JURNAL KESEHATAN

DOI: 10.63323/jkes.v14i01.215
ISSN: 2302-0911; e-ISSN: 2685-1040

OPEN ACCESS

Original article

Effectiveness of Butterfly Pea Flower Extract (*Clitoria ternatea*) as a Natural Alternative Dye to Replace Methyele Blue Diffquick Coloring in Buccal Smear Cytology Preparations

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Abstract

Introduction: Diffquick dying is one the methods of cytological dying. The diffquick method's components is methylene blue. Methylene blue is toxic and non-biogradable so it requires natural dyes that do not have harmful properties for the body. *Clitoria ternatea* is a natural colorant that has an anthocyanin content and produces blue pigment and has been tested to color the nucleus of epithelial cells.

Method: This research for the influence of temperature factors and long storage of the solution of clit extract as a nutarl colour subtitutes for methylene blue in the coloring of nucleus cells diffquick method. Research design is experimental with purposive sampling. The research was conducted in January-March 2024 at the sitohistotechnology laboratory.

Results: The Mann-whitney (sig.) test result 1.0>0.05 or H0 was received so there was no difference in the core color power of the cell with a 16% concentration of oak flower extract stored at 7-11ºC dan 25ºC as a substitute for the methylene blue dye in diffquick dyeing.

Conclusion: This shows that there is no difference in the color power of the cell from the solution of the butterfly pea extract that are stored at showcase and room temperature

Keywords: Butterfly pea flower, Extract, Diffquick, Methylene Blue, Buccal Smear.

Received April 15, 2025; Received in revised form April 17, 2025; Accepted April 19, 2025; Available online April 21, 2025

INTRODUCTION

Cytology examination is a study of the normality of cell morphology obtained from various parts of the body (Mutoharoh et al, 2020). One of the functions of cytology examination is as an early detection of precancer in the oral cavity (Permasutha, 2021). The accuracy of the diagnosis depends on the quality of the preparation that has been produced based on the quality of the preparation, preparation preparation, coloring, and interpretation of the preparation. One of the cytology staining techniques is staining with the method diffquick (Mutoharoh et al, 2020).

Method diffquick is a derivative of dye romanovsky which is used to stain cells derived from blood and non-gynecological samples (Mutoharoh et al, 2020). The advantages of staining diffquickthat is, it only requires a soaking time of 15 minutes and can have quite clear staining results between the cytoplasm and nucleus of the oral mucosal epithelial cells (Aini et al, 2023). The composition of the reagents used by staining diffquick are methanol, eosin and methylene blue (Mizan et al, 2021). Dye methylene blue is a cationic dye or has positive ions in it Methylene blue used to stain cell nuclei. Methylene blue is an azo compound and a benzene group derivative which has properties non- biodegradableand toxic so that it can cause genetic mutations, respiratory irritation if inhaled and cause skin irritation (Azka et al, 2021).

Based on the problems and impacts caused by methylene blue then it is necessary to conduct research on search for natural alternative dyes as substitute dyes methylene blue. One of the natural ingredients that has the potential to be an alternative dye to replace methylene blue namely butterfly pea flower extract. butterfly pea flower extract carried out at a concentration of 1:5 soaking for 15 minutes can color the mucosal epithelium better or more contrastingly than soaking for 2 minutes and 5 minutes (Azka et al, 2021). Research on the effectiveness of butterfly pea flower extract on cytology preparations buccal smearhas been carried out at a concentration of 1:5 using the method diffquick. However, previous research has only conducted a few tests on the stability and optimum storage temperature of flower extract

solutions as substitute dyes. methylene blueon the method diffquick.Therefore, in this study, a test will be carried out on the stability of the coloring ability of butterfly pea flower solution with storage temperature. Showcase and storage at room temperature 25ºC and testing of anthocyanin content in the coloring of the butterfly pea flower extract solution. This is because if the purpose of the butterfly pea flower solution telang as a substitute for solution methylene bluethen the solution must also have long-term stability in coloring epithelial cells so that it functions as a substitute for the core dye in the method.diffquick more efficient and environmentally friendly.

METHOD

Research Hypothesis is a difference in the color strength of the cell nucleus of the butterfly pea flower extract solution with a concentration of 16% which is stored at a storage temperature of 7-11ºC and a temperature of 25ºC as a substitute for dye. methylene blue on the coloring method diffquick. This type of research is the relationship between quantitative variables. The design of this study is experimental. The selection of this research design was chosen because the researcher intervened in storage temperature on the quality of core color in epithelial cells.

The research sample was taken in the laboratory 304 STIKes Mitra Keluarga in the form of oral mucosal swabs. Extraction, coloring, and observation were carried out microscopic on cytology preparations buccal smear conducted in laboratory 304 STIKes Mitra Keluarga. This research was conducted from January to March 2024. The sample of this research is a sample buccal smear taken from final year students of STIKes Mitra Keluarga DIII Medical Laboratory Technology study program with a total of 15 samples. The independent variable in this study is the storage temperature of the butterfly pea flower extract solution. The dependent variable in this study is the epithelial cell profile as seen from the score of cell shape, cytoplasm color intensity and cell nucleus.

The data collection technique in this study was carried out by means of observation. Observation is a data collection technique carried out through observation, accompanied by records of the condition or behavior of the target object. The researcher will conduct direct observation of the cytology preparation buccal smear.

RESULTS

Butterfly Pea Flower Extract Solution

Butterfly pea flower extract that has been made from butterfly pea flower powder using aquades solvent which is left for 24 hours. The extract results are filtered using filter paper. The filtered extract results have a blue color (Figure 1).

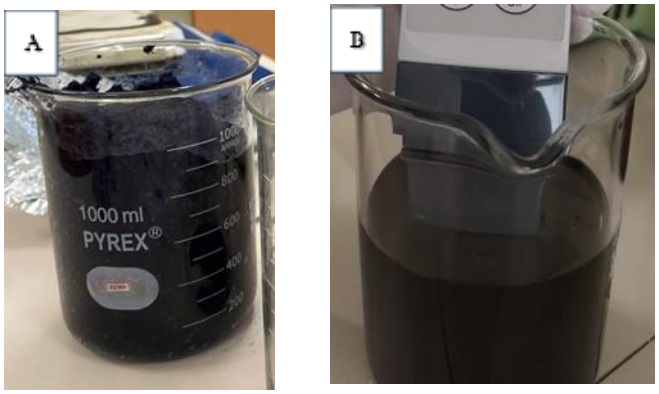

Figure 1. Results of butterfly pea flower extract, (A) Results of butterfly pea flower extract, (B) Results of filtered butterfly pea flower extraction.

pH Test of Butterfly Pea Flower Extract

The butterfly pea flower extract solution that has been made with a concentration of 16% is stored in a refrigerator or showcase and the pH is measured for 5 days using a pH meter. The results of the butterfly pea flower extract that have been measured using a pH meter show that the average pH of the extract solution stored at showcase temperature has an average pH of 4.67 and the butterfly pea flower extract solution stored at room temperature has an average pH of 4.43. So it is concluded that the pH of the two butterfly pea flower extract solutions each has a pH of 4 or acidic in Table 1.

Table 1. Results of pH measurements of extract solutions stored at showcase temperature and room temperature.

Storage days	Temparature Showcase (℃)	Room Temperature (℃)

First Day	4.93	4.14
Second Day	4.54	4.55
Third Day	4.64	4.21
Fourth Day	4.66	4.62
Fifth Day	4.62	4.64
Average pH	4,67	4,43

Flavonoid Test

The results of the butterfly pea flower extract solution were tested for flavonoids. The results obtained in the extract were the formation of a yellow fluorescent color. The extract solution was reacted with 10% NaOH, the formation of the yellow fluorescent color indicated the presence of anthocyanin flavonoid compounds in the butterfly pea flower extract solution in Figure 2.

Figure 2. The presence of anthocyanin flavonoid compounds

Test Preparation Assessment Results

Butterfly pea flower extract solution as a substitute for methylene blue dye that can color the cell nucleus so that it is pink. Testing was carried out on 90 buccal smear preparations using the Diffquick method which was carried out at the Cytohistotechnology Laboratory of Mitra Keluarga Health Science College Table 2.

Table 2. Data from the results of assessing the quality of test preparations using butterfly pea flower extract coloring.

Day	Preparat	Storage Temperature of Butterfly Pea Flower Extract		Control
		Showcase Temperature	Room Temperature	
1	A1	3	3	4
	A2	3	3	4
	B1	3	3	4
	B2	3	3	4
	C1	3	2	4
	C2	3	3	4
2	D1	3	2	4
	D2	3	2	4
	E1	2	3	4
	E2	2	3	4
	F1	3	3	4
	F2	3	3	4
3	G1	3	4	4
	G2	3	4	4
	H1	3	3	4
	H2	3	3	4
	I1	3	3	4
	I2	3	3	4
4	J1	3	3	4
	J2	3	3	4
	K1	3	3	4
	K2	3	3	4
	L1	3	3	4
	L2	3	3	4

5	M1	3	3	4
	M2	3	3	4
	N1	3	3	4
	N2	3	3	4
	O1	3	3	4
	O2	3	3	4
Average		2.93	2.96	4

Buccal Smear Preparation Image in Test Solution

The results of the observation of the quality of the first day's preparation in Figure 3. (A) using a solution of butterfly pea flower extract as a substitute for methylene blue with a concentration of 16% stored at showcase temperature with the description of the quality of the cell shape clearly visible cytoplasm and nucleus, the intensity of the cytoplasm color is clear with a pink color and the cell nucleus has a clear color intensity with a thicker pink color. Figure 3. (B) the researcher conducted a test using a solution of butterfly pea flower extract as a substitute for methylene blue with a concentration of 16% stored at room temperature with the description of the cell shape clearly visible cytoplasm and cell nucleus, the intensity of the cytoplasm color is clear with a thin pink color and the intensity of the cell nucleus color is clear with a thick pink color. Figure 3. (C) epithelial cells are seen using diffquick staining as a control. The quality of the control solution is seen in the cell shape and purplish blue color, epithelial cell fragments are very clear because the background of the epithelial cells of the preparation looks faint, the intensity of the cell nucleus color is very clear with a dark purple color.

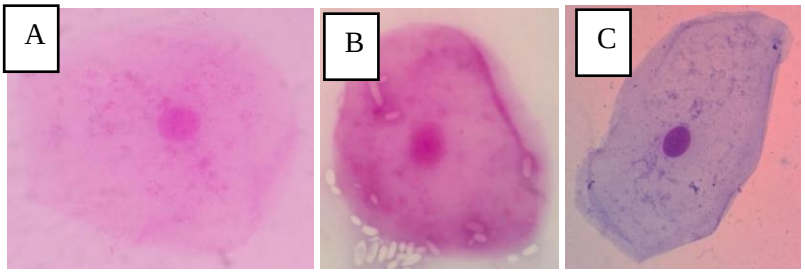

Figure 3. The intensity of the cytoplasm color

The results of the observation of the quality of the preparation on the second day in Figure 4. (A) using a solution of butterfly pea flower extract as a substitute for methylene blue with a concentration of 16% stored at showcase temperature on the second day with the description of the quality of the cell shape is not clear, the presence of cytoplasm and nucleus, the intensity of the color of the cytoplasm and cell nucleus is not clear. Figure 4. (B) The researcher used a solution of butterfly pea flower extract that had been stored at room temperature on the second day with the description of the cell shape clearly visible cytoplasm and nucleus, the intensity of the color of the cytoplasm is clearly visible with a thin pink color and the intensity of the color of the cell nucleus is clearly visible with a thicker pink color. Figure 4. (C) shows buccal smear epithelial cells using control staining have a quality preparation, the cell shape is very clear with cell fragments very clear because the background of the epithelial cells looks faint, the intensity of the color of the cell nucleus is very clear with a dark purple color.

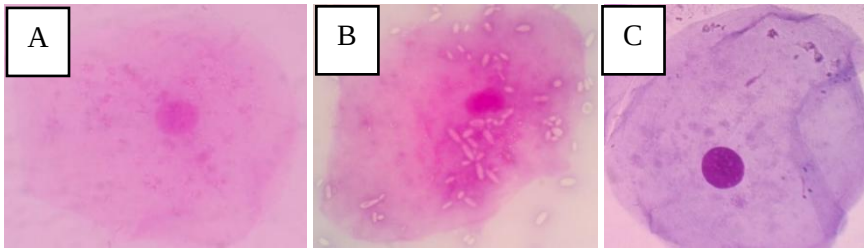

Figure 4. Buccal smear staining results on the second day, (A) Staining results of the butterfly pea flower extract preparation stored at showcase temperature on the second day, (B) Staining results of the butterfly pea flower extract preparation stored at room temperature on the second day, (C) Staining results of the buccal smear preparation using the diffquick method

The results of the observation of the quality of the third day's preparation in Figure 5. (A) using a 16% concentration of butterfly pea flower extract solution stored at showcase temperature on the third day with

the description of the quality of the cell shape clearly visible cytoplasm and cell nucleus, the intensity of the cytoplasm color is clear with a pink color and the intensity of the cell nucleus color with a thicker pink color. Figure 5. (B) the researcher used an extract solution with a concentration of 16% stored at room temperature on the third day with the description of the quality of the cell shape clearly visible cytoplasm and cell nucleus, the intensity of the cytoplasm color is clear with a thin pink color and the intensity of the nucleus color is clear with a thicker pink color. Figure 5. (C) buccal smear epithelial cells are seen using diffquick staining as a comparison or control. The quality of the preparation from the control solution shows a very clear cell shape with a purplish blue color, epithelial cell fragments are very clear because the background of the epithelial cells of the preparation looks faint, the intensity of the cell nucleus color is very clear with a dark purple color.

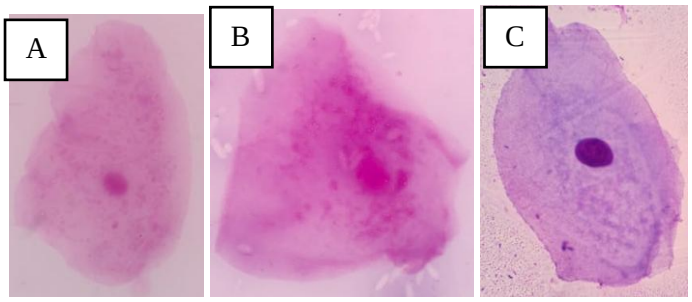

Figure 5. Buccal smear staining results on the third day, (A) Staining results of the butterfly pea flower extract preparation stored at showcase temperature on the third day, (B) Staining results of the butterfly pea flower extract preparation stored at room temperature on the third day, (C) Staining results of the buccal smear preparation using the Diffquick method.

The results of the observation of the quality of the fourth day's preparation in Figure 6. (A) using a test solution, namely a solution of butterfly pea flower extract as a substitute for methylene blue with a concentration of 16% stored at showcase temperature on the fourth day with information on the quality of the cell shape, the cytoplasm and nucleus are clearly visible, the intensity of the cytoplasm color is clear with a pink color while the nucleus is thicker pink. Figure 6. (B) the researcher used a test solution, namely butterfly pea flower extract as a substitute for methylene blue with a concentration of 16% stored at room temperature on the fourth day with information on the quality of the cell shape, the cytoplasm and nucleus are clearly visible, the intensity of the cytoplasm color is clear with a thin pink color and the color of the cell nucleus is clearly visible with a thick pink color. Figure 6. (C) buccal smear epithelial cells are seen using diffquick staining as a comparison or control. The quality of the control or comparison solution is very clear in the cell shape with a purplish blue color, epithelial cell fragments are very clear because the background of the epithelial cells of the preparation looks faint, the intensity of the nucleus color is very clear with a dark purple color.

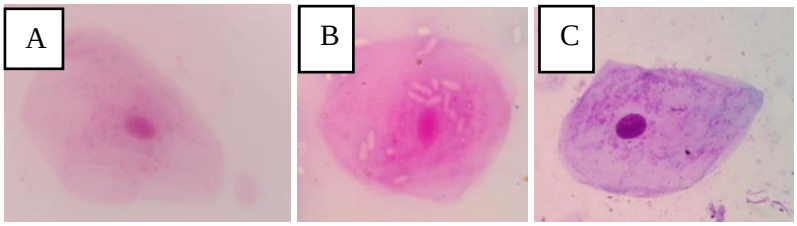

Figure 6. Buccal smear staining results on the fourth day, (A) Staining results of the butterfly pea flower extract preparation stored at showcase temperature on the fourth day, (B) Staining results of the butterfly pea flower extract preparation stored at room temperature on the fourth day, (C) Staining results of the buccal smear preparation using the Diffquick method.

The results of the observation of the quality of the fifth day's preparation in Figure 7. (A) using a 16% concentration of butterfly pea flower extract solution stored at showcase temperature on the fifth day with the description of the quality of the cell shape clearly visible cytoplasm and cell nucleus, the intensity of the cytoplasm color is clear with a pink color and the intensity of the cell nucleus color is clearly visible with a thicker pink color. Figure 7. (B) the researcher used a 16% concentration of butterfly pea flower extract solution stored at room temperature on the fifth day with the description of the quality of the cell shape clearly visible cytoplasm and cell nucleus, the intensity of the cytoplasm color is clearly visible pink while the intensity of the cell nucleus color is clearly visible pink thicker. Figure 7. (C) buccal smear epithelial

cells are seen using diffquick staining as a control. The quality of the control solution shows the cell shape is very clear with a purplish blue color, epithelial cell fragments are clearly visible because the background of the epithelial cells of the preparation looks faint, the intensity of the cell nucleus color is very clear with a dark purple color.

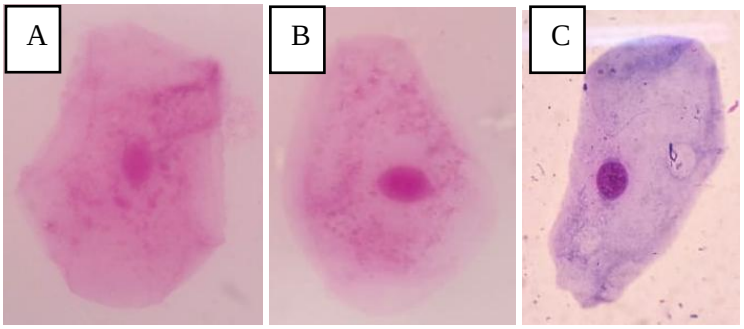

Figure 7. Buccal smear staining results on the fifth day, (A) Staining results of the butterfly pea flower extract preparation stored at showcase temperature on the fifth day, (B) Staining results of the butterfly pea flower extract preparation stored at room temperature on the fifth day, (C) Staining results of the buccal smear preparation using the diffquick method.

Normality Test and Non-Parametric Test of Data

The study conducted statistical testing, namely the normal distribution test using the Kolmogorov-smirnov test or Shapiro-Wilk. Based on table 3, a significant value of 0.0001 at room temperature and 0.001 at showcase temperature was obtained. Sig in the table shows <0.05, which means that Ha is rejected or the data is not normally distributed so that the test cannot be continued using the T test. This is because the T test has a requirement for normally distributed data. Then the data will be continued with a non-parametric test, namely the Mann Whitney test.

Table 3. The results of the normality distribution test of the pH value of the butterfly pea flower extract solution with a concentration of 16% stored at showcase temperature and room temperature using the Kolmogorov-Smirnov test.

	Storage temperature	N	P-value
PH Solution Extract	Showcase temperature	15	0.0001
	Room temperature	15	0.001

The test was continued with the Mann Whitney test. Based on the Mann Whitney test on the data, Asymp.sig 1.0 was obtained, which showed >0.05, which means that Ho is accepted or there is no difference in the color power of the cell nucleus of the butterfly pea flower extract solution with a concentration of 16% stored at a storage temperature of 7-11 ℃ and 25 ℃ as a substitute for methylene blue dye in the diffquick method for buccal smear cytology in Table 4.

Table 4. Mann Whitney test results for each buccal smear preparation treatment using the diffquick method

	Treatment	N	Mean Rank	P-value
Coloring Results	Showcase temperature	15	15.50	1.0
	room temperature	15	15.50	
	Total	30		

The test was continued to determine the effect of storage temperature on the pH of the solution using the Spearman test. Based on the Spearman test, it showed a sufficient effect (r = -0.525) and a negative pattern, meaning that the higher the storage temperature, the lower the pH of the solution. The statistical results showed that there was no significant effect between storage temperature and pH of the solution (*p-value* = 0.003).

Table 5. Testing the effect of storage temperature on solution **pH.**

Variable	R	*P*-Value
Storage temperature	-0,525	0,003

Pre-study Research

The extract results that have been made are given 1 M NaOH to provide a basic atmosphere. The butterfly

pea flower extract solution in a basic atmosphere cannot color the cell nucleus well.

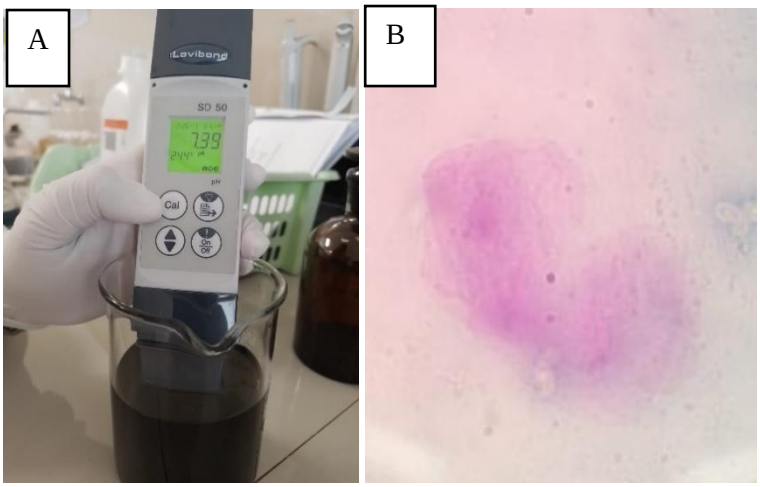

Figure 8. Pre-study results (A) Measurement of pH of Extract Solution (B) Observation Results.

DISCUSSION

Butterfly pea flower extract is known to contain anthocyanin compounds that tend to give a blue color in acidic conditions (pH <5), if anthocyanin in an alkaline solution (pH> 7) will affect the anthocyanin pigment to turn green. The solvent used is distilled water. Distilled water can extract the anthocyanin content of butterfly pea flowers. This is because anthocyanin compounds dissolve in polar solvents. The pH test was carried out using a pH meter, the results obtained were the pH of the butterfly pea flower extract stored at showcase storage temperatures (7-11℃) and room temperature (25ºC) obtained an extract pH of 4 or acidic conditions (Sofiah et al, 2022). Methylene blue staining is a basic staining that can color the cell nucleus blue, but in this study in acidic conditions the butterfly pea flower extract staining was able to color the cell nucleus clearly pink while in alkaline conditions the cell nucleus could not be colored clearly (Mutoharoh et al, 2020).

Maceration is carried out for 1 day or 24 hours. The extraction time greatly affects the resulting compounds. Too short a maceration time can result in not all compounds being dissolved in the solvent (Amelinda et al, 2018). Aquades is the solvent used in butterfly pea flower extract. Aquades solvent is used because it has more polar properties than ethanol (Sofiah et al, 2022). The extracted butterfly pea flowers are added with 1% anhydrous aluminum chloride solution and 1.2% ferric chloride solution which functions as a mordant or preservative solution (Azka et al, 2021). The filtered butterfly pea flower extraction is tested for anthocyanin using 10% NaOH and forms a yellow color, the yellow color obtained indicates the presence of flavanols and flavones in the butterfly pea flower extract (Suryanto et al, 2016).

Testing the quality of butterfly pea flower extract compounds can be affected by irradiation, storage temperature and storage time. Butterfly pea flower extract in this study was stored in a dark bottle, so that the butterfly pea flower extract is stable in coloring epithelial cells. The results of pH measurements can be seen that the solution stored at showcase temperature on the first day pH 4.93, second day pH 4.54, third day pH 4.64, fourth day pH 4.66, and fifth day pH 4.62. While the butterfly pea flower extract solution stored at room temperature was measured using a pH meter obtained on the first day pH 4.14, second day pH 4.55, third day pH 4.21, fourth day pH 4.62 and fifth day pH 4.64. Based on pH measurements in both treatments, it can be seen that the average pH of the showcase storage temperature and room temperature are 4.67 and 4.43 so that the pH of the extract solution is stable at acidic pH.

Damage to the extract solution used as a dye is often caused by several factors, including temperature and pH during storage. Changes in pH can disrupt the electrostatic properties and hydrogen bonds contained in the material which can cause changes in the chromophore structure. Temperature can also affect pigment degradation and can accelerate the reaction between the dye and free oxygen so that the oxidation process will occur faster (Suryanto et al, 2016). This study tested whether there was an effect of storage temperature and pH of the solution in table 5 using the Spearman test. The test results showed that there was a sufficient effect (0.525) and a negative pattern, meaning that the higher the storage temperature, the lower the pH of the solution. So that the storage temperature greatly affects the pH because the higher the storage temperature, the lower the pH of the solution, although not significantly.

Based on Table 3, the results of the normality test if the probability (sig.) > 0.05 then H0 is accepted, if the probability (sig.) <0.05 then H0 is rejected. The results obtained (sig.) <0.05 or 0.0001 <0.05 then H0 is rejected or the data is not normally distributed. Because the data is not normally distributed, the data is

tested using the Mann Whitney test. Based on Table 4, the results of the Mann Whitney test if the probability (sig.) > 0.05 then H0 is accepted, if the probability (sig.) <0.05 then H0 is rejected. The table obtained the results (sig.) 1.0 > 0.05 or H0 is accepted, then there is no difference in the color power of the cell nucleus of the butterfly pea flower extract solution with a concentration of 16% stored at a storage temperature of 7-11 ℃ and 25 ℃ as a substitute for methylene blue dye in diffquick staining.

The results of the study at the storage temperature in the showcase (7-11ºC) obtained an average of 2.93, at room temperature storage an average of 2.96 was obtained, and at the control an average of 4 was obtained. The average value of the showcase temperature was the lowest, and the highest average value was at room temperature storage. This is because at room temperature the coloring solution has a more acidic nature so that the staining results found many cell shapes and the intensity of the cell nucleus color was clear, epithelial cell fragments were very clear because the background of the epithelial cells looked faint, and the intensity of the cytoplasm color was clear. However, in the results of the study, the butterfly pea flower extract stored at room temperature had many impurities due to the influence of increasing temperature. The results of the study are in accordance with the usefulness of diffquick staining, which is able to color cells well in a relatively short time.

CONCLUSION

The conclusion is a research has been conducted on the effectiveness of butterfly pea flower extract (Clitoria ternatea) as a natural alternative dye methylene blue Staining method diffquick In buccal smear cytology preparations, the results of the Man Whitney test showed that there was no difference in the color strength of the cell nucleus from the butterfly pea flower extract solution with a concentration of 16% stored at showcase temperature and room temperature, which was able to replace methylene blue staining in staining.diffquick. The butterfly pea flower extract solution has a significant effect between the storage temperature and the pH value of the butterfly pea flower extract solution.

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