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Potential of *Clitoria ternatea* L. flower extract as a safe and effective alternative to methylene blue stain

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Abstract

The efficacy of *Clitoria ternatea* L. flower (Pukingan) aqueous extract for use in staining cheek cells was studied to obtain non-toxic, environmentally friendly and low-cost dyes for use in staining. *C. ternatea* L. has a sparsely pubescent stem that sub-erect and woody at the base and may be up to 5 m long. The flower extract of *C. ternatea* L. have anthocyanins which are categorized as ternatins. A number of histological techniques have been identified to be used to provide a nuclear stain consist of natural phenolic compounds, structurally related to anthocyanins. The objective of this study is to determine the efficacy of aqueous extract of Pukingan on cheek cells considering several parameters. Dye extracts from *C. ternatea* L. were used to stain cheek cells using the existing standard staining procedures with little modification. One Way Analysis of Variance was used to analyze differences among the mean scores. A significant difference was determined using a post hoc analysis which is Tukey's test using SPSS and the level of significance was set at 0.05. From the results of the descriptive, parameters for all sample populations have almost similar interpretations. The nucleus was stained satisfactorily, and the sharpness and contrast were excellent. The cytoplasm of the cheek cells was stained intensely, and the stained areas are homogeneous. The prepared extracts had affinity for the cell membrane and nucleus. Therefore, this study shows that dye extracts from *C. ternatea* L. could be used for cheek cell staining as alternative to Methylene blue stain.

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Introduction

The complexity of tissue can be observed in the varied shapes, sizes, and arrangements of cells, and stains are advantageous in revealing these details and more (Nguyen, 2017). Dyes and stains are commonly used in biology to highlight cellular structures in different tissues. While microscopes allow for the magnification of minute anatomical features, dyes provide a clearer and more emphasized view of the specimens. Staining can also be used to highlight metabolic processes or differentiate between live and dead cells, and to enumerate cells to determine biomass in a given environment. Methylene blue is the preferred stain for animal cells, and is often used to view cheek cells in laboratory settings. However, it is toxic when ingested and causes skin and eye irritation (Chew Weng Cheng *et al.*, 2014). Plant extracts are a potential source of natural dyes that produce varying color intensities from yellow to red to black, and are safe for users and the environment due to their herbal and organic nature.

The *Clitoria ternatea* L., also known as butterfly pea or Pukingan, is a tropical legume that can grow vigorously as a trailing, scrambling, or climbing plant. Its stems are sub-erect and sparsely pubescent, and may be woody at the base, reaching up to 5 meters in length. The plant only roots at the tips of its stems, according to studies by Cook *et al.* (2005) and Staples (1992). The leaves are pinnate, with 5-7 elliptical leaflets that are about 3-5 centimeters in length. The flowers are typically solitary or paired, with a diameter of about 4 centimeters and can be either deep blue or pure white in color.

Generally, this study aims to determine the efficacy of aqueous extract of Pukingan (*Clitoria ternatea*) aqueous extract on cheek cells. Specifically, this study aims to compare the mean staining scores of cheek cells stained with: (1) Stain 1, 5gram Pukingan flower/150ml distilled water subjected to 5minute boiling (2) Stain 2, 5gram Pukingan flower/150ml distilled water subjected to 10minute boiling (3) Stain 3, 10gram Pukingan flower/150ml distilled water subjected to 5minute boiling and (4) Stain 4, 5gram Pukingan flower/150ml distilled water subjected to 10minute boiling based on the following parameters: Nuclear staining; Nuclear Detail; Cytoplasmic staining and Uniformity of Stain.

Since many synthetic dyes pose a hazard to the health of students and those who prepare them, this study also aims to make use of a natural alternative to Methylene blue stain. Roots, Fruits, leaves and flowers from dye-producing plants can hypothetically provide an effective stain. An abundant number of potential plants with staining properties sprout throughout the Philippine setting, and one of them is *Clitoria ternatea* L. (Pukingan). The Fabaceae family in general has anthocyanins, which are water-soluble, vacuolar pigments capable of staining cellular structures. Therefore, *Clitoria ternatea* L. (Pukingan) presents a promising alternative as a non-toxic, accessible and cost-efficient organic chemical stain.

Materials and methods

Study Area

The research was carried out at the Science Department within Carigara National High School, between the months of September and October 2018.

Collection of Plant Materials

Pukingan (*Clitoria ternatea L.*) were collected at the Beengo Farm at San Vicente; Tunga, Leyte, Philippines. Flowers of (*Clitoria ternatea* L.) Pukingan were brought to a Biology class of Carigara National High School and was further verified through the International Society for Taxonomic Explorations page and the paper of Deshmukh and Desai of Department of Botany, The New College, Kolhapur, India.

Preparation of Flower Extract

Dye extracts were prepared by boiling the flowers with the use of distilled water as a solvent. At the end of each extraction procedure, the extracts were purified by a filtration process. Filtration was achieved by passing the extract through No. 2 Whatmann grade filter paper. The supernatant was then transferred into four (4) plastic bottles and labeled as Stain 1, 2, 3 & 4. Set-up 1 (Stain 1), 5grams of flower with 5minutes of boiling, Set-up 2 (Stain 2) 10grams of flower with

10minutes of boiling, Set-up (Stain 3) 3 5grams of flower with 10minutes of boiling and Set-up 4 (Stain 4) 10grams of flower with 5 minutes of boiling.

Staining Procedure

Cheek cells used in the experiments were obtained from the researchers by scraping the inner buccal area of the cheeks using wooden flat sticks. The cheek cell staining was administered to the prepared slides following the standard protocol of staining cheek cells. Observation of all the prepared slides were made and evaluated microscopically using a Compound Light Microscope with 40x magnification and their staining intensity was identified. Photographs of selected preparations were taken using Vivo Y53 camera.

Statistical Analysis

High-definition pictures of stained cells were provided and were graded by selected 5 Biology teachers who have good background in cellular staining and have been teaching Biology and Science related subjects for more than 5 years. Scores from all score sheets were tallied. The mean scores from each test group, were obtained. One Way Analysis of Variance was used to analyze differences among the mean scores. A significant difference was determined using a post hoc analysis which is Tukey's test using SPSS and the level of significance was set at 0.05. To interpret the results, the following hypothetical mean ranges and their corresponding interpretations are proposed:

3.01-4.00 Chromatin clear and sharp; nuclear border visible; Nuclei stain intensely; sharpness and contrast of nucleus excellent; Cytoplasm stains intensely; sharpness and contrast of cytoplasm excellent; Stained areas homogeneous; unevenly stained areas non-existent

2.01-3.00 Chromatin somewhat clear and somewhat defined; nuclear border somewhat delineated; Nuclei stain satisfactorily; sharpness and contrast of nucleus satisfactory; Cytoplasm stains satisfactorily; sharpness and contrast of cytoplasm satisfactory; Stained areas show some heterogeneousity

1.01-2.00 Chromatin unclear and poorly defined; nuclear border ill defined; Nucleistain poorly; sharpness and contrast of nucleus non-existent; Cytoplasm stains poorly; sharpness and contrast of cytoplasm non-existent; Absence of homogeneousity

0.00-1.00 Chromatin and nuclear border not visible; Nuclei not stained; Cytoplasm not stained; All of the slide is stained unevenly

Results and discussion

The examination of cells under microscopes after grading revealed that the use of *Clitoria ternatea* flower extract resulted in the staining of cheek cells. Tables 1 to 4 were included to provide descriptive results of the groups involved in the study. These tables were interpreted based on the hypothetical mean ranges of each parameter, and their interpretations were presented in the preceding section. The results of the study are presented in Tables 1, 2, 3, and 4, which provide a descriptive analysis of the slides stained with Stains 1, 2, 3, and 4, respectively. The parameters for all sample populations have similar interpretations. The chromatin of the cheek cells appears to be somewhat clear, while the nucleus is satisfactorily stained. The cytoplasm of the cheek cells was also satisfactorily stained, with satisfactory sharpness and contrast. However, some heterogeneity is observed in the stained areas.

Table 1. Descriptive results of stain 1.

	Nuclear Detail	Nuclear Staining	Cytoplasmic Staining	Uniformity of Stain
Rater 1	1.00	2.00	2.67	1.00
Rater 2	3.00	2.33	3.00	3.00
Rater 3	2.00	2.33	2.33	2.33
Rater 4	2.67	2.67	3.00	3.00
Rater 5	2.00	3.00	4.00	3.00
Average	2.13	2.47	3.00	2.47
	Chromatin somewhat clear and somewhat defined; nuclear border somewhat delineated	Nuclei stain satisfactorily; sharpness and contrast of nucleus satisfactory	Cytoplasm stains satisfactorily; sharpness and contrast of cytoplasm satisfactory	Stained areas show some heterogeneousity

Table 2. Descriptive results of stain 2.

	Nuclear Detail	Nuclear Staining	Cytoplasmic Staining	Uniformity of Stain
Rater 1	2.00	2.00	2.67	1.33
Rater 2	3.33	3.33	3.00	4.00
Rater 3	3.00	3.00	3.00	3.00
Rater 4	2.33	2.67	3.00	2.67
Rater 5	2.00	2.67	2.67	4.00
Average	2.53	2.73	2.87	3.00
	Chromatin somewhat clear and somewhat defined; nuclear border somewhat delineated	Nuclei stain satisfactorily; sharpness and contrast of nucleus satisfactory	Cytoplasm stains satisfactorily; sharpness and contrast of cytoplasm satisfactory	Stained areas show some heterogeneousity

Table 3. Descriptive results of stain 3.

	Nuclear Detail	Nuclear Staining	Cytoplasmic Staining	Uniformity of Stain
Rater 1	1.00	2.00	1.33	1.00
Rater 2	4.00	4.00	4.00	4.00
Rater 3	2.33	2.67	2.33	2.33
Rater 4	2.67	2.00	2.33	2.00
Rater 5	1.00	1.00	2.00	2.00
Average	2.20	2.33	2.40	2.27
	Chromatin somewhat clear and somewhat defined; nuclear border somewhat delineated	Nuclei stain satisfactorily; sharpness and contrast of nucleus satisfactory	Cytoplasm stains satisfactorily; sharpness and contrast of cytoplasm satisfactory	Stained areas show some heterogeneousity

Table 4. Descriptive results of stain 4.

	Nuclear Detail	Nuclear Staining	Cytoplasmic Staining	Uniformity of Stain
Rater 1	1.67	1.67	1.67	1.00
Rater 2	3.33	3.00	3.33	4.00
Rater 3	2.00	2.00	2.00	2.00
Rater 4	2.00	2.00	2.00	2.33
Rater 5	2.00	2.00	2.00	2.00
Average	2.20	2.13	2.20	2.27
	Chromatin somewhat clear and somewhat defined; nuclear border somewhat delineated	Nuclei stain satisfactorily; sharpness and contrast of nucleus satisfactory	Cytoplasm stains satisfactorily; sharpness and contrast of cytoplasm satisfactory	Stained areas show some heterogeneousity

Fig. 1. *Clitoria ternatea L.* Flower.

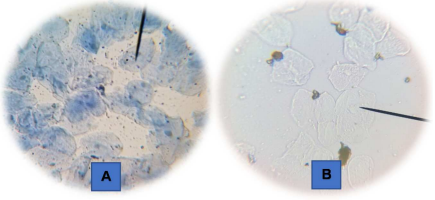

Fig. 2. Micrograph of cheek cells with *Clitoria ternatea* L. flower extract (A) and without stain (B).

Table 5, which displays the ANOVA table, indicates that there is a noteworthy difference in the average ratings of the raters across various parameters. This is supported by the p-value of 0.01, which is lower than the level of significance set at 0.05. In fact, the different groups exhibited significant differences in comparison to each other, which suggest that the number of grams used and the boiling time for making the extract are influential factors in staining cheek cells.

Table 6 clearly shows that the performance of Stains 2-3 and Stain 2-4 is markedly different from that of Stain 1. It is worth noting, however, that Stains 3 and 4 exhibit similar performance and have no statistical difference as indicated by p-values greater than 0.05.

Table 5. One Way ANOVA for the comparison of mean ratings.

Source	sum of squares SS	degrees of freedom *vv*	mean square MS	F statistic	p-value
Treatment	0.8004	3	0.2668	5.9232	0.01
error	0.5406	12	0.045		
total	1.341	15			

Table 6. Post hoc analysis for piecewise comparison of mean ratings.

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
Stain 1 vs Stain 2	2.4972	0.3351122	insignificant
Stain 1 vs Stain 3	2.0496	0.4952046	insignificant
Stain 1 vs Stain 4	2.9919	0.2030736	insignificant
Stain 2 vs Stain 3	4.5467	0.0326061	* p<0.05
Stain 2 vs Stain 4	5.4891	0.0101606	* p<0.05
Stain 3 vs Stain 4	0.9423	0.8999947	insignificant

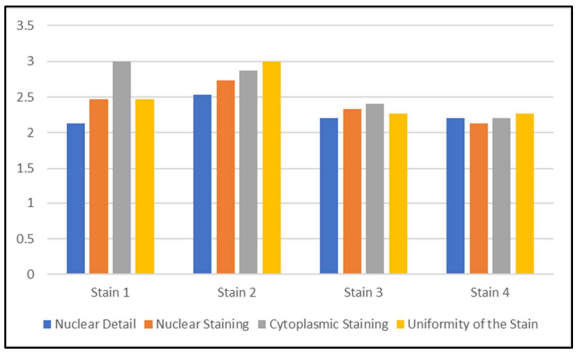

Fig. 3. Comparison of factor averages between groups.

Clitoria ternatea L. flowers contain anthocyanins, which are plant pigments responsible for producing red, violet, and blue colors in plants (Vidana *et al.*, 2021). When staining cells, the concentration of anthocyanins can become high enough to cause the pigment to separate in a crystalline or amorphous form, allowing the anthocyanins to be absorbed by the materials within the cell (Onslow, 2014). Anthocyanins are also structurally related to several powerful intercalators that tend to bind to purines (Webb *et al.*, 2008). Both DNA and RNA can act as potent co-pigments for natural anthocyanins (Mistry *et al.*, 1991). The efficiency of flower extract as source of dye for staining animal sections was narrowly identified. The present findings on the competence of dye extract of *Clitoria ternatea* L. recognized the fact that dye extract stain of *Clitoria ternatea* L. could be successfully utilized for cheek cell staining.

Conclusion and recommendation

The study revealed that the extract of *Clitoria ternatea* L. flower (Pukingan) can be utilized to stain cheek cells, effectively highlighting their cellular structures such as the cytoplasm and nucleus. Interestingly, this micro technique procedure did not involve the use of any solvents, reducing the involvement of synthetic chemicals. The increased concern worldwide regarding the use of hazardous synthetic chemicals has led to a growing interest in cost-effective, eco-friendly, and biodegradable materials, with natural dyes receiving significant attention among scientists (Eom *et al.*, 2001). The identification of the exact dye component in *Clitoria ternatea* L. that can stain animal cells could lead to promising avenues for future research.

Improvements to the staining protocols and conditions are necessary to enhance nuclear staining of specimens, particularly for cheek cells. The addition of a mordant could be used to make the stain more stable. However, the use of domestically available and easily prepared dye extracts from *Clitoria ternatea* L. is a feasible option for staining cheek cells and can be adopted in any science classroom to facilitate simple staining procedures.

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