Original Article

Butterfly pea flower extract as an alternative dye in cytological canine mast cell tumor staining

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Abstract

Mast cell tumors (MCTs) are the most common skin tumors in dogs. Laboratory diagnosis of MCTs has been established by many techniques. The dyes generally used for tissue staining, including MCTs, are synthetic dyes, however, the global interest in using eco-friendly natural dye has become a significant matter. We focused on the butterfly pea (*Clitoria ternatea L.*) which is locally available. Limited studies are known about their efficacy as a tissue stain. The aim of this study was to investigate the staining ability of butterfly pea flower extract in cytological canine MCTs. The dried petals were ground into powder and dissolved in acidified ethanol to obtain the crude extraction. After filtration, this was added with mordant, 2.5% aluminium chloride, before staining. The methanol-fixed cytological samples of diagnosed MCTs were stained with the dye extract for 30 minutes, washed with distilled water and observed under light microscope initially, and after the counterstaining, with hematoxylin. The slides were stained with Giemsa, Toluidine blue and Diff-Quick stains to compare with the dye extract. The other round cell tumors including canine transmissible venereal tumor (TVT) and melanoma were also stained with the dye extract to compare with the MCTs. The results revealed a bright pink color specifically stained in the MCT granules. However, Giemsa, Toluidine blue and Diff-Quick staining shown their specific stain colors in the granules in larger numbers. The dye extract staining was not detected in either TVT or melanoma. Improvement of the dye extraction and staining conditions may provide additional staining outcomes, thus it may be possible to serve as an alternative natural dye for future application in cytological canine MCT diagnostics.

Keywords: Butterfly pea extract, *Clitoria ternatea*, Stain, Mast cell tumor, Canine

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Introduction

Mast cell tumors (MCTs) or mastocytomas are round cell tumors. They are the most common skin tumors in dogs (London and Thamm, 2013). A low incidence of the tumors has been reported in other organs such as the intestine, liver and spleen (Dorn *et al*., 1968). Middle-aged to older and some breeds of dogs, including Boxers, Labrador, Golden retrievers, Shar-pei and Pugs are at risk and predisposed to the tumors. However, they are not sex-related (Goldschmidt and Shofer, 1992). MCTs are widely variable in appearance and biological behavior, leading to challenges in treatment and making a prognosis. Diagnosis and prognosis are based on historical, physical, cytological and histological examination. Additional detection of some prognosis indices such as Ki67 expression, KIT and tryptase staining pattern may be performed by immunohistochemistry to achieve a more accurate outcome that allows for the selection of the most suitable therapy (Kiupel *et al*., 2004; Dos Santos Costa Poggiani *et al*., 2012). At present, it has been reported that cytological evaluation of MCTs that classify them into low-grade and high-grade tumors is by the novel Kiupel grading system that has a high correlation with histological grading. This scheme eliminates the difficult to predict grade II category, thus, it can be more helpful to assist clinical decisions (Camus *et al*., 2016; Scarpa *et al*., 2016).

There are various kinds of dye used for tissue staining. Most of them are synthetic dyes, including those for MCT cytological staining. Toluidine blue or Romanowsky-type stain such as Wright's stain, Giemsa's stain, Wright-Leishman stain and Diff-Quick are common dyes for such staining. With the worldwide concern for human health and the ecosystem, the use of natural dyes, especially plant dyes, has attracted interest, since several synthetic dyes cause allergic-like symptoms or are carcinogenic (Ratna and Padhi, 2012).

Butterfly pea (*Clitoria ternatea L.*) or blue pea is a climber which belongs to the Fabaceae family. It is native to equatorial Asia, including Southeast Asia. Various parts of the plant are applied in traditional medicine. The medication has potential due to its pharmacological activities such as antimicrobial, anti-inflammatory, antioxidant, analgesic, antidiabetic and vascular smooth muscle relaxing properties (Chakraborthy *et al*., 2018). The blue color of its flowers is the result of anthocyanins that are classified as ternatins (Terahara *et al*., 1998). Anthocyanins are flavonoids of phenolic compounds available in a large variety of fruit, vegetables, flowers and other plant tissues. They are water-soluble pigments that can be used as a natural pH indicator when their colors change to red in acidic solution and in neutral aqueous and basic solution when they remain blue (Khoo *et al*., 2017). Thus, they are also the predominant choice for natural food colorants. At present, plant dyes have been studied for their potential use in various tissues staining in order to substitute chemical synthetic dyes (Suebkhampet and Naimon, 2014; Mohandas *et al*., 2019). There are some anthocyanin rich plant extracts such as red cabbage, dahlia, roselle and turmeric which have been investigated for theirs staining abilities in diverse tissues (Lillie *et al*., 1975; Haddar *et al*., 2018; Rosemary *et al*., 2012; Avwioro *et al*., 2007). However, the dye extracted from butterfly pea flowers has been little reported (Suebkhumpet and Sotthibandhu, 2012). The purpose of this study was to investigate the efficacy of butterfly pea flower extract as an alternative dye on cytological canine MCT staining which is the first report.

Materials and Methods

***Sources of butterfly pea flowers and extraction*:** The fresh butterfly pea flowers were obtained from a market place in Meuang Chachoengsao District, Thailand. Their petals were collected and air-dried in the shade. The dried petals were then ground into fine pieces using a blender before extraction (Suebkhumpet and Sotthibandhu, 2012). The ground petals were dissolved in acidified ethanol with phosphoric acid (pH 4.60) which was chosen as the extraction solvent (Barnes *et al*., 2009; Metivier *et al*., 1980). The pH was measured using a CyberScan 1000 pH meter (Eutech Instruments Pte Ltd, Singapore). The mixture of butterfly pea powder and acidified ethanol at a ratio of 1:10 was then heated at 56 Celsius and centrifuged at the highest speed on a hot plate stirrer machine (Bixby sterilin Ltd, England) for 2 hours (Zou *et al*., 2011; Cacace and Mazza, 2003). During heating and stirring, the glass beaker of mixture was covered with aluminum foil in order to protect the extract from light exposure. Then, filtration of the crude extract was done coarsely in a filtered plastic cone and subsequently in filter cloth.

***Processing of butterfly pea dye for staining*:** The filtrate was added with 2.5% aluminium chloride anhydrous as a mordant (Suebkhumpet and Sotthibandhu, 2012) in a fume hood cupboard (Genconlab, GenconEngineering Co.,Ltd., Thailand). The dye solution was thoroughly mixed and filtrated with No.1 Whatman grade filter papers (Whatman, 1001-150). After filtration, the pH of dye solution was measured and its pH was strongly acidic in the range of - 0.25 to 0.90 (or 0.58 on average). Hence, the dye solution was ready for cytological staining.

***Cytological samples*:** The cytological samples of 7 diagnosed canine MCTs obtained from the Small Animal Teaching Hospital of Faculty of Veterinary Medicine, Mahanakorn University, Bangkok, Thailand were evaluated. The samples were included if they had been subjected to clinical, cytological and histological evaluation. Cytological slides of the tumors were prepared by fine needle aspiration technique (FNA). The cells were air-dried and fixed in absolute methanol for 1 minute. The surgically removed tumors were sent to the Exclusive Veterinary Professional Lab Center Co., Ltd., Bangkok, Thailand in order to perform the histological evaluation. They were classified into 3 low grade and 4 high grade MCTs according to the Kiupel grading system (Kiupel *et al*., 2011). The samples included dogs, aged from 9 to 13 years old (9.9 ± 2.0; mean ± SD). They had cutaneous MCTs that occurred in different parts. Signalment, excerpts of clinical data

and histological grades of individual dog are summarized in Table1. In addition, the cytological samples of 3 diagnosed canine transmissible venereal tumors (TVT) and 1 melanoma and the other types of round cell tumors were also collected to compare with the TVT staining with the dye extract.

Table 1 Signalment, clinical data and histological grade of 7 dogs with cutaneous MCTs.

Case no.	Breed	Sex	Age (yr)	Tumor location	Tumor grade
1	Labrador Retriever	F	9	Abdomen, mammary gland	Low
2	Labrador Retriever	F	9	Inguinal region	High
3	Thai Dog Breed	F	8	Shoulder	Low
4	Thai Bangkaew	F	8	Hind limb, hip, lymph node	High
5	Mixed	M	10	Ear pinna	Low
6	French Bulldog	M	13	Ear pinna, scrotal sac	High
7	Mixed	F	12	Humerus, leg	High

***Cytological staining*:** Cytological slides of the tumors were dipped in the dye solution for 30 minutes at room temperature (25 Celsius) and washed in distilled water. Then, the slides were counterstained with hematoxylin for 15 seconds and washed by soaking in distilled water and, then, in tap water. Dehydration was done in graded series of isopropanol (70% once, 80% once, 95% 3 times and 100%, 3 times, 5 minutes each). Finally, the slides were put in xylene 3 times for 5 minutes each for tissue clearing. After air drying, the slides were observed under light microscope without mounting. All the samples were treated in duplicate. The cytological samples were also stained with Giemsa (VWR international Ltd., Lutterworth, UK), Diff-Quick (Dade Behring Inc., Newark, NJ, USA) and Toluidine blue (BDH Laboratory Supplies, Poole, UK) stains in order to compare with the butterfly pea dye staining. The staining of the last three stains were conducted according to the manufacturer's protocol. The cytological slides of diagnosed canine TVTs and melanoma were also stained with the dye extract to compare with the MCT staining. All the stained slides were examined and images were taken under light microscope using Axio Vision software.

Results

***Cytological staining of canine MCTs with butterfly pea dye*:** Both low grade (n=3) and high grade (n = 4) MCT samples were stained with the dye extract in the tumor cell granules (Fig.1d-h). A larger number of the stained granules were detected in the low grade MCT samples than that of the high grade MCT samples corresponding to the larger number of granules in the low grade tumor cells. The tumor cells were stained bright pink color in theirs granules with the dye extract. The nuclear stain was also detected in their nuclei when the slides were counterstained with hematoxylin which assisted the cellular location (Fig.1d-e, g-h). The slides without counterstaining still revealed specific dye extract stained in the granules (Fig.1f). The dye extract was detected in some of the granules in both conditions. Pictures of low grade MCT (panel a-g) were taken from the same sample. The high grade MCT revealed quite a degree of specific granular staining with the dye extract (panel h). The staining with Giemsa, Toluidine blue and Diff-Quick revealed their stain colors in the granules in larger numbers than the dye extract although they were the same grade MCT (panel a-c). The dye extract staining was not detected in either TVT or melanoma samples (panel i-j).

Discussion

Cellular structures are selectively stained by several dyes. The conditions prevailing in this study reveal particular dye extract staining in the MCT granules. There are many factors associated with the staining such as dye chemical structure and its concentration, kind of solvent, pH and mordant. The dye extract from butterfly pea petals contains mainly ternatin anthocyanins which are water-soluble and pH sensitive pigments. They are derivatives of flavylium cation that can form a series of secondary structures according to a different acid-base, hydration and tautomeric reactions in water (Basílio and Pina, 2016). Their color changes with pH; pink or red in acidic solutions, purple in neutral solutions, blue to greenish-yellow in alkaline solutions. Thus, the acidic dye extract and microenvironment in the granules affects the coloring. It is possible that the granular staining is caused by the chemical structure interaction between cationic ternatins in the dye extract and anionic heparins rich in the granules. The acidic pH was also the optimal condition to protect the stability of the anthocyanins (Laleh *et al*., 2006). This relates to our study in that the staining remained in the MCT granules after the washing step. This was different from our prior study that used aqueous dye extract to stain the animal blood film when their staining disappeared after washing (Suebkhumpet and Sotthibandhu, 2012). However, there were many other factors affecting the staining.

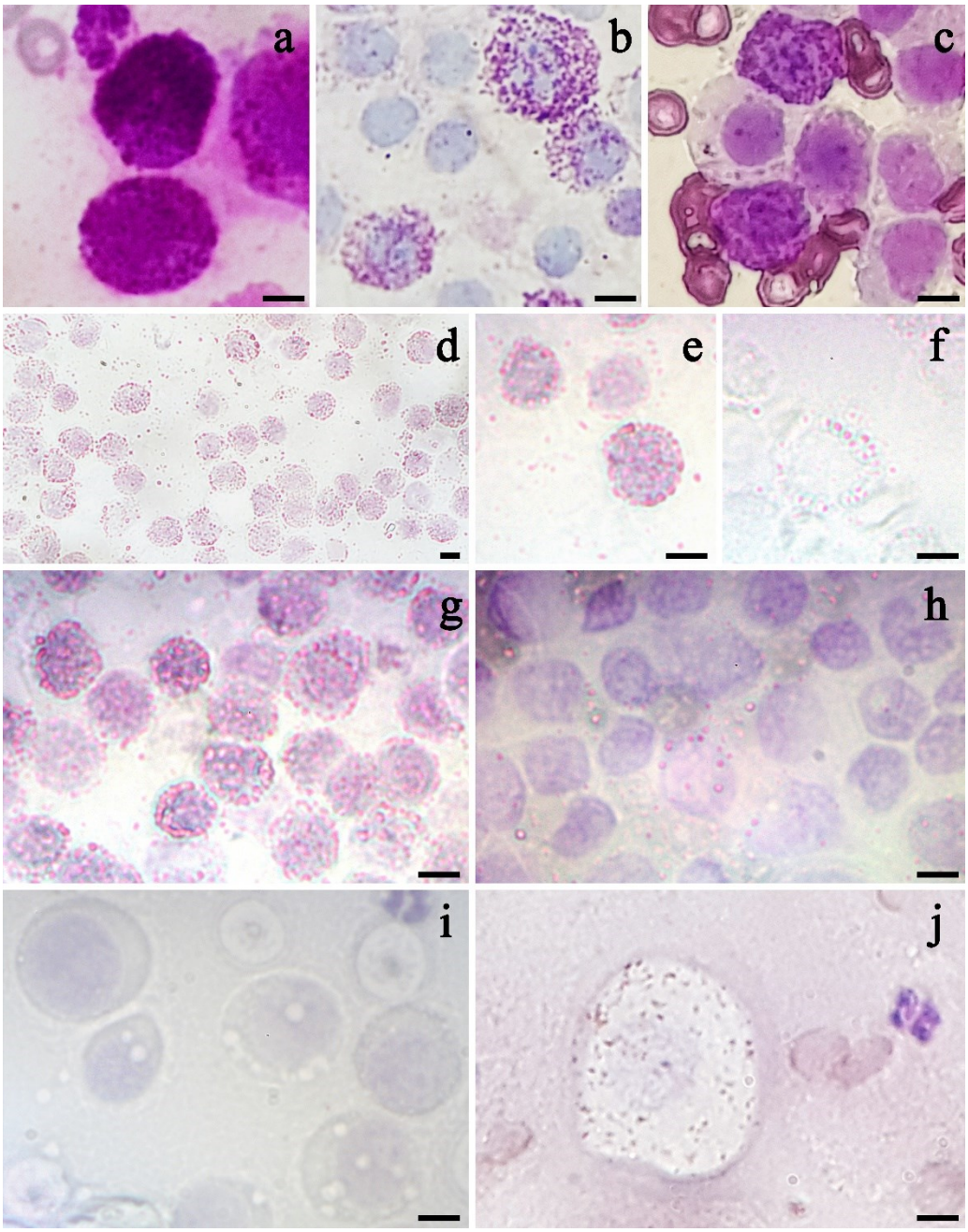

Figure 1 Cytologic low grade of canine cutaneous MCTs stained with Giemsa (a), Toluidine blue (b), Diff-Quick (c), butterfly pea flower extract counterstained with (d-e, g) or without (f) hematoxylin. Cytologic high grade of the MCT stained with the dye extract counterstained with hematoxylin (h). Pink color of the dye extract staining was detected in the MCT granules of both grades. Cytologic canine TVT (i) and melanoma (j) stained with the dye extract and counterstained with hematoxylin. The dye extract staining was not detected in either of these tumors. Scale bar = 5µm.

Mordants commonly included in the natural dye staining protocols affected the dye color on the tissues. Different mordants gave different colors with the same dye. They made the dye colorfast by forming a coordination complex with the dye, then attached to the tissue (Bancroft and Gamble, 2008). Aluminium chloride anhydrous as a mordant used in this study also possessed dye brightening properties (Prabhu and Bhute, 2012). Therefore, the bright pink color in the granules was a consequence of these factors. However, the staining could not be detected in some of the granules. Staining properties of MCTs varied and tended to be associated with the number and size of cytoplasmic granules and the degree of cell differentiation. Poorly differentiated tumors contain lower amounts of heparin, the main component in the granules. Inadequate staining might reflect low or lack of production or storage of the granular contents (Simoes and Schoning, 1994). This is in the same trend as Diff-Quick staining where sometimes MCT granules are not stained with Diff-Quick so it is recommended that they be stained with Wright's stain or Toluidine blue (Sirois, 2020). However, if eosinophils are seen along with large round cells that lack granules, suspicion should be raised for a MCT. The staining in the MCT nuclei was not detected when they were stained with the dye extract, although anthocyanins were known to bind to DNA and RNA through

intercalation between the stacked DNA/RNA bases (Mistry *et al*. 1991). Thus, we counterstained with hematoxylin to more clearly indicate cell location. We did it to avoid any interference to the dye extract staining. It was different from the previous study on animal blood smears stained with the aqueous extract of butterfly pea flowers that revealed faint acidophilic staining in the nuclei of white blood cells and avian red blood cells (Suebkhumpet and Sotthibandhu, 2012). This was probably caused by molecular diversity differences in tissue. These may depend on conditions and chemicals used in the protocols such as solvent, pH, type and amount of mordants being different and affecting their structural conformation and binding ability to the cell components. Other factors such as source of plant cultivation, harvesting season and method, age of the plant used in each batch affecting the amount of anthocyanins in the dye extract (Saptarini and Suryasaputra, 2018; Oguis *et al*. 2019). All of these factors should be figured out to get more effective staining.

Recently, the co-pigmentation effect of catechin, a natural compound found in tea, cocoa and berries, on the stability of anthocyanins in butterfly pea flower extract has been reported. It also intensifies the color extract. Co-pigmentation protects anthocyanins (i.e. flavylium ion) from the nucleophilic addition of water by pretreatment with co-pigments, such as alkaloids, amino acids, organic acids, metals, phenolics and anthocyanins themselves; otherwise, the flavalium ion becomes pseudobase or colorless (Charurungsipong *et al*. 2020). Therefore, this is another issue to be considered for improvement during the dye extract processing.

The number of stained granules with the dye extract in the low grade and high grade MCTs differed to some extent. This was related to the cell granularity and the other factors as mentioned previously. The cytological MCT evaluation needed more criteria that included the presence of mitotic figures, anisokaryosis, binucleation or multinucleation, or nuclear pleomorphism (Camus *et al*. 2016), thus in a real situation, where we do not know the type of tumors before the dye extract staining, the nuclear counterstaining should be also adjusted to make them clearer for observation.

The staining of the dye extract was not detected in TVT and melanoma samples. Thus, in this situation, the dye extract staining could differentiate the cytological MCTs from those round cell tumors. Nevertheless, the other tumors such as lymphoma, plasmacytoma and histiocytoma should also be considered.

Most canine MCTs are easily diagnosed with cytological FNA. However, the histological grading scheme is still one of the most accepted prognostic indicators of their behavior. Nowadays, a 2-tier histological grading system based on morphological characteristics of neoplastic cells, including karyomegaly, multinucleation, nuclear pleomorphism, and mitotic figures has been devised to eliminate the nebulous Patnaik grade II category and to allow for improved interobserver variation (Sabattini *et al*. 2015).

In addition to cytological staining with the dye extract, further study of histological staining should be performed to give more chance to get other diagnostic or prognostic information.

In conclusion, the dye extract can identify canine MCTs in cytological staining. Therefore, it should be developed and applied as an alternative natural dye for cytological MCT staining. The utilization of locally available natural plant dyes for a variety of biological tissue staining will decrease the expense of purchasing synthetic dyes and reduce their effect on humans and the environment.

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