EFFECT OF USING AQUEOUS CRUDE EXTRACT FROM BUTTERFLY PEA FLOWERS *(CLITORIA TERNATEA L.)* AS A DYE ON ANIMAL BLOOD SMEAR STAINING

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Abstract

Butterfly pea *(Clitoria ternatea L.)* is a climber which commonly grows in tropical areas. It is known to store ternatin in its blueish petals. Ternatins are a group of delphinidin glycosides, types of anthocyanin pigment, which can be easily dissolved in water and given different colors according to the pH. An aqueous extract of butterfly pea flowers is traditionally used in food colorants and hair dying. Nowadays, the utilization of natural dyes to replace the use of synthetic dyes in many fields is favored because of public concerns about the safety of synthetic dyes. The objective of this study was to determine the effectiveness of the crude extract from butterfly pea flowers on animal blood smear staining. The crude extract was prepared by soaking the petal powder in distilled water at 4o C overnight and filtering it with gauzes and filter paper, respectively. The filtrate was adjusted to pH 0.2 and treated with a mordant before staining. The methanol-fixed blood smears of 4 different animal species (chicken, pigeon, dog, and horse) were used for the experiments. Preliminary results revealed that faint acidophilic staining was found in the nuclei of nucleated cells in the blood smears of all species. The cytoplasm of red blood cells stained grayish pink with differences of shading. Additionally, dull acidophilic staining was detected in the granules of the chicken heterophils and also the eosinophils of all species. The results indicated that using a crude extract from butterfly pea flowers for blood smear staining was able to differentiate the blood cells. The variation of acidophillic shades on different blood cells might be caused by the ability of the butterfly pea crude extract to change its color according to the pH level. However, the intensity of the staining should be improved by adjusting both the extraction protocols, in order to get more concentration of the crude extract, and also the staining conditions. Thus, it may be modified as a substitute for routine blood smear staining.

Keywords: Butterfly pea, *Clitoria ternatea*, blood smear, staining

Introduction

Butterfly pea or *Clitoria ternatea L.* is a member of the Fabaceae family. It widely grows in tropical areas including Southeast Asia. Its flowers can be white, blue, or purple (Figure 1(a)).

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The blue color of butterfly pea flowers comes from anthocyanins which are classified as ternatins (Terahara *et al*., 1998). Several flavonoids together with quercetin and robonin are also found in the butterfly pea flowers (ILDIS, 1994). The abundant usefulness of the butterfly pea has been documented. It is used as a companion crop, an ornamental plant, or animal feed (Morris, 2009). The physiological actions of butterfly pea in traditional uses and the potential to have valuable nutraceutical (Rao *et al*., 2003; Lau *et al*., 2005; Edwards *et al*., 2007) and pharmaceutical traits (Malabadi *et al*., 2005; Zhang *et al*., 2005; Nothlings *et al*., 2007) have been reported. In Southeast Asia, the flowers are used to color food or are used as food.

The blue color of butterfly pea flowers that represents the existence of anthocayanin is used for colouring food or other things depending on the application. This is consistent with anthocyanin's property that it is easily dissolved in water due to its chemical structure (Figure 1(b)). The difference in chemical structure that occurs in response to changes in pH is the reason why anthocyanins are often used as a pH indicator, as they change from blue in an alkaline solution to red in an acidic solution.

It is known that several histological techniques which are used to provide a nuclear stain consist of natural phenolic compounds, structurally related to anthocyanins. These include carminic acid (used in carmine stain) and hematoxylin. Anthocyanins from red cabbage and dahlia were also used as histological stains (Lillie *et al*., 1975). In addition, a method substituting anthocyanin BB from blackberry juice was developed when a world deficiency of hematoxylin occurred in the 1970s (Al-Tikritti and Walker, 1977). Thus, the anthocyanins in butterfly pea flowers may perform the same results.

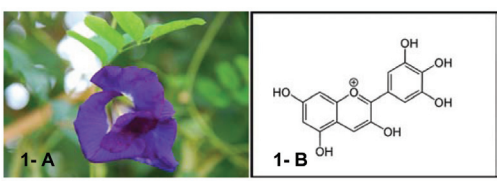

Figure 1. 1-A. Butterfly pea flower, 1-B. Chemical structure of delphinidin

The aim of this study was to determine the effectiveness of the aqueous crude extract from butterfly pea flowers on blood smear staining. The conventional stains used for examination of peripheral blood smears are Giemsa or Wright-Giemsa stains. Alternatively, the Diff-Quick stain is currently favoured by the medical technologist since it can be done by making easy, fast protocols with a good stain quality. However, the commercial dyes are expensive and may contain hazardous chemical components, whereas natural dyes are safe for use, unsophisticated, and harmonized with nature.

Materials and Methods

Preparation of Crude Extract from Butterfly Pea Flowers

Butterfly pea petals were air-dried in the shade and ground into a fine powder. The powder was gradually dissolved in distilled water at a ratio 1:10 at room temperature. The crude extract was collected by initially filtering with gauzes and subsequently with No. 1 Whatman grade filter papers (Whatman, 1001-150). The pH of the crude extract was measured using a CyberScan 1000 pH meter (Eutech Instruments Pte Ltd, Singapore).

Preparation of Butterfly Pea Dye for Staining

The filtered crude extract of butterfly pea petals was added to 1.0% aluminium chloride anhydrous and 1.2% Iron (III) chloride hexahydrate. The dye solution wasmixed well and filtered using No. 1 Whatman grade filter papers. The pH was adjusted to 0.2 using concentrated HCl.

Staining on Blood Smear

The staining process was designed to be conducted on blood smears of chicken, pigeon, dog, and horse. The smears were prepared from EDTA-blood on glass slides. The slides were air-dried and fixed in Diff-quick fixative reagent which contained methanol and triaryl-methane dye for 30-60 sec. The slides were stained with the butterfly pea dye for 30 min.

The staining process was stopped by covering the slides with glass covers without washing and counter-staining. Images of the stained smears were taken under an Axiolab microscope using Axio Vision software. All of the process was conducted under light-protected conditions.

Results and Discussion

The results revealed that faint acidophilic staining was found in the nuclei of the nucleated cells in the blood smears of all the examined species which were stained with the crude extract. However, the nuclei of the chicken and pigeon red blood cells were stained more intensely than the staining found in the white blood cell nuclei (Figure 2). The nuclear staining was strongest in the pigeon red blood cells. The cytoplasm of the red blood cells of all species stained grayish pink with differences in the shading.

The types of the white blood cells can be differentiated by observing the shape of their nuclei and the presence or absence of specific cytoplasmic granules (Bacha and Bacha, 2000), as they can be classified by using conventional dyes. The specific granules of eosinophils were stained in various shades of dark gray or dull acidophilic staining (Figure 2(a1)-2(d1)). The granules of the eosinophils of the horse (Figure 2(d1)-2(d2)) which were large and round to oblong were stained more intensely than those of the other species. The heterophils in the chicken contain rod-shaped or spindle-shaped granules. Their centers sometimes contain a distinctive, spherical granule (Bacha and Bacha, 2000). The magenta in the heterophil granules of chicken was revealed only in the spherical granules (Figure 2(a1)-2(a2), while the grayish stain in the granules of pigeon was found in the spindle-shaped granules (Figure 2(b2)). These may depend on the species-specific properties of the heterophil granules on the dye staining. We did not present the results of the basophil staining since only a small percentage of leukocytes in mammals were basophils, and thus they were not often found in the blood smears. However, basophils of avians, which were more numerous than in mammals, were still hardly differentiated.

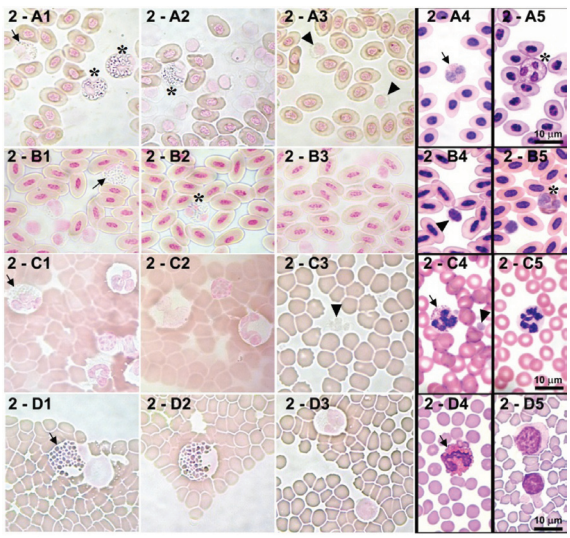

Figure 2. Peripheral blood smear stained with butterfly pea flower crude extract (2-A, -B, -C, -D: 1-3) and Dip-Quick stain (2-A, -B, -C, -D: 4-5). 2-A1 to 2- A5: chicken blood smear, 2-B1 to 2-B5: pigeon blood smear, 2-C1 to 2-C5: dog blood smear and 2-D1 to 2-D5: horse blood smear. Arrows point to eosinophils with various shades of their specific granules dyeing. Asterisks represent heterophils in chicken (2-A1, 2-A2 and 2-A5) and pigeon (2-B2 and 2-B5). The arrow heads in 2-C3 and 2-C4 point to dog platelets and those in 2-A3 and 2-B4 point to chicken and pigeon thrombocytes, respectively

The granules of platelets and chicken thrombocytes (Figure 2(c3) and 2(a3)) were not stained with the crude extract. However, they still can be distinguished from the other blood cells by their whole structure.

Anthocyanins are structurally related to several potent intercalators and are known to bind to purines (Mas *et al*., 2000). Both DNA and RNA can act as strong effective co-pigments for natural anthocyanins (Mistry *et al*., 1991). Since anthocyanins contain cationic in their chemical structure (Figure1 (b)), the interaction between anthocyanins and polynucleotide molecules in the nucleus occurs. Thus, the staining detected in the nucleated blood cells that were stained with the anthocyanins containing the crude extract may have the same reaction. However, the staining intensity was lower than that of the staining with the Diff-Quick stain in the blood smears of all the examined species (Figure2). One reason that might explain this was that we did not add any counter-stain which can help to increase the tissue contrast in our staining procedure. However, the extraction protocol should be adjusted, for instance by using a rotary evaporator to evaporate the solvent in order to get more dye concentration which may improve the staining. In addition, all steps of the experiments should be concerned about light, temperature, and pH which can all affect the stability of the anthocyanin pigments.

The crude extract used in our experiment was adjusted to the acidic pH that was the optimal condition to protect the stability of the anthocyanins (Laleh *et al*., 2006). This may correspond to our preliminary results (data not shown) that the staining was not significantly detected in any blood cells at a pH higher than 0.2. The variation of the acidophillic shades on different blood cells might be caused by the ability of the anthocyanins in the crude extract to change the color according to the pH level. The blood smears were stained without washing since the crude extract containing the anthocyanins was easily washed out by the aqueous solution. However, the background staining was not detected. The mordants (aluminium chloride and ferric chloride) were added into the crude extract as followed in the study by Al-Tikritti and Walker (1977). They used anthocyanin BB from blackberries as a nuclear stain for a hematoxylin substitute. Mordants are regularly included in the dyeing protocols when natural dyes are used in order to fix or intensify the stains in cell or tissue preparations. They are used to set dyes on tissue by forming a coordination complex with the dye which then attaches to the tissue (IUPAC, 1997; Llewellyn, 2005). Different kinds of mordants give a different hue to the staining dye in the cell or tissue. This corresponds to our preliminary results on the blood smears staining. In addition, various kinds and amounts of mordants included in the staining procedure showed the different staining patterns and dye stability on the blood smear (data not show). This is our ongoing work.

Conclusions

The results suggested that using the aqueous crude extract from butterfly pea flowers as a dye could be used to differentiate blood cells in different animal peripheral blood smears as compared with the staining with the Diff-Quick stain (Figure2). However, nuclear staining of the nucleated blood cells should be improved, including adjustments of the extraction protocols and staining conditions. Counter-staining with the cytoplasmic stain and changing the mordant included in the aqueous crude extract before staining may enhance the contrast and color hue of the tissue, respectively. Thus, the aqueous crude extracts from butterfly pea flowers which are domestically available and easy to prepare may be used as an alternative stain for routine blood smear staining. Since the aqueous crude extract might be easily contaminated with microorganisms, the conditions for preserving the aqueous crude extract should be further investigated.

Acknowledgments

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