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RESEARCH ARTICLE

Applicability of Butterfly Pea Flower Extract as an Alternative Natural Dye in Histopathological Canine Mast Cell Tumor Diagnosis

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A B S T R A C T

Mast cell tumors (MCTs) are the most common skin tumors in dogs. Clinical data and cytological examination typically suffice for the initial diagnosis. However, prognosis and appropriate treatments necessitate histopathological examination. Amid global concerns regarding hazardous chemicals, the utilization of natural dyes has garnered increased attention. In this context, we focused on using butterfly pea flower (BPF) extract, rich in anthocyanins, as a dye for canine histopathological MCT staining. The dried petal powder was dissolved in distilled water (DW), filtered to obtain a crude extract, and then subjected to a freeze-drying process to preserve its quality. The BPF-dye was prepared by adding DW and 2.5% NaCl into the dried extract. The paraffin serial sections of 32 diagnosed MCTs were stained with the dye for 2 hours. Additionally, the sections were stained with Hematoxylin and Eosin, and Toluidine blue (TB) routine dyes for MCT diagnosis. Sections of non-MCT round cell tumors were also stained with BPF-dye. The results revealed pink-reddish specific staining in the neoplastic mast cell granules, with different staining levels corresponding to the degree of cytoplasmic granulation, similar to TB staining. However, TB staining exhibited blue or metachromasia, indicating differences in staining color. Additionally, the BPF-dye could differentiate MCT from non-MCT round-cell tumors, providing an alternative method of differentiation.
The dye maintained its staining ability for 60 days when stored at 4°C. Accordingly, it holds potential as an alternative dye for MCT diagnosis, being locally available and eco-friendly. Further study should focus on improving the stability and longevity of the dye by preserving its anthocyanin content with natural co-pigments for practical application

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INTRODUCTION

Mast cell tumors (MCTs) are the most common skin tumors in dogs (De Nardi *et al.*, 2022). Although they typically form skin nodules, they also affect other organs such as the liver, spleen, and gastrointestinal tract with a low incidence (Pakhrin *et al.*, 2007). Older individuals of certain breeds, including Boxer, Bulldog, and Labrador are susceptible to MCT development (Shoop *et al.*, 2015; Śmiech *et al.*, 2019). A wide variability of MCT biological behaviors, such as variations in anatomical location, the presence of lymph nodes and distant metastases and the occurrence of paraneoplastic syndromes, complicate clinical management for veterinarians and challenge their ability to provide accurate prognoses to pet owners. This can be assessed by clinical staging and histopathological

grading (Kiupel and Camus, 2019). Histopathological grading is commonly used in canine cutaneous MCTs including Patnaik and Kiupel systems. The 3-tier grading system of Patnaik *et al*. (1984) classifies MCTs based on histopathological features as grade I (monomorphic well-differentiated mast cells), grade II (moderately pleomorphic mast cells), and grade III (poorly differentiated mast cells). However, the novel Kiupel (2-tier) grading system includes high-grade MCT that exhibit any of the following criteria: at least 7 mitotic figures in 10 high-power fields (hpf); at least 3 multinucleated cells in 10 hpf; at least 3 bizarre nuclei in 10 hpf; and karyomegaly. The absence of those characteristics indicates low-grade MCT. High-grade tumors were significantly associated with a poor prognosis and shorter survival times (Kiupel *et al*., 2011). The samples used in this study underwent assessment based on the Kiupel system. To enhance the prognosis accuracy, it is recommended to incorporate immunohistochemistry or molecular techniques in the MCT biological behavior evaluation. This could entail examining factors like proliferation activity or the mutation status of c-Kit. (Gobbo *et al.*, 2021; Sabattini *et al.*, 2015).

Hematoxylin and eosin (H&E) are frequently utilized for histopathological staining. Nevertheless, toluidine blue (TB) or the Romanowsky-type dye such as Giemsa's stain and Wright's stain are recommended for identifying mast cells, as they exhibit metachromasia in their granules (Ribatti, 2018). Most of the dyes currently employed are synthetic, despite their effectiveness. However, these synthetic dyes are expensive, present environmental hazards, and are harmful to health, particularly for laboratory personnel (Ratna and Padhi, 2012). Consequently, there has been increasing attention toward the study of natural dyes as an alternative. Plant anthocyanins, found in various flowers and fruits, are potential candidates for natural dyes due to their vivid blue, red, and purple colors (Khoo *et al.*, 2017). It belongs to flavonoids, similar to hematin, a natural pigment found in hematoxylin (Dapson *et al.*, 2010). Numerous studies have demonstrated the utilization of anthocyanin-rich plants in histological staining protocols (Jasphin *et al.*, 2021). However, butterfly pea flowers (BPF) have received limited attention.

Butterfly pea (*Clirotia ternatea* L.) is indigenous to tropical equatorial regions (Hasanah *et al.*, 2023). Its various parts have been used in traditional medicine due to its richness in biologically active compounds (Chakraborthy *et al.*, 2018; Dhangar *et al.*, 2023). It is now widely cultivated due to the growing popularity of butterfly pea tea, a traditional herb utilized in Southeast Asia as a healthful beverage (Sofyan *et al.*, 2021). BPF-extract is employed as natural food colorants (Roy and Rhim, 2021: Noola *et al.*, 2022). The extract is abundant in ternatin anthocyanins, a group of delphinine glycosides known for their water solubility. These anthocyanins possess a natural pH indicator property, wherein they exhibit a red color in acidic solutions, transition to purple in neutral solutions, and turn blue to greenish-yellow in alkaline solutions (Thuy *et al.*, 2021; Saptarini *et al.*, 2015). Although extensive studies have been conducted on the use of BPF-extract in food manufacturing (Shirodkar *et al.*, 2023), there is limited research regarding its application for tissue staining. Our group has studied the utilization of BPF-extract in animal blood smears and cytological canine MCT staining, which can differentiate blood cell types and identify MCTs, respectively (Suebkhampet *et al.*, 2020; Suebkhampet and Sotthibandhu, 2012). Although most MCTs are easily diagnosed with cytological evaluation, histopathological grading remains crucial. This contributes further diagnostic or prognostic details, assisting in precise treatments. The present study focuses on investigating the staining ability of BPF-extract in histopathological canine MCTs. The longevity of the dye extract after storage was also investigated to determine its potential for further practical application.

MATERIALS AND METHODS

Aqueous extraction of butterfly pea flowers (BPF): The fresh flowers were purchased from Talaad Thai, the wholesale market for agricultural products in Thailand. The petals were air-dried overnight, heat-dried (60ºC) for 5 hours, and finely ground using a blender. The petal powder was dissolved in distilled water (DW) in a 1:10 ratio (Suebkhampet and Sotthibandhu, 2012). Then, the petal powder solvent was centrifuged for 2 hours at 56ºC. The aqueous extract was press-filtered through gauze in filter cloth on a plastic filter funnel and subsequently filtered using Whatman grade 1 qualitative filter paper on a glass funnel.

Freeze-drying process of BPF-extract: The process was implied to preserve the quality and increase the extract concentration. The filtered extract in a glass beaker was covered with punctured aluminum foil (for light protection and sublimation during freeze drying), and frozen at -40ºC)Sanden, SNQ-0205P, Thailand(for 24 hours. Then, it was rapidly moved to the freeze dryer at -107ºC (Labogene Scanvac CoolSafe, FRE4502, Denmark) for 72 hours and stored at -20ºC until used.

Preparation of the BPF-dye: The BPF-dye was prepared by mixing DW and freeze-dried extract (1:5), followed by adding 2.5% NaCl as a mordant. The dye solution was filtered with Whatman grade 1 qualitative filter paper, and the pH was measured using a CyberScan 1000 pH meter (Eutech Instruments Pte Ltd, Singapore). The pH was adjusted to 1.5 using phosphoric acid. This freshly prepared dye solution was classified as day 0 of BPF-dye preparation.

Measurement of total anthocyanin content: Total anthocyanin of the fresh extract and BPF-dye were measured by a pH-differential method using a UV-Vis spectrophotometer (Mapada, V1100D, United States). This was conducted based on the method described by Lee *et al*. (2005). The samples were diluted in KCl (0.025M, pH 1.0) and CH_3CO_2Na (0.4 M, pH 4.5) buffer solutions (1:20), respectively. The absorbance was measured at 520 nm and 700 nm wavelengths. The obtained values were used to calculate total anthocyanin content, expressed as cyanidine-3-glucoside equivalents in mg/L, as follows:

$$\text{Anthocyanin content (mg/L)} = A \times MW \times DF \times 10^3$$

$$\varepsilon \times 1$$

Where A) = $A_{520nm} - A_{700nm}$(pH 1.0) $-A_{520nm} - A_{700nm}$(pH 4.5; MW) molecular weight = (449.2 g/mol for cyanidin-3-glucoside; DF = dilution factor; l= pathlength in cm; ε = 26900 molar extinction coefficients, in L × mol^{-1} × cm^{-1} for cyanidin-3-glucoside; and 10^3 = factor for conversion from g to mg.

Histological samples: The paraffin-embedded samples of 32 diagnosed canine cutaneous MCTs, aged 6 to 17 (10.3 ± 2.8; mean ± SD), were obtained from the Vet and Vitro Central Laboratory and Animal Diagnostic Laboratory Center (ADLC), Mahanakorn University of Technology, Bangkok, Thailand. They were classified according to the Kiupel grading system. Excerpts of clinical data and tumor grades were summarized in Table 1. Non-MCT round cell tumors; histiocytoma, lymphoma (n=2), plasmacytoma, and transmissible venereal tumors (TVT) (n=3), were included to compare with MCTs. All samples were prepared as paraffin serial sections (2 µm of thickness).

Table 1: Clinical data and histological grade regarding the thirty-two dogs with cutaneous MCTs.

Variable	Dogs (n =32)
Sex	
Female	15
Male	11
Unknown	6
Breed	
Thai Ridgeback	8
French Bulldog, Pit Bull, Pug	4
Labrador and Golden Retriever	4
Bang Kaew	3
Shih-Tzu	2
Poodle, Chihuahua	2
Crossbreed	3
Unknown	6
Age (years)	
Adults (2-8)	8
Elderly (>8)	16
Unknown	8
Location	
Limb	8
Head, neck, shoulder	4
Mammary gland	3
Genital area	3
Inguinal area	2
Axilla, frank	2
Skin (not indicate area)	7
Histological grade	
Low-grade	14
High-grade	18

Staining process: The sections were deparaffinized in xylene, and rehydrated through a graded isopropanol concentration (100%, 95% and 70%), and then rinsed in DW. Serial sections were separately stained with H&E, TB and BPF-dye, respectively. The slides were dripping with 200 µl of the BPF-dye for 2 hours in a moist chamber at room temperature (RT). The sections were quickly washed in DW twice, air-dried (RT), dipped in xylene, and mounted using a Permount mounting medium. H&E (Leica Biosystems, Hematoxylin 560 MX, and alcoholic Eosin Y 515, Germany) and TB (Sigma-Aldrich, O T360-5G, Germany) staining were conducted according to the manufacturer's instruction. Sections of non-MCT round cell tumors were stained with BPF-dye as a negative control. The MCT sections were also stained with BPF-dye and stored for 30 and 60 days (at 4ºC) to evaluate the durability of the dye. All stained slides were observed and imaged using a light microscope (ZEISS, Primostar3, Germany) and Labscope version 3.3.1 software.

RESULTS

Total anthocyanin content: Total anthocyanin detected in fresh extract and BPF-dye were 236.46 mg/L and 370.72 mg/L, respectively. The increased anthocyanin content in BPF-dye probably resulted from blending freeze-dried extract with DW in a ratio, which was decreased from 1:10 in the fresh extract to 1:5 in BPF-dye. This enhanced the efficiency of the staining.

BPF-dye staining: The staining results revealed that the BFP dye specifically stained the neoplastic mast cell granules at different levels, varying with the degree of cytoplasmic granulation. It was similar to the TB staining pattern; however, it had a distinct color. BPF-dye staining revealed a pink-reddish color in the granules, while TB staining resulted in a blue or metachromatic appearance. Faintly pink staining was also detected in the tumoral nuclei and collagenous stroma. The same stained areas were observed in MCT serial sections stained with H&E, TB, and BPF-dye, respectively, indicating the specificity of the BPF-dye staining (Fig. 1). Both low-grade and high-grade MCTs with well-differentiated tumoral cells revealed numerous tumor cells stained with BPF-dye. In contrast, high-grade MCTs with poorly differentiated cells exhibited markedly fewer stained tumor cells with the BPF-dye (Fig. 2). The pattern observed was consistent with TB staining. Infiltration of eosinophils was noted within the tumoral tissues, with their nuclei appearing pale pink when stained with the BPF-dye; nonetheless, their granules remained indistinct. (Fig. 2G-, J-white arrowhead).

With H&E staining, well-differentiated tumor cells displayed many finely basophilic granules (Fig. 1L and Fig. 2F). Tumor cells in low-grade MCT were observed clustered or rows between collagen bundles, exhibiting minor nuclear changes (Fig. 2C). In contrast, high-grade MCT displays moderate cellular pleomorphism. In contrast, high-grade MCT displays poorly differentiated tumor cells with moderate cellular pleomorphism, high mitotic activity, and inconspicuous cytoplasmic granules (Fig. 2R). Although the results indicate that BPF-dye is effective in identifying mast cells, it is not suitable for tumor grading, unlike H&E staining. Nevertheless, it acts as a special dye in conjunction with H&E staining for MCT diagnosis.

The BPF-dye maintained its staining ability for about 60 days when stored at 4ºC. On days 30 and 60 of the dye preparation, the staining pattern was similar to that on day 0. However, the intensity was slightly higher, and some dye precipitates were detected in the tissues (Fig. 3A-F). The BPF-dye staining was not detected in histiocytoma, lymphoma, plasmacytoma, and TVT samples. (Fig. 3I, J, K and L).

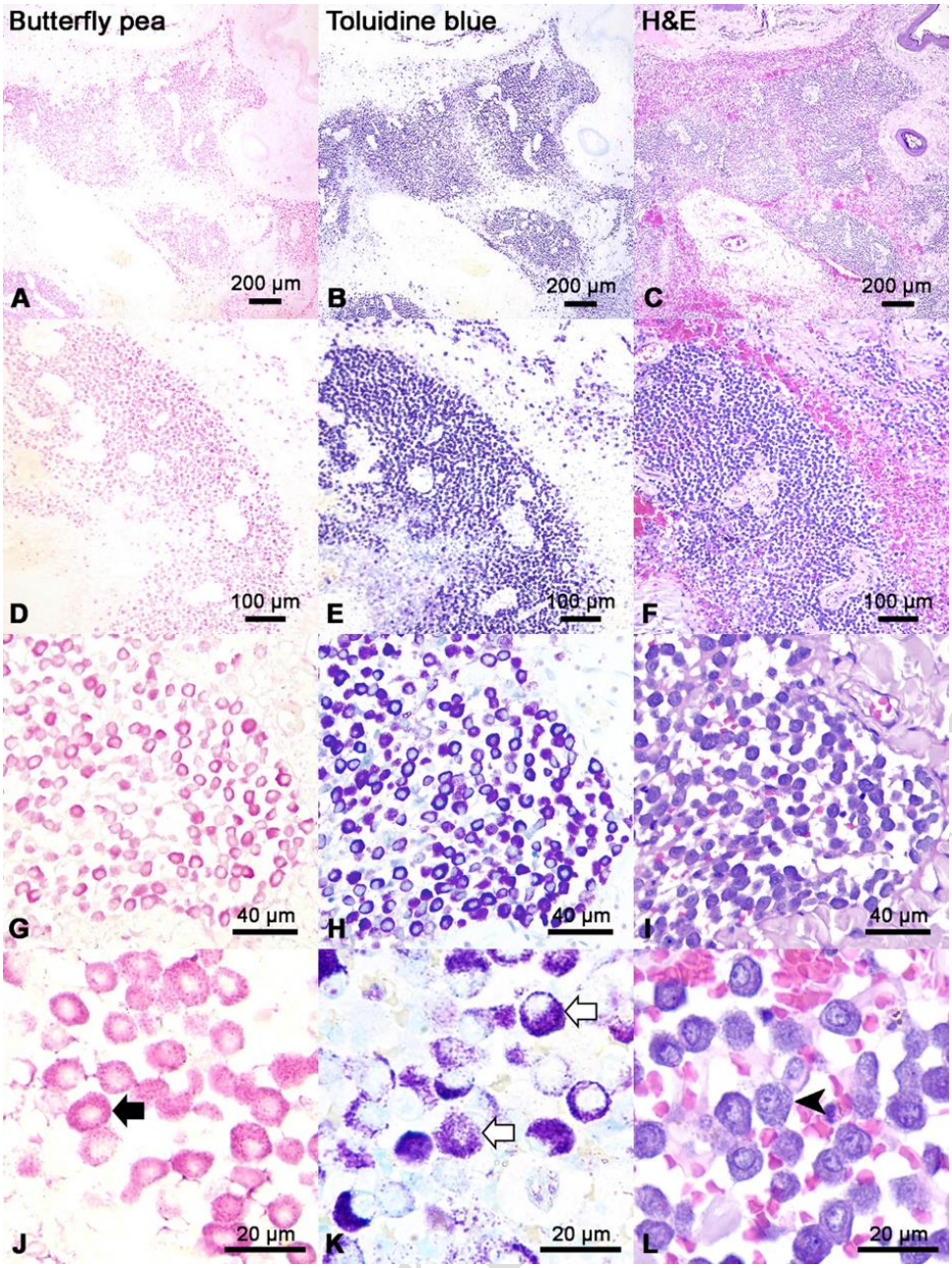

Fig. 1: Low-grade canine MCTs were stained with BPF-dye (A, D, G, J), Toluidine blue (TB) (B, E, H, K), and H&E (C, F, I, L) at different magnifications. The BPF-dye specifically bound to MCT granules, appearing as a pink-reddish color (black arrow). The nuclei and collagenous stroma stained faintly pink. The granules stained blue, or metachromatically with TB staining (white arrows), while finely basophilic granules were detected with H&E staining (black arrowhead). Each dye exhibited a consistent staining pattern within the same area across the serial sections.

DISCUSSION

Different dyes are applied to selectively stain cellular structures. The present study highlights specific staining of the BPF-dye, resulting in a pink-reddish color in the MCT granules. This is possibly due to the binding between the flavylium cations of ternatin anthocyanins in the acidic dye solution and the polyanionic heparins rich in the MCT granules (Ribatti, 2018). As anthocyanins possess the ability to change color with pH (Chu *et al.*, 2016), the acidic dye solution and the microenvironment within the granules affect the coloring. Moreover, anthocyanin aggregations, complexation by organic molecules, and the presence of metal ions have been identified as factors influencing coloration (Quina *et al*., 2009).

The BPF-dye staining was also detected in the MCT nuclei and tumor stroma. This staining pattern may be attributed to the properties of anthocyanins, which have been documented to exhibit binding capabilities to biological membranes, proteins, and DNA. However, their color was lighter than it was in the granules. This may be attributed to electrostatic interactions between the hydroxyl groups of anthocyanins and the polar groups of these macromolecules (Dudek *et al.*, 2023; Jaldappagari *et al.*, 2008). Eosinophils can also be identified to some extent by their pale pink, bilobed nuclei. They are often seen in MCT due to chemotactic factors released by mast cells (Galietta *et al.*, 2023).

MCT serial sections stained with H&E, TB, and BPF-dye revealed consistent staining in the same areas, whereas no staining was observed in non-MCT round cell tumors. These findings affirm the specificity of BPF-dye staining. The staining was highlighted in the MCT granules and showed a similar pattern to TB staining, indicating its potential in identifying mast cells. However, it yielded a different color tone. The staining of MCT with BPF-dye and TB appears to be correlated with the amount and size of the granules and the degree of cellular differentiation. In poorly differentiated tumors, contain diminished concentrations of heparin, which is the main substance to interacts with the dye.

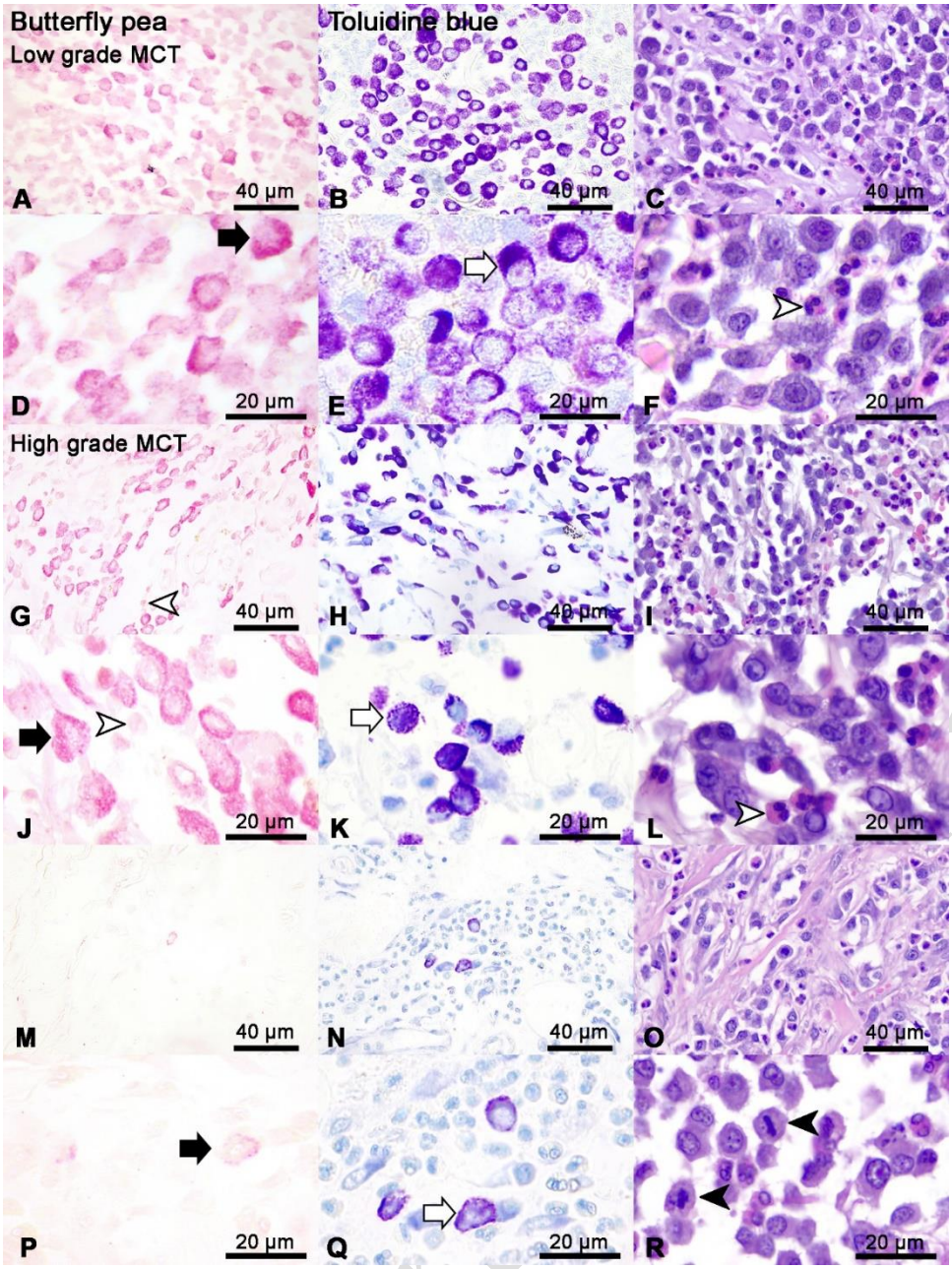

Fig. 2: Low-grade and high-grade canine MCTs were stained with BPF-dye (A, D, G, J, M, P), TB (B, E, H, K, N, Q) and H&E (C, F, I, L, O, R). The BPF-dye staining revealed a pink-reddish color in the MCT granules of both grades (black arrows), exhibiting varying intensity corresponding to the degree of cytoplasmic granulation. This revealed the same trend as TB (white arrows). High-grade MCTs with poorly-differentiated cells revealed a few tumor cells stained with both dyes (M, N). In this case, mitotic figures were occasionally observed with H&E stain (black arrowheads). Eosinophils were frequently seen in MCTs (white arrowheads).

Poor staining may indicate low production of the granular contents (Simoes *et al.*, 1994). Unlike H&E staining, neither of them provided sufficient detail of cell and tissue structures for tumor grading. Therefore, the BPF-dye is suitable to be used as a special stain, like TB, for confirming mast cells in addition to the routine H&E staining.

The results appear to be consistent with our previous study on cytological staining using ethanolic BPF-dye (Suebkhampet *et al*., 2020). However, the number of stained granules in cytological staining was fairly lower compared to histopathological staining using aqueous BPF-dye. Furthermore, no staining has been observed within the MCT nuclei. These variations may arise due to various factors, such as the type of solvent and mordant, dye concentration, pH level, and tissue preparation process all of which influence the staining capability (Jasphin *et al.*, 2023). Some authors have noted that aqueous BPF-extract yields significantly higher anthocyanin content than ethanolic-extract (Netravati *et al.*, 2022; Vidana Gamage *et al.*, 2021), indicating possible benefits in enhancing dyeing efficiency. Considering the advantages of using an aqueous dye, it would be interesting to investigate whether this formula can also effectively stain MCT cytology. Using water as a solvent is both safe and cost-effective.

We included NaCl as a mordant in the BPF-dye similar to TB. Both dyes have pH levels of 1.5 and 2.5, respectively. In contrast, the previous study on MCT cytology used aluminum chloride as a mordant, resulting in a dye with a very low pH of 0.58. Therefore, it is plausible that the similarity in staining patterns observed between the BPF-dye and TB in this study is due to using the same mordant. NaCl is considered safer than other metallic mordants, which are known to be relatively toxic. (Suebkhampet and Naimon, 2014).

Staining remained effective with the BPF-dye, despite being stored at 4°C for 60 days, demonstrating its longevity and potential for reuse. However, we observed dye precipitation in the tissue, prompting the recommendation to filter it with a paper filter before use. Storing the dye at

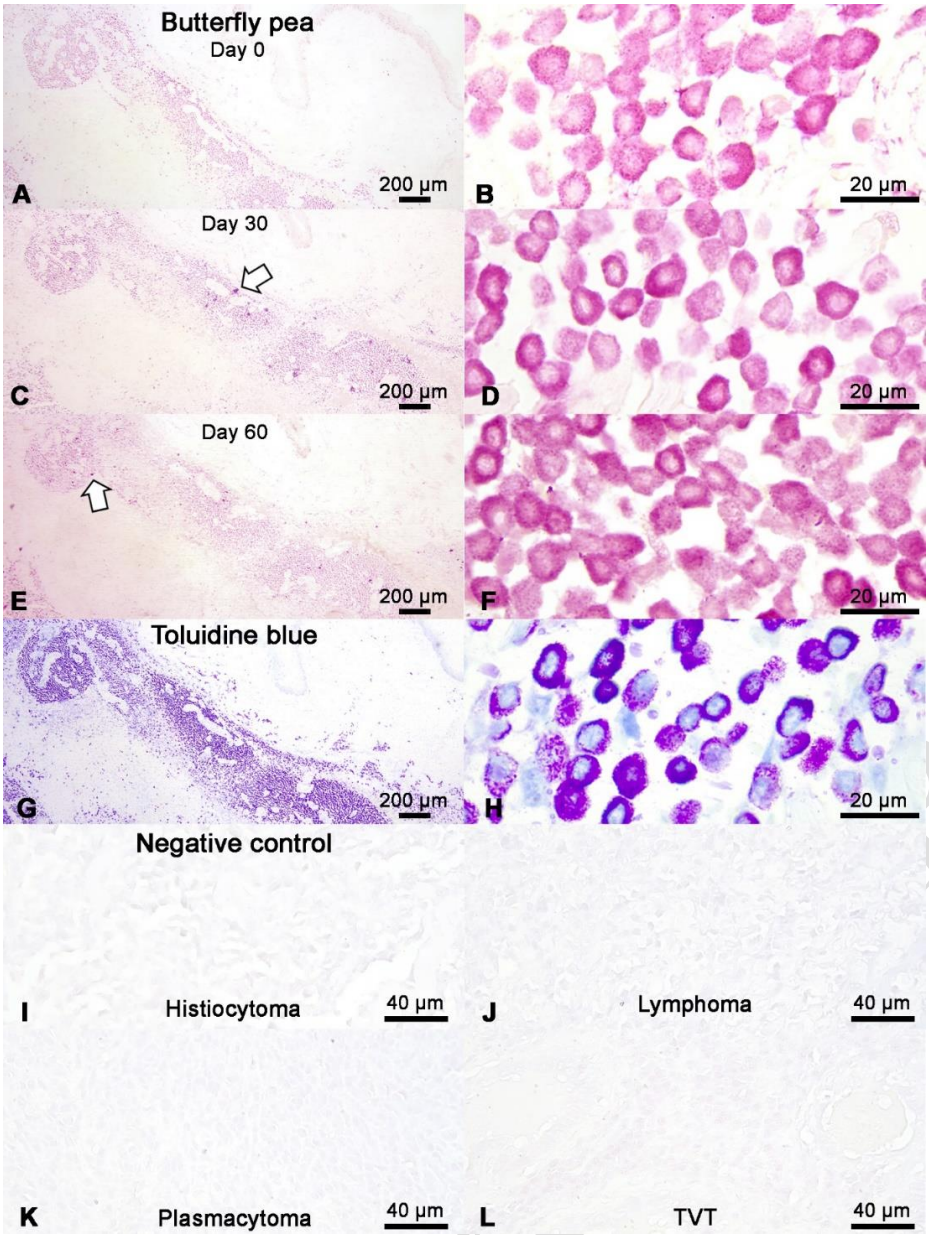

Fig 3: Canine MCTs were stained with BPF-dye on day 0, 30, and 60 of preparation (A-F). The staining pattern observed on days 30 and 60 closely resembled that of day 0 (B) but showed a slight increase in intensity (D, F), accompanied by the detection of some dye precipitates within the tissues (white arrows). TB staining revealed the consistent staining pattern specific to the tumor cells (G-H). No specific staining of the BPF-dye was observed in histiocytoma (I), lymphoma (I), plasmacytoma (K) or TVT (L) samples.

4°C may help minimize anthocyanin degradation due to light and higher temperatures (Vidana Gamage *et al*., 2023). In addition, the acidic dye condition is suitable for its stabilization (Sachdev *et al.*, 2021). Furthermore, freeze-drying helps to retain the anthocyanin content of the BPF-extract. Anthocyanins may be degraded due to a combination of many factors such as light, temperature, pH, solvents, and co-pigmentation (Hou *et al.*, 2013). Co-pigmentation with organic acid can improve anthocyanins' stability by protecting them from the nucleophilic addition of water; otherwise, the flavalium ion becomes pseudobase or colorless (Luo *et al.*, 2022). After 6 months of staining, the tissues were also preliminary assessed to evaluate for the BPF-dye fastness. It found that color intensity had decreased compared to the initial staining, with the color shifting from pink-reddish to a dull purple. This may be due to the alteration or degradation of anthocyanins stained on the tissues. However, it remains adequate for identifying mast cells.

Although the BPF-dye demonstrates effective staining, it requires a longer staining duration compared to TB staining. Further enhancement is achievable through specific developments. For instance, pretreating the dye with natural co-pigments such as catechin or tannin, which are known to bolster anthocyanin stability and color intensity (Charurungsipong *et al*., 2020; Khoo *et al*., 2017), could be explored. Additionally, encapsulating the dye using substances like maltodextrin or Arabic gum has proven effective in preserving anthocyanins, with a half-life of 14 to 19 months, in freeze-dried powder during storage (Hamzah *et al*., 2013; Fuzetti *et al*., 2022). These approaches may improve the preservation of the BPF-dye's anthocyanin content and enhance staining efficacy, leading to a reduction in staining time and consequently making the staining process more efficient. However, a significant limitation is the unstable consistency of the anthocyanin content in the BPF-dye extract from batch to batch. Factors such as plant cultivation, harvesting season, and plant age significantly affect the anthocyanin content in the extract (Andrzejewska *et al*., 2015; Dabravolski and Isayenkov, 2023). Therefore, it is imperative to establish a standardized anthocyanin concentration in the BPF-dye to ensure its practical applicability.

Conclusions: The current investigation presents an innovative method for employing the BPF-dye as an alternative dye in histopathological diagnosis of canine cutaneous MCTs. It offers the advantages of specificity and eco-friendliness. Future research should prioritize enhancing the dye's stability and longevity to facilitate practical application. Additionally, this might broaden its applicability to diverse tissues, including visceral MCTs, consequently enriching diagnostic capabilities within veterinary pathology.

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Authors contribution: MP, PS, and SP performed experiments. AS provided supervision, developed the initial concept, and wrote the article. AS and PS designed the experiments and interpreted the data. PS made the figures and legends. WM advised on the BPF extraction and freeze-drying process. HB compiled samples, and clinical data and interpreted H&E staining. All the authors critically revised the manuscript.

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