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ORIGINAL ARTICLE

Ethanolic Extract of *Vitis vinifera* (Black grapes) Skin as a Safer Alternative to Hematoxylin and Eosin Stain

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KEYWORDS

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ABSTRACT: Hematoxylin and eosin (H&E) is the gold standard stain in routine histopathological staining. However, the worldwide shortage of hematoxylin, the substandard quality of the stain, its infrequent supply, and increasing cost have troubled pathologists across the globe. Besides, the treatment with acidic solutions in trichrome and other stains leads to diminished nuclear staining. This combined with the detrimental effects of eosin, which is a synthetic dye, has encouraged the development of newer, eco-friendly, organic dyes from natural derivates. The present study aimed to evaluate the staining efficacy of ethanolic extract of *Vitis vinifera* (Black grapes) skin in routine histopathological staining compared to H&E stain. Dye components from *Vitis vinifera* skin were extracted using absolute ethanol. The ethanolic extract of *Vitis vinifera* skin and H&E stain were used to stain tissue blocks containing mucosa, muscle, gland, lymph nodes, and decalcified bone. The slides were evaluated for the nuclear details, nuclear staining, cytoplasmic staining, and uniformity by a blinded pathologist. A chi-square test was done to check the statistical significance. Ethanolic extract of *Vitis vinifera* skin demonstrated nuclear details comparable to H&E. However, the difference in staining between the stains was not found to be statistically significant (p=0.088). This study showed that dye components can be extracted from *Vitis vinifera* skin. The nuclear staining of histological tissue with ethanolic extract of *Vitis vinifera* skin was comparable to the H&E stain.

INTRODUCTION

Over the years, numerous stains have been used in histopathology, employing a wide variety of chemicals ranging from carmine, trichrome stains, and differential stains [1]. While some stains have been discontinued because of their complex manufacturing process, toxicity, and unavailability, some stains like Hematoxylin and Eosin (H&E) have stood the test of time [1]. This simple dye combination highlights the various components of the tissues ranging from extracellular matrix to the fine structure of cells and tissues and plays a significant role in routine diagnostics [2].

Despite all the above merits, H&E has drawbacks that have created a need to explore other stains. Firstly, eosin is hazardous to human and animal health [3-5]. Repeated exposure to eosin may lead to systemic poisoning, impaired vision or blindness, and defatting of the skin [6]. Though an excellent cytoplasmic stain for most tissues, eosin still does not provide sufficient contrast between cellular and extracellular structures and is unable to stain mucins, reticular fibers, and basement membranes as well as cannot define cell borders [7].

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Hematoxylin does not have staining properties unless it is oxidized to hematein by the use of mordants. The major disadvantage of hematoxylin is that it is pH-sensitive. This affects its staining properties in the application of acidic staining solutions like van Gieson and other trichrome stains [8]. Another drawback of hematoxylin is that the color of the staining reaction depends on the constituents of the staining solution and the type of mordant used. Hematoxylin with alum as a mordant imparts a blue color to the nucleus, whereas black nuclear staining is observed with Iron hematoxylin [4]. Additionally, the shortage of hematoxylin and prudent increase in the prices has encouraged the development of newer, eco-friendly, organic dyes from natural sources [9].

Vitis vinifera (Black grapes) is cultivated worldwide and is the world's largest fruit crop [10]. Its skin extract contains anthocyanins, tartaric acid, tannins, sugars, and minerals [11]. Anthocyanins are pigments that impart color to the grape skin and are an active dye component [12]. Several studies that have assessed the use of *Vitis vinifera* extracts in the textile industry, leather industry, and as food coloring agents [12-14]. However, these extracts have not been tested for staining of histological tissues.

Hence, the present study aimed to evaluate the staining efficacy of ethanolic extract of *Vitis vinifera* (Black grapes) skin in routine histopathological staining compared to standard H&E staining.

MATERIALS AND METHODS

The study was designed to extract the dye components from *Vitis vinifera* skin to check their efficacy in staining various histopathological tissues and to compare them with H&E stain to assess their effectiveness.

Study design

Comparative study

Sample size calculation

The sample size calculation was done using G power software based on a previous study conducted by et al. Keeping the expected proportion as 0.316, precision as 0.6%, and confidence interval of 95%, the sample size was calculated as 20, with 4 in each sub-group.

Study groups

Group I: Stained with ethanolic extract of *Vitis vinifera* skin (n=20)
Group II: Stained with ethanolic extract of *Vitis vinifera* skin and counterstained with light green (n=20)
Group III: Stained with H&E stain (n=20)

Both the groups contained 5 subgroups; four samples in each subgroup

Subgroup 1: Oral mucosal tissue (n=4)
Subgroup 2: Salivary gland tissue (n=4)
Subgroup 3: Skeletal muscle tissue (n=4)
Subgroup 4: Lymph node tissue (n=4)
Subgroup 5: Decalcified bone tissue (n=4)

Eligibility Criteria

Sections of oral mucosa, salivary gland, lymph node, muscle, and decalcified bone were randomly selected after microscopic examination of previously stained H&E slides by a pathologist.

Ethanolic extract preparation with Vitis vinifera skin

500 grams of fresh *Vitis vinifera* was procured from the local market. The fruit was washed in tap water and dried. 100 grams of the fruit was weighed using a weighing scale. The skin from the fruit was separated from the pulp by peeling it off manually. The pulp was discarded. The skin was crushed using a mortar and pestle. After initial crushing, 10 mL of absolute ethanol was added and the crushing was continued. Further, 40mL of absolute ethanol was added and crushed until a uniform dark purple colored mixture was obtained. The contents of the mortar were transferred to a beaker and filtered using a Whatman filter paper. The filtrate was stored in a sterile plastic container at 4°C.

Preparation of Light Green Counterstain

The Light green stock solution was prepared by adding 0.2 g light green powder to 100 ml of distilled water. To this, 0.2 ml of Glacial Acetic Acid was added. The stock solution was stored in a sterile plastic container at 4^0C.

The working solution was prepared just before staining by adding 10 ml of Light Green stock solution to 50 ml of distilled water.

Preparation of Mordant solution

The mordant used was Potassium aluminum sulfate dodecahydrate (Potash alum). The mordant solution was prepared just before staining by adding 5g of Potassium aluminium sulphate dodecahydrate powder to 100 ml of distilled water.

Staining protocol

All slides were deparaffinized in Xylene for 20 minutes followed by isopropyl alcohol for 10 minutes. Forty test group sections were washed in distilled water and treated with a mordant solution (5% potassium aluminum sulfate dodecahydrate) for 10 minutes. The sections were then stained with the ethanolic extract of *Vitis vinifera* skin for 30 minutes. Twenty slides were rinsed in running tap water and placed in a light green solution for 1 minute. The control group sections were stained with H&E stain as per routine procedure. All the slides were rinsed in running tap water and air-dried. The slides were cleared in Xylene and mounted with a coverslip using Dibutylphthalate Polystyrene Xylene (D.P.X.)

Assessment of the stained sections:

All the slides were labeled correctly. The slides were observed by a blinded pathologist using a binocular light microscope. The slides were graded based on nuclear details, nuclear staining, cytoplasmic staining, and uniformity of staining. The following scores were assigned to each criterion:

1 – Poor
2- Fair
3- Good
4 – Excellent

Statistical analysis

Statistical analysis was performed using the SPSS Version 23. Chi-square test was used in this study to compare between the groups. Further comparison was done among the different tissues to find which stain was better among individual tissues. A test was also done to compare the artefacts among the groups. Significance was fixed as 5% ($\alpha = 0.05$). $p < 0.05$ was considered statistically significant.

Results

Ethanolic extract of Vitis vinifera skin

A total of 20 sections were stained with ethanolic extract of *Vitis vinifera* skin, which stained the nucleus dark purple and the cytoplasm light purple (Figure 1). Among the sections, nuclear details were good in 50%, fair in 35%, and poor in 15% of the sections. The nuclear staining was good in 55%, fair in 30%, and poor in 15% of the sections. The cytoplasmic staining was fair in 70%, good in 20%, and poor in 10% of the sections. The uniformity of staining was good in 55%, fair in 30%, and poor in 15% of the sections.

Ethanolic extract of *Vitis vinifera* skin was a better nuclear stain than a cytoplasmic stain. Among all the tissues, the lymph node and salivary gland showed better nuclear staining and uniformity when stained with ethanolic extract of *Vitis vinifera* skin. Among all the parameters evaluated, ethanolic extract of *Vitis vinifera* skin demonstrated nuclear details comparable to H&E. However, the difference in staining between the stains was not found to be statistically significant (p=0.088).

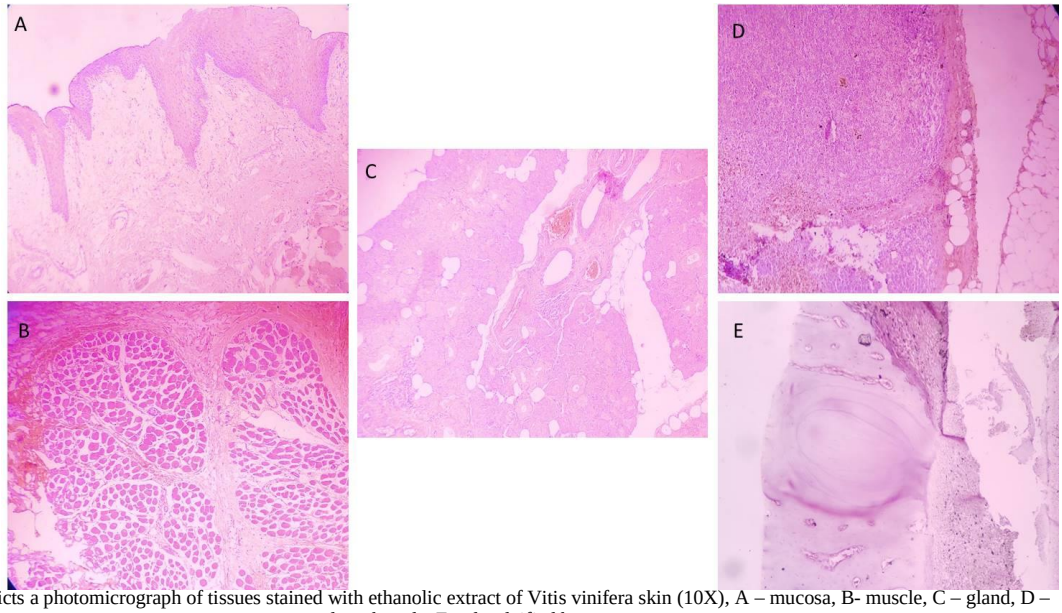

Figure 1. depicts a photomicrograph of tissues stained with ethanolic extract of Vitis vinifera skin (10X), A – mucosa, B- muscle, C – gland, D – lymph node, E – decalcified bone.

Ethanolic extract of Vitis vinifera skin with Light green as counterstain

When light green was used as counterstain with ethanolic extract of *Vitis vinifera* skin, the nucleus was stained light purple and the cytoplasm appeared green, masking the light purple color of *Vitis vinifera* (Figure 2). Among the 20 sections that were stained, excellent nuclear details were seen in 10%, good nuclear details were seen in 25%, fair nuclear details were seen in 50%, and 15% of cases showed poor nuclear details. The nuclear staining was excellent in 10%, good in 25%, fair in 50%, and poor in 15%. The nuclear staining, though had improved in 10% of cases, the addition of light green had reduced the nuclear staining efficacy of *Vitis vinifera*.

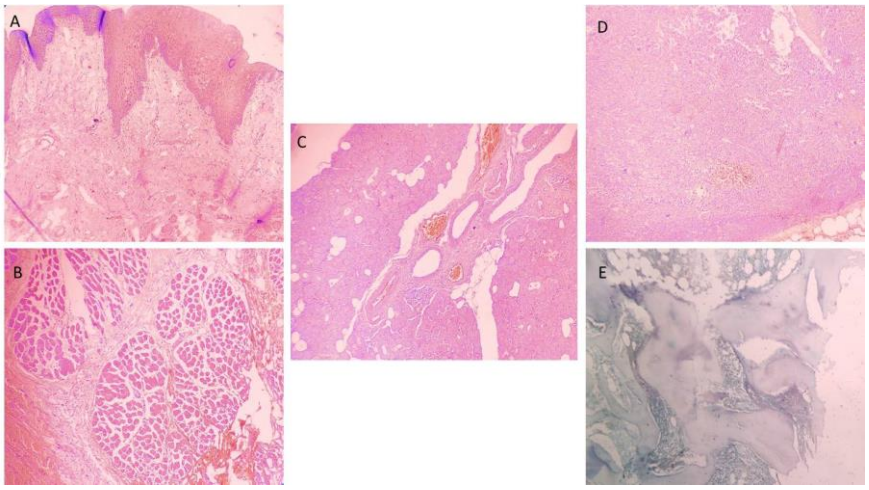

Figure 2. depicts a photomicrograph of tissues stained with ethanolic extract of *Vitis vinifera* skin and light green as counterstain (10X), A – mucosa, B- muscle, C – gland, D – lymph node, E – decalcified bone.

The cytoplasmic staining was good in 15%, fair in 75%, and poor in 10% of cases. The uniformity of staining was good in 35%, fair in 50%, and poor in 15% of cases. The lymph node and salivary gland showed better nuclear staining and details among all the tissues. Between *Vitis vinifera* alone and *Vitis vinifera* with light green, the nuclear staining of tissues was better with *Vitis vinifera* stain and the results were comparable to hematoxylin and eosin as the staining between the groups was not found to be statistically significant. The summary of the results is tabulated in Table 1.

Table 1. Comparison of ethanolic extract of *Vitis vinifera* skin with and without light green as counterstain with Hematoxylin and Eosin stain

Criteria	*Vitis vinifera*		*Vitis vinifera* + Light Green		Control (H & E)		p-value
	n	%	n	%	n	%	
Nuclear details							
Poor	3	15	3	15	1	5	0.088
Fair	7	35	10	50	6	30	
Good	10	50	5	25	7	35	
Excellent	0	0	2	10	6	30	
Nuclear staining							
Poor	3	15	3	15	1	5	0.061
Fair	6	30	10	50	6	30	
Good	11	55	5	25	7	35	
Excellent	0	0	2	10	6	30	
Cytoplasmic staining							
Poor	2	10	2	10	0	0	0.003
Fair	14	70	15	75	5	25	
Good	4	20	3	15	14	70	
Excellent	0	0	0	0	1	5	
Uniformity of staining							
Poor	1	5	3	15	0	0	0.159
Fair	11	55	10	50	6	30	
Good	8	40	7	35	13	65	
Excellent	0	0	0	0	1	5	

- statistically significant

Hematoxylin and Eosin stain

The 20 control sections were stained with hematoxylin and eosin stain (Figure 3). The nuclear details were excellent in 30%, good in 35%, good in 30%, and poor in 5% of cases. The nuclear staining was excellent in 30%, good in 35%, fair in 30%, and poor in 5% of cases. The cytoplasmic staining was excellent in 5%, good in 70%, and fair in 75% of cases. The uniformity of staining was excellent in 5%, good in 65%, and fair in 30% of cases. Among the different tissues, lymph nodes and muscles showed better nuclear staining. The comparison of staining efficacy of the stains among various tissue types has been summarized in Table 2.

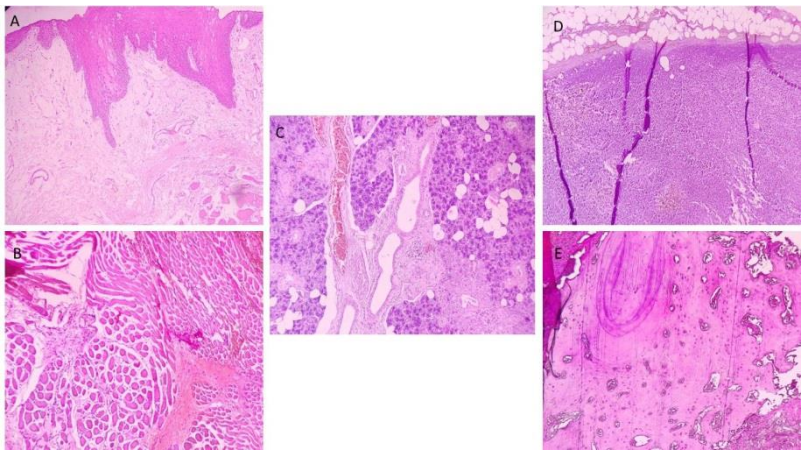

Figure 3. depicts a photomicrograph of tissues stained with H&E (10X), A – mucosa, B- muscle, C – gland, D – lymph node, E – decalcified bone.

Table 2. Comparison of Ethanolic extract of *Vitis vinifera* skin with and without light green counterstain with H & E stain among various tissue types.

Criteria	Mucosa				Muscle				Salivary gland				Lymph node				Bone			
	Vitis vinifera	***Vitis vinifera* + Light Green *n (%)***	**H & E *n (%)***	**p-value**	***Vitis vinifera* *n (%)***	***Vitis vinifera* + Light Green *n (%)***	**H & E *n (%)***	**p-value**	***Vitis vinifera* *n (%)***	***Vitis vinifera* + Light Green *n (%)***	**H & E *n (%)***	**p-value**	***Vitis vinifera* *n (%)***	***Vitis vinifera* + Light Green *n (%)***	**H & E *n (%)***	**p-value**	***Vitis vinifera* *n (%)***	***Vitis vinifera* + Light Green *n (%)***	**H & E *n (%)***	**p-value**
Nuclear details																				
Poor	0(0)	0(0)	0(0)	0.041	2(50)	1(25)	0(0)	0.006	0(0)	0(0)	0(0)	0.014	0(0)	0(0)	0(0)	0.001	1(25)	2(50)	1(25)	0.015
Fair	1(25)	2(50)	0(0)		0(0)	3(75)	2(50)		3(75)	3(75)	1(25)		0(0)	0(0)	0(0)		3(75)	2(50)	3(75)	
Good	3(75)	2(50)	3(75)		2(50)	0(0)	0(0)		1(25)	0(0)	3(75)		4	3(75)	1(25)		0(0)	0(0)	0(0)	
Excellent	0(0)	0(0)	1(25)		0(0)	0(0)	2(50)		0(0)	1(25)	0(0)		0(0)	1(25)	3(75)		0(0)	0(0)	0(0)	
Nuclear staining																				
Poor	0(0)	0(0)	0(0)	0.001	2(50)	1(25)	0(0)	0.067	0(0)	0(0)	0(0)	0.075	0(0)	0(0)	0(0)	0.009	1(25)	2(50)	1(25)	0.026
Fair	1(25)	2(50)	0(0)		0(0)	2(50)	2(50)		3(75)	2(50)	1(25)		0(0)	2(50)	0(0)		2(50)	2(50)	3(75)	
Good	3(75)	2(50)	2(50)		2(50)	1(25)	0(0)		1(25)	1(25)	3(75)		4	1(25)	2(50)		1(25)	0(0)	0(0)	
Excellent	0(0)	0(0)	2(50)		0(0)	0(0)	2(50)		0(0)	1(25)	0(0)		0(0)	1(25)	2(50)		0(0)	0(0)	0(0)	
Cytoplasmic staining																				
Poor	0(0)	0(0)	0(0)	0.003	2(50)	1(25)	0(0)	0.016	0(0)	0(0)	0(0)	0.001	0(0)	0(0)	0(0)	0.001	0(0)	1(25)	0(0)	0.020
Fair	3(75)	4	0(0)		1(25)	2(50)	1(25)		4	3(75)	1(25)		3(75)	4	1(25)		3(75)	2(50)	2(50)	
Good	1(25)	0(0)	3(75)		1(25)	1(25)	3(75)		0(0)	1(25)	3(75)		1(25)	0(0)	3(75)		1(25)	1(25)	2(50)	
Excellent	0(0)	0(0)	1(25)		0(0)	0(0)	0(0)		0(0)	0(0)	0(0)		0(0)	0(0)	0(0)		0(0)	0(0)	0(0)	
Uniformity of staining																				
Poor	0(0)	0(0)	0(0)	0.019	1(25)	1(25)	0(0)	0.004	0(0)	0(0)	0(0)	0.025	0(0)	0(0)	0(0)	0.017	0(0)	2(50)	0(0)	0.006
Fair	1(25)	2(50)	1(25)		3(75)	2(50)	1(25)		2(50)	3(75)	1(25)		2(50)	3(75)	1(25)		4	1(25)	2(50)	
Good	3(75)	2(50)	2(50)		0(0)	1(25)	3(75)		2(50)	1(25)	3(75)		2(50)	1(25)	3(75)		0(0)	1(25)	2(50)	
Excellent	0(0)	0(0)	1(25)		0(0)	0(0)	0(0)		0(0)	0(0)	0(0)		0(0)	0(0)	0(0)		0(0)	0(0)	0(0)	

Statistically significant

DISCUSSION

Vitis vinifera is an important source of red to violet-colored pigment called anthocyanins [15]. Considerable amounts of anthocyanin-containing waste are produced by the food and beverage industry [16]. The skin from waste *Vitis vinifera* fruit is an excellent source of anthocyanins, which have been identified as 3-glucosides, 3-acetylglucosides, and 3-p-coumaroyl-glucosides of malvidin (Mv), peonidin (Pn), delphinidin (Dp), petunidin (Pt) and cyanidin (Cy). During extraction, a proportion of these monomeric anthocyanins become degraded and condensed with other flavonoids to form polymeric pigment [17]. They are frequently used in the food industry as a potent food coloring agent and in the textile industry for the dyeing of wool and cotton fabrics and can be a potential source of dye for staining histopathological tissues [13, 14].

In the present study, the nuclear staining was good in 55%, fair in 30%, and poor in 15% with alcoholic extract of *Vitis vinifera* skin. This could be due to the presence of anthocyanin, which is a weak acid with a pH of 5-6 and has an affinity to the acidic more acidic component of the cell [18]. The nucleus is the acidic component of the cell, due to the presence of nucleic acid within them. Hematoxylin, which is a basic dye, has an affinity to the nucleus, which is acidic, resulting in nuclear staining. This could be a similar mechanism that occurs in *Vitis vinifera* staining [1]. The cytoplasmic staining was poor compared to the nuclear staining. The cytoplasmic staining was fair in 70%, good in 20%, and poor in 10% of cases. This could be due to the basic nature of the cytoplasm, which has an affinity for a more acidic dye [8].

In previous dyeing experiments using grape pomace, intensively colored extracts were obtained during the extraction step, but color yield was unsatisfactory using the standard dyeing process [19]. Mordanting procedures are used to increase the affinity of natural dyes to the textile substrate. They increase absorptivity via hydrogen bonding and van der Waals forces. In the textile industry, the addition of mordants like potassium dichromate improved the light fastness of the dye [13]. The same principle is applied for staining in routine histology. In our study, potash alum was used as a mordant to increase the nuclear affinity as the aluminum ions in the alum have a considerable affinity for DNA and increase the selectivity of nuclear staining [20].

Light Green was used as a counterstain along with *Vitis vinifera* stain to improve the contrast. The addition of light green reduced the nuclear staining efficacy of *Vitis vinifera* by 15%. This could be due to dye-dye interactions, though the effect of anthocyanin and aniline dye interaction is not known at this time [1].

Ethanolic extract of *Vitis vinifera* skin can be used over a wide range of tissues like mucosa, muscle, gland, lymph nodes, and decalcified bone. However, when used to stain a sample with multiple types of tissues, it is questionable whether it would stain all the tissues parallel to that of Hematoxylin and Eosin and hence needs to be explored further. Also, the procedure of extraction of dye needs to be standardized. The affinity of dyes towards various tissues, molecular sizes, and chemical behavior need further exploration. Also, the stain has to be tried in a setting of other stains like Van Geison and Masson's trichrome stain. The entire dyeing capabilities or exact mechanism is not known. Further studies need to be done to substantiate the findings of this study. The role of various additives needs to be evaluated to enhance stain commercialization

This is the first study of its kind to use ethanolic extract of *Vitis vinifera* skin as histological stains. The fact that these sources are rather inexpensive and easily available makes it even better. The complex nature of the dye and its interactions with various tissues has to be further analyzed to use the stain regularly on a large scale. Further studies using different mordants and applying specific staining conditions are needed to check if the stain stands the test of time.

CONCLUSIONS

Based on the results obtained, we conclude that the dye component can be extracted from *Vitis vinifera* skin. This study showed that nuclear staining of histological tissue with ethanolic extract of *Vitis vinifera* skin was comparable to Hematoxylin. Further studies with larger sample size and diverse tissue sections can help formulate a standardized staining protocol. The addition

of counterstain reduced the staining efficacy. Among the tissues, lymph nodes and glands showed the best staining while decalcified bone showed the weakest staining.

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ETHICAL CONSIDERATION

The study was approved by the Institutional Scientific and Ethical Review Board (SRB/SDMDS04/18/OMP/03).

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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