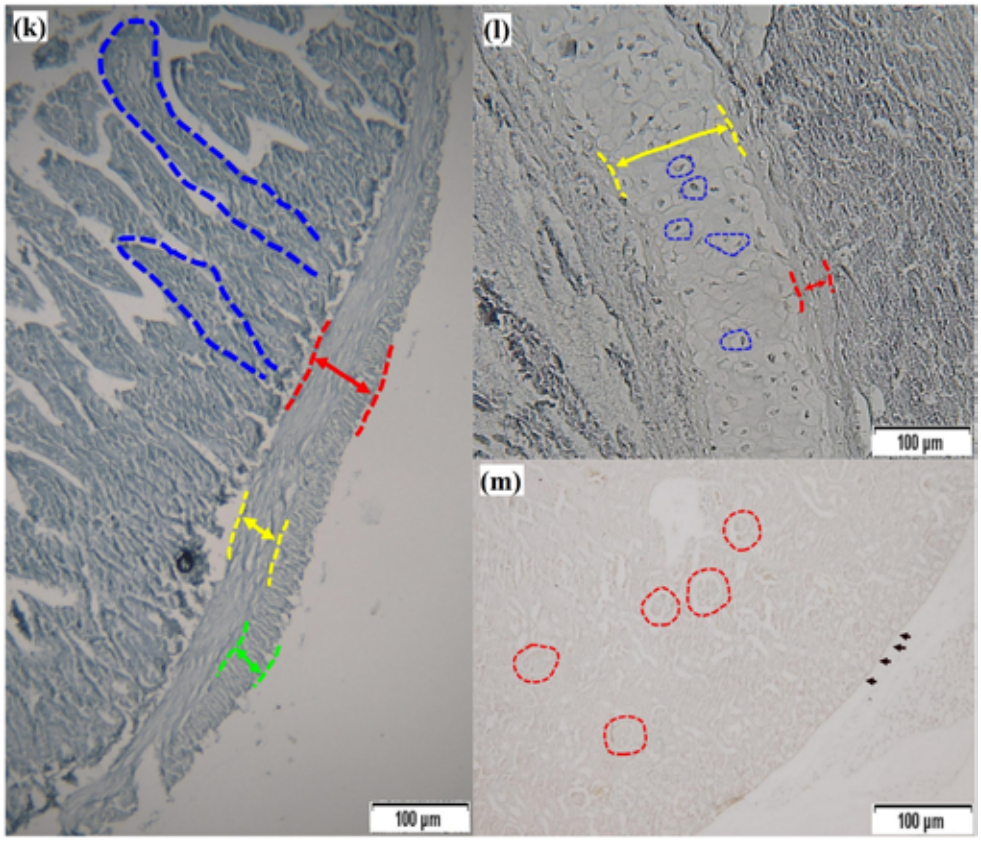

Figure 6. Tissue images stained with solvent B

k) Intestinal villi (blue broken lines), the range of red broken lines: Intestinal mucosal muscle, inner circular muscle layer (area of yellow broken lines, outer longitudinal muscle layer (green broken lines); l) Hyaline cartilage (broken yellow line area), chondrocytes (circled by blue broken lines), and red broken lines, perichondrium; m) Kidney capsule (black arrow) and circle broken lines kidney glomerulus (x400 magnification)

structure of anthocyanins is referred to as the flavylium cation. The flavylium form predominates and remains stable when diluted in low pH values less than 5 (Mendoza et al., 2018; Çoruh et al., 2022). Alum was used because of its capability to form stable complexes with anthocyanins and the Al^{3+} metal ion. Al $(SO_4)_2$. $12H_2O$, commonly referred to as alum, belongs to a group of hydrated double salts that do not pose any health risks. By combining with other substances, alum can alter the color of anthocyanins from red to blue (Mollaamin et al., 2021). Anthocyanins contain hydroxyl (-OH) and carbonyl (C=O) groups, which can establish robust chemical bonds when they interact with alum, along with the amino (-NH2) and -OH groups found in proteins. The presence of alum facilitates the creation of a more stable complex (Kusculu & Eser, 2022) (Figure 7).

The findings indicated that solution A exhibited a more stable color than solution B, attributed to alum and a lower pH. A colorful complex might result when protein and dye functional groups combine during tissue staining without using a mordant (Figure 8).

The pH values of the stain determine its ability to color various structures within the tissue. It is generally true that acid dyes stain essential elements (cytoplasm), and basic dyes stain acidophilic materials (nucleus) (Chukwu et al., 2011). In processing and preparing plant-based colors, monitoring the acidity level is essential. With a slight change in pH, the amount of color absorption of the tissue changes completely, and the desired color may lose efficiency. The application of acid and alum in stains has been proven to enhance the staining potential of tissues (Kiernan, 2018). The experiment's findings demonstrated that the natural stains derived from black carrot effectively colored the cytoplasmic components of the tissue, producing histological staining results comparable to those achieved by combining H&E. Therefore, based on the current study, the extract of black carrot can be referred to as cytoplasmic stains and can be utilized an alternative stain. Various natural and synthetic dyes selectively stain tissue structures (Kusculu & Eser, 2022; Chukwu et al., 2011; Daryani et al., 2011; Ajileye et al., 2015; Sk et al., 2021). Black carrots have been utilized as a natural food coloring agent. Interestingly, without amalgamating it with other dyes, black carrot dye alone

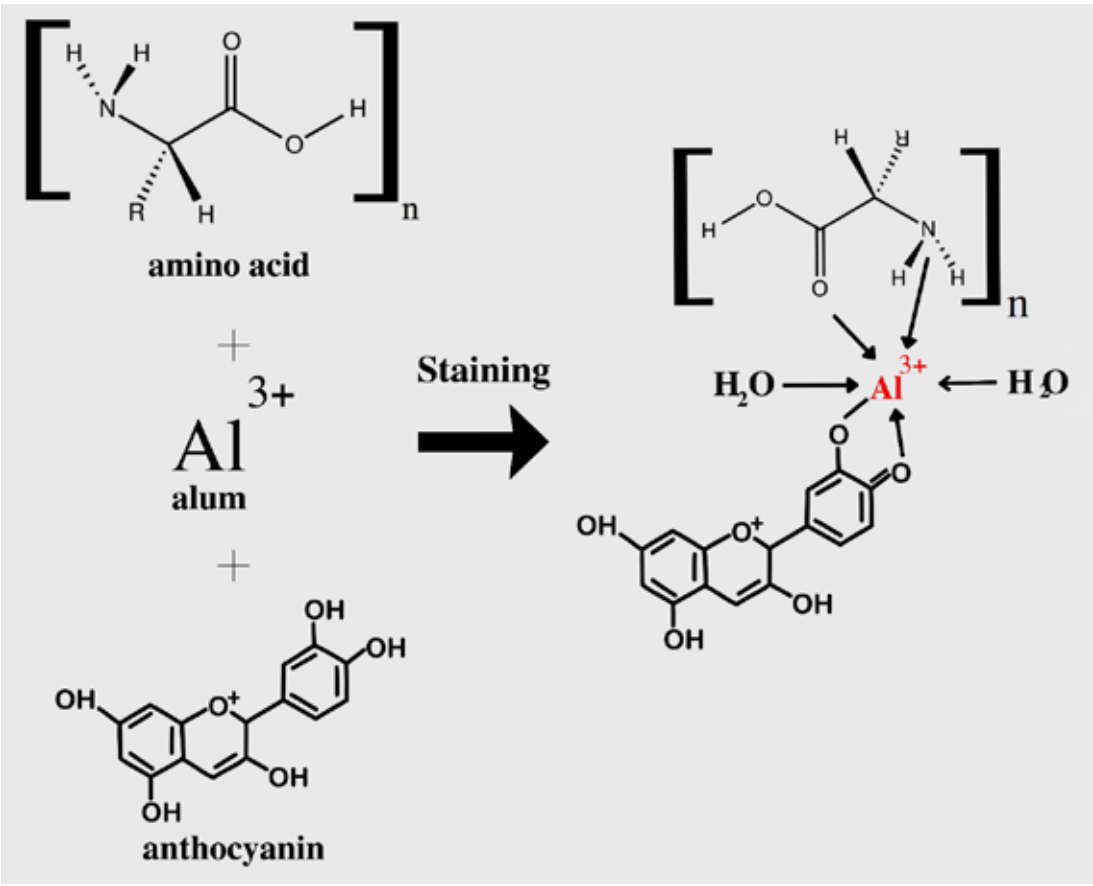

Figure 7. Staining mechanism of cytoplasm with black carrot extract in the presence of alum

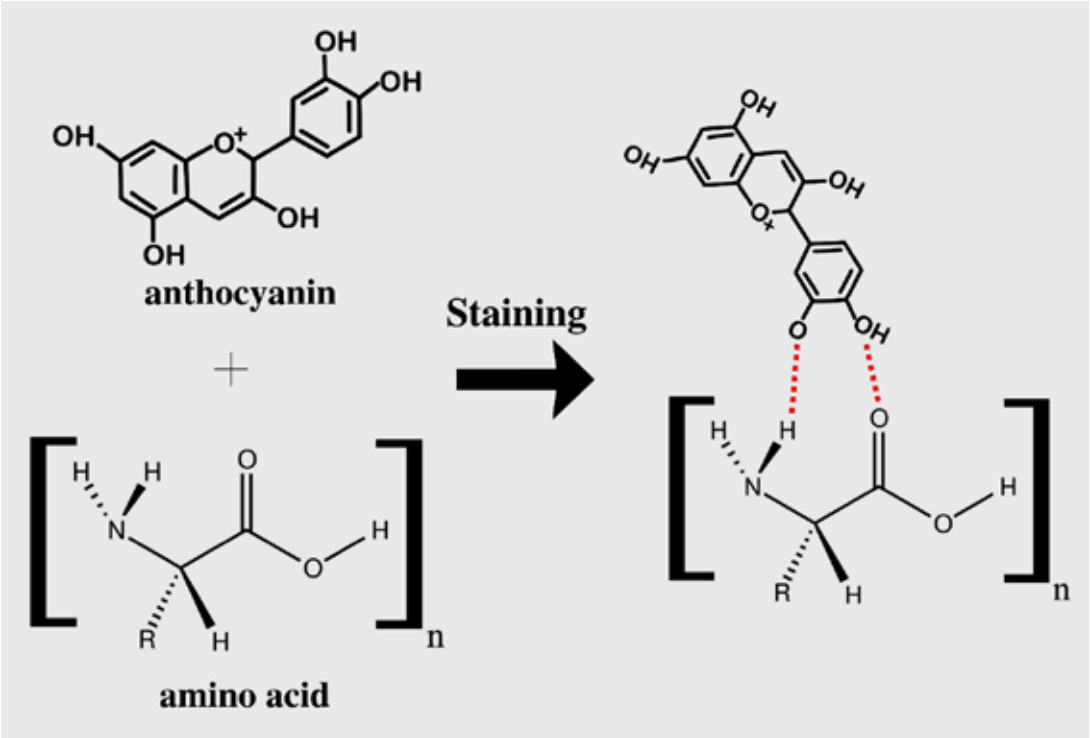

Figure 8. Staining mechanism of cytoplasm with black carrot extract without alum

yielded satisfactory outcomes for tissue diagnosis. Black carrots are renowned for their high content of phenolic compounds, including acyl anthocyanins, which contribute to color stability (Barba-Espín et al., 2020). Utilizing this dye for staining offers several advantages, such as cost-effectiveness, environmental friendliness, and absence of carcinogenic effects. The results demonstrated that the dye could effectively stain tissues without causing any damage, and notably, tissues stained with black carrot dye retained color after several days. However, further research is required to enhance the shelf life and establish standardized formulas for utilizing natural dyes. Achieving consistent and reproducible staining results is a crucial aspect of the field. Additionally, there are limitations in collecting these dye plants due to specific growth areas, climate conditions, and soil requirements.

Conclusion

In conclusion, black carrot extract showed excellent potential as an alternative stain in clinical laboratory studies for evaluations such as distinguishing normal and abnormal tissues and cells.

Ethical Considerations

Compliance with ethical guidelines

All procedures were approved by the Ethics Scientific Committee of the Ferdowsi University of Mashhad (Code: IR.UM.REC.1400.131).

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Authors' contributions

Study design and methodology: Abbas Parham and Mohammad Taghi Vajed Ebrahimi; Experiments: Mohammad Taghi Vajed Ebrahimi and Farhad Mohammadi Gheshlagh; Making coloring agent: Mohammad Taghi Vajed Ebrahimi; Preaparing tissue samples: Farhad Mohammadi Gheshlagh; Writing the original draft: Mohammad Taghi Vajed Ebrahimi; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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مطالعه پژوهشی

استفاده از عصاره هویج سیاه به‌عنوان رنگ طبیعی جایگزین در رنگ‌آمیزی بافت

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چکیده

زمینه مطالعه: رنگ‌آمیزی بافت یک فرآیند ضروری در بافت‌شناسی و آسیب‌شناسی بافتی است و نقش بسزایی در تشخیص نوع بافت و بیماری‌ها دارد. استفاده از رنگ‌های گیاهی به‌دلیل عدم تولید پساب‌های سمی، با محیط زیست سازگار هستند و سلامت کارکنان آزمایشگاه و عموم مردم را تأمین می‌کنند، علاوه‌براین، بسیار مقرون به‌صرفه می‌باشند.

هدف: این مطالعه اثر رنگ‌آمیزی بافت‌های مختلف موش ازجمله کبد، کلیه، روده و غضروف، با استفاده از رنگ استخراج‌شده از هویج سیاه را بررسی کرده است.

روش کار: عصاره اتانولی ۲۰۰ گرم هویج سیاه تازه(Daucus Carota L.) در اتانول ۹۵ درصد اشباع‌شده با ۲ حلال مختلف در ۲۰۰ میلی‌لیتر آب مقطر تهیه شد. مقاطع بافتی آماده‌شده از بافت موش نر به‌مدت ۲۰ دقیقه در عصاره رنگی غوطه‌ور شدند و درنهایت با میکروسکوپ نوری مورد ارزیابی قرار گرفتند. رنگ‌آمیزی هماتوکسیلین-ائوزین به‌عنوان شاهد استفاده شد.

نتایج: رنگ استخراج‌شده از هویج سیاه با زاج و اسید استیک بافت‌های غضروف، کلیه، روده و کبد را به رنگ آبی مایل به خاکستری درآورده است. غربالگری فتوشیمیایی وجود آنتوسیانین را در بافت هویج سیاه تأیید کرد.

نتیجه‌گیری نهایی: رنگ تهیه‌شده از هویج سیاه به‌راحتی می‌تواند بافت‌ها را رنگ کند و در آزمایشگاه‌های بافت‌شناسی و آسیب‌شناسی بافتی به‌عنوان جایگزین روش معمول هماتوکسیلین-ائوزین استفاده شود.

کلیدواژه‌ها: بافت‌شناسی، رنگ‌آمیزی، رنگ گیاهی، هویج سیاه، بافت

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