Research Article

Evaluation of Duhat (*Syzygium cumini*) Leaves as Cytological Stain

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ABSTRACT

Context: Staining is a vital histology process and synthetic stains, notably Hematoxylin and Eosin, are widely used in identifying tissues in the Histopathology section. Despite their remarkable efficiency in staining, these synthetic dyes pose a health hazard to laboratory workers, especially with frequent exposure. Hence, creating an alternative natural stain is imperative and has become of great interest in the research field. **Objectives:** This study aimed to develop a natural stain from the leaf extract of *Syzigium cumini.* Furthermore, to determine if the extract can be an alternative to Eosin as a stain for cytological specimens like urine and buccal samples, along with efficiency in staining, the study intends to determine the stain's viability when stored over time at different storage conditions. **Data sources:** The ethics committee approved the research before starting and followed specific protocols for the participants' data and safety. 180 slides were prepared from the participants' buccal and urine specimens collected. The slides were then stained using the stain produced from the *S. cumini* extract and then viewed and graded under the microscope by four registered Medical Technologists. **Results:** The results demonstrated that distilled water and acetone, leaves extract, and acetone extract showed no significant difference compared to Eosin's staining efficiency. On the other hand, distilled water leaf extract can also stain; however, it is inferior to Eosin.

Keywords: Alternative stain, Buccal, Urine, Hematoxylin and eosin, Chlorophyll, Duhat, *S. cumini.*

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INTRODUCTION

The fundamental purpose of staining specimens is to improve the cells or specific cellular components' appearance under the microscope and to highlight the specimen's noteworthy features.[8] It is one of the most clinically essential laboratory methods to help the doctor diagnose properly. Adding dye to emphasize a particular structure or irregularity in the sample enables viewing and identifying various bacteria, cells, and tissues under the microscope. Histological stains are used in the laboratory's histopathology department to stain tissues for further assessing and identifying pathological disorders.[4] Hematoxylin and Eosin constitute some of the most used stains in this section. However, notwithstanding their potency and efficacy, these stains pose health hazards, putting the safety of laboratory personnel at risk, especially when exposed frequently. However, natural stains prepared from non-toxic compounds typically derived from plants diminish the potential health risks associated with staining.[5] For these reasons, developing effective and economical natural stains as an alternative to synthetic dyes has become vital.

Syzygium cumini, commonly known as "Java Plum" or "Duhat," is a fruit-bearing tree native to the Philippines that provides a broad range of therapeutic and clinically essential health advantages.[6] The fruit of *S. cumini* is vital in research because of its phytochemical content. It can also be used as an alternative stain due to anthocyanin.[2]

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This research focuses on *S. cumini* leaves' extract, precisely its staining properties on cytological samples. With these in mind, the researchers intend to determine the staining ability of Duhat (*Syzygium cumini*) leaves in staining cytological smears, specifically buccal swabs, and urine sediments. This study will evaluate *S. cumini* leaves as a cytological stain. Medical technologists and students will primarily benefit from this study since preparing natural dyes and stains like *S. cumini* can offer safer techniques. Medical students can apply their theoretical learning to staining. In addition, this study will benefit laboratories because Duhat leaves' extract as a cytological stain can be more economical and easier to use. Moreover, this study will be advantageous to future researchers since it can be utilized as a source of supplementary information for future research focused on natural staining products.

Materials and METHODS

Study Design

This study utilized quantitative research methodology, particularly experimental. This design is the most appropriate for determining the relationship between the staining capacity of the *S. cumini* leaves' extract on the cytological specimens (buccal swab and urine), and the varying concentrations of the *S. cumini* leaves' extract, the storage temperature, and the length of storage time, given that the goal of the study is to determine the relationship among the dependent variables and independent variables.

Study Setting

The leaves of *S. cumini* used to produce chlorophyll originated in the city of Binan in the province of Laguna. Then, the Herbarium Department of Far Eastern University–Manila authenticated the foliage. At the Philippine College of Health Sciences, the extract underwent spectrophotometric analysis. The sample collection was conducted by research assistants in locations other than Far Eastern University–Manila. Consequently, the Science Building Room 508 (SB 508) and the Medical Technologists' Room at Far Eastern University - Manila on Nicanor Reyes Street in the Sampaloc neighborhood of Manila, 1008 were utilized for sample processing, evaluation, and analysis.

Sample Collection

The researchers gathered participants for the experiment by publishing recruitment notices on Facebook and disseminating information about the experiment in the form of a Google form that outlined the steps to be taken. Participation in this study is entirely voluntary, and to maintain the participants' anonymity, a research assistant was assigned and oversaw contacting and assisting the participants in collecting the samples. Participants who agreed to participate in the study were asked for their contact numbers as the mode of communication and notification about the day and location where their specimens were collected. The researchers evaluated and screened all the submitted forms, excluded the individuals who did not meet the criteria, and picked 30 people randomly from those that are qualified. The chosen participants were 18-to-25-year-old non-FEU students residing in Metro Manila. Written consent forms were distributed to each participant, and a revised version of the health declaration form of the University of Visayas Health Service Unit (n.d.) was used as the screening tool. Additionally, a physician evaluated the participants to assess and ascertain that the participants were in their best health condition. To maintain anonymity among participants, they were scheduled differently, with time intervals, and the specimen collection was done outside the university.

The research assistant transported the collected specimen to the researchers within a given time for processing and staining.

Data Collection and Procedure

The *S. cumini* leaves collected from Biñan, Laguna, were authenticated at Far Eastern University's National Herbarium Department. After confirming that it is indeed *S. cumini* leaves, they were dried using the modified air/shade-drying technique, wherein the Duhat leaves are kept in a chamber with sufficient ventilation but are shielded from direct sunshine for 7-10 days.[18] The leaves were dried, and chlorophyll was obtained from them using a mixture of 90% acetone and 10% distilled water, a technique based on those devised by Kwartiningsih *et al*. and Sasadara *et al*. but with modifications.[13,14] On the extraction of chlorophyll pigment using distilled water as a solvent, the dried leaves were blended with distilled water in a 1:1 ratio (50 g leaves: 50 mL distilled water (DE 1:1)) and in a 1:2 ratio (50 g leaves: 100 mL distilled water (DE 1:2). The same formulations were performed using acetone as a solvent: 1:1 ratio (50 g leaves: 50 mL 90% acetone (AE 1:1) and in a 1:2 ratio (50 g leaves: 100 mL 90% acetone (AE 1:2) however, the dried *S. cumini* leaves were ground 90% acetone using a mortar and pestle. After being transferred into separate Erlenmeyer flasks, each formulation's extract was filtered through Whatman filter paper No. 3. After filtering the extracts, they were left at room temperature

for 1 hr, then refrigerated for three days, and then stored at 4°C for one week.

The obtained solutions of Duhat mixed with 90% acetone and distilled water was then subjected to spectrophotometric analysis for chlorophyll measurement in the Philippine College of Health Sciences. The wavelengths used were 645 and 663 nm to determine the absorbance, and the total chlorophyll content was calculated using Arnon's equation.[10]

$$Chl.a\ (\mu g) = 12.72(A_{663}) - 2.59(A_{645}) \times dilution\ factor$$

$$Chl.b\ (\mu g) = 22.9(A_{645}) - 4.67(A_{663}) \times dilution\ factor$$

$$Chl.t\ (\mu g) = 20.31(A_{645}) - 8.05(A_{663}) \times dilution\ factor$$

The analysis was conducted on 3 separate days: the day of the extract extraction, 3 days after, and 1 week after the extraction. 4 urine samples and 8 buccal swabs were needed for each day. Each buccal swab was used in preparing 3 slide replicates. Participants who opted to donate buccal swabs were first instructed to gargle with distilled water and their inner cheek area was swabbed at least 5 times. Before rolling buccal swabs onto glass slides, albumin was rolled onto the slides for optimal sample adhesion. The samples' DNA (Deoxyribonucleic Acid) was also maintained by the scientists by employing ethanol (95% concentration).[10] For the urine specimen, the first-morning sample was collected, and 95% ethanol was also used for the same reason as the buccal swab.

The same numeric code and letter designation are used for all samples obtained from the same person. 60 slides were prepared from the urine and buccal swab. 12 slides were prepared that served as controls for comparison: 6 buccal slides stained with H&E (Hematoxylin and Eosin), 3 of which are stored at room temperature and the remaining 3 stored at 4°C. The same number of urine slides were prepared and stored under the same conditions. The staining process for the control slides was modified based on Ali *et al.*[1] The specimen was first fixed on the slide using albumin and was stained with hematoxylin. After 5 min, it was washed with distilled water and stained with Eosin for 10 sec; afterwards, the slides were washed with distilled water. New 12 urine slides were allotted for staining using the extracts stored at room temperature: 6 slides were stained using DE 1:1 and 6 slides were stained using DE 1:2. Similarly, 12 urine slides were allotted for the extracts stored at refrigerator temperature (4°C): 6 slides were stained using AE 1:1. 6 slides were stained using AE 1:2. Another 12 buccal slides were also allotted for staining using the extracts stored at room temperature: 6 slides were stained using DE 1:1 and 6 slides were stained using DE 1:2. Lastly, 12 buccal slides were allotted for the extracts stored at refrigerator temperature (4°C): 6 slides were stained using AE 1:1 and 6 slides were stained using AE 1:2. The process of staining for these slides was identical to H&E, with the exception that the different concentrations of extract stored at both room temperature and at 4°C was used as the counterstain in replacement of Eosin.

With the assistance of four (4) medical technologists (microscopists) and a modified criterion based on the research of Surendra *et al.* (2018) and Sudhakaran *et al.* (2018), the researchers graded the stain on various specimens following staining.[17] Newly stained slides were evaluated by the same observers three (3) days and one (1) week after staining with the Duhat extract to determine if the strength of the stain had reduced.

As recommended by OSHA (Occupational Safety and Health Administration) and required by local regulatory agencies, all specimens used in slide preparation were disposed of appropriately. The waste from the buccal swab samples was disposed of in a biohazard container or garbage can. In accordance with the OSHA standard for bloodborne pathogens, urine samples were disposed of in the laboratory sink, while urine containers containing no visible blood were disposed of in biohazard-red labelled containers. The Medical Technology Office at Far Eastern University was given a puncture-resistant receptacle for the disposal of used slides.

Data Processing and Analysis

The researchers utilized Two-way Analysis of Variance (Two-way ANOVA) to determine the varying concentrations of *S. cumini* extract and comprehend how the different storage conditions and concentrations of the extracts influence the outcome of the prepared smears. The researchers determined the optimal concentration to achieve the desired results by identifying the significantly varied concentration levels.

The researchers used $F = \frac{MST}{MSE}$ and $F = \frac{MSB}{MSE}$ as formula, where *F* stands for the coefficient of ANOVA, *MST* is for the mean square of treatments, *MSB* for the mean sum of squares between the groups, and *MSE* for error mean sum of squares.

In addition, Paired *t*-tests were utilized to study to determine the significant difference between the distilled water extract and acetone.

$$t = \frac{\Sigma d}{\sqrt{\frac{n(\Sigma d^2) - (\Sigma d)^2}{n-1}}}$$

Ethical Considerations

In the determination of the potential of *Syzygium cumini* as a cytological stain, certain chemicals were in the process. The procedures done were under Biosafety Level 1. The procedures were done in a laboratory equipped with proper apparatus under the guidance of an adviser and other medical professionals to ensure the integrity of the procedures and especially the safety of the researchers.

Furthermore, the researchers applied for ethical clearance and submitted a research proposal to the Ethics Review Committee of Far Eastern University before collection of samples. The researchers ensured that all personal information is confidential. All procedures considered the ethics surrounding sample collection from qualified individuals.

RESULTS

This study developed a natural stain from *S. cumini* leaf extract to see if it might replace Eosin in staining cytological tissues like buccal and urine. The dye was made by mixing dried *S. cumini* leaves with distilled water and 90% acetone at 1:1 (50 g: 50 mL) and 1:2 (50 g: 100 mL). To examine stain viability over time, the solution was held at room temperature and 4°C for 1 hr, 3 days, and 7 days after extraction. Spectrophotometric analysis determined the chlorophyll content in solutions, which affects staining.

Chlorophyll Content of S. cumini Leaf Extract

Chlorophyll is the chemical constituent contributing to the staining ability of the dye extracted from *S. cumini* leaves. Table 1 shows that the amount of amount of chlorophyll in the leaf extract with distilled water and 90% acetone as solvents, each with a ratio of 1:1 and 1:2, respectively, is then measured spectrophotometrically.

The absorbances are measured at wavelengths 645 and 663 nm. Absorbances were then computed using Arnon's (1949) equation of quantification of total chlorophyll. Presented in the table below are the absorbances and corresponding total chlorophyll yield (in µg/mL^{-1}) of the extracts:

S. cumini leaf extract diluted 1:2 in distilled water yielded twenty-fold more total chlorophyll than its 1:1 counterpart. Likewise, 1:2 dilutions in 90% acetone produced more chlorophyll content than the 1:1 ratio. Moreover, based on the results, acetone yielded more chlorophyll than distilled water in all the dilutions in the extract. To summarize, the dilution that yielded the least total chlorophyll is the one that was diluted 1:2 in distilled water, and the dilution that produced the most is the one that was made 1:2 in acetone.

Table 1: Absorbances and total chlorophyll of *S. cumini* leaf extracts with their respective diluents and ratios.

	Absorbance at 645 nm	Absorbance at 663 nm	Total Chlorophyll (µg/mL^{-1})
1:1 Distilled Water	0.429	0.425	121.34
1:2 Distilled Water	0.025	0.022	6.85
1:1 90% Acetone	0.382	0.568	123.31
1:2 90% Acetone	0.436	0.856	157.46

Table 2: Result of the paired T-test comparing Distilled water and Eosin.

Parameter	Value
P-value	0.000983
t	13
Average of differences ($\bar{x}$d)	1.625
SD of differences (Sd)	0.25
Effect size (d)	6.5

Significant Difference of Extracts to Eosin

A paired T-test was used to analyze the data obtained to test if there is a statistically significant difference between the control Eosin and the alternative stain made from *S. cumini*.

Table 2 shows that the Results obtained in the paired t-test comparing distilled water and Eosin, as demonstrated in Table 4, show a p-value equal to 0.000983. The p-value is less than the 0.05 significance level. Hence, it strengthens the decision to reject the null hypothesis stating that there is a significant difference between Eosin and the *S. cumini* extract regarding staining. The data instead supports the alternative hypothesis, that Eosin's efficiency in staining is not equal. The observed effect size (d) obtained which is 6.5 indicates that the observed magnitude of difference among the 2 groups is large further proving that *S. cumini* extract when prepared with distilled water is incomparable with Eosin's staining efficiency.

Results from the paired *t*-test comparing 90% Acetone and Eosin as observed in Table 3 demonstrated a *p*-value of 0.1343 that is greater than the 0.05 significance level

Table 3: Result of the paired T-test comparing 90% Acetone and Eosin.

Parameter	Value
p-value	0.1343
t	2.0381
Average of differences (x̄d)	0.75
SD of differences (Sd)	0.736
Effect size (d)	1.02

Table 4: Results of Two-way ANOVA in comparison of the staining effect of *S. cumini* between room temperature and refrigerated temperature of 1:1 formulation.

Source	Sum of Square (SS)	Mean Square (MS)	F Statistic (df_1, df_2)	*p*-value
Factor A - rows (A)	0.02083	0.02083	0.07843 (1,8)	0.7865
Factor B - columns (B)	0	0	0 (1,8)	1
Interaction AB	0.02083	0.02083	0.07843 (1,8)	0.7865
Error	2.125	0.2656		
Total	2.1667	0.197		

hence, this indicates that there is compelling evidence supporting the null hypothesis. Therefore, we fail to reject the possibility that there is indeed no significant difference between Eosin's staining efficiency and the *S. cumini's* efficiency in staining when prepared with 90% acetone. This implies that the observed difference between the control, Eosin, and the 90% acetone-prepared *S. cumini* stain is not big enough to be considered as statistically significant which is further supported by the small observed effect size (d) of 1.02.

Significant variation in the Staining Effect of *S. cumini* Extracts on the Buccal and Urine Specimens

Distilled water

Stains are natural or artificial substances that add color to cells or tissues. These are used in histology laboratories to add color to tissue samples to develop a contrast between the components of a cell. The use of stains makes tissue and cellular structures visible for assessment. The effect of *S. cumini* extract on buccal and urine specimen were evaluated using 1:1 formulation and 1:2 formulation with distilled water as solvents.

Table 5 presents that a two-way anova was performed to analyze the effect of room temperature and refrigerated temperature on the staining capability of duhat extract. The results revealed that there is no significant difference

Table 5: Results of Two-way ANOVA in comparison of the staining effect of *S. cumini* between room temperature and refrigerated temperature of 1:2 formulation.

Source	DF	Sum of Square (SS)	Mean Square (MS)	F Statistic (df_1, df_2)	*p*-value
Factor A - rows (A)	1	0.6302	0.6302	1.4235 (1,8)	0.267
Factor B - columns (B)	1	2.2969	2.2969	5.1882 (1,8)	0.05225
Interaction AB	1	0.4219	0.4219	0.9529 (1,8)	0.3576
Error	8	3.5417	0.4427		
Total	11	6.8906	0.6264		

(*p*=0.7865) between the staining effect of *S. cumini* extracts on the buccal and urine specimen when used with distilled water 1:1 ratio. The test statistic F_A equals 0.07843 which is in the 95% region of acceptance. Likewise, the test statistic F_B which is in the 95% region of acceptance equals 0.

Using Two-Way ANOVA, the *S. cumini* extracts with a 1:2 formulation of distilled water at room and refrigerated temperature showed no significant difference ($p = 0.267$) when used on buccal and urine specimens. In indicates that the difference between the sample averages of all groups is not big enough to be statistically significant.

90% acetone

This section discusses the results and statistics of the Evaluation of *S. cumini* as a cytological stain using 90% acetone. There are two concentrations that are used in this experiment, 1:1 concentration which has 50 g of *S. cumini* leaves and 50 mL of 90% acetone. The other stain has 1:2 concentrations which have 50 g of *S. cumini* leaves and 100 mL of 90% acetone. Moreover, the temperature where the stain is stored is also controlled in this study; the stains are placed at room temperature and refrigerator temperature.

Table 6 shows the two-way ANOVA results for the staining effect of acetone regarding 1:1 formulation. The computed *p*-value of Factor A is equal to 0.4726 ($P(x \leq 0.5682) = 0.5274$), and the computed *p*-value for Factor B is 0.6631 [$P(x \leq 0.2045) = 0.3369$]. Since the *p*-values for the factors, respectively, are greater than α, H0 cannot be rejected, and the averages of all groups are assumed to be equal. Furthermore, the computed p-value of the interaction AB is equal to 0.7865 ($P(x \leq 0.07843) = 0.2135$). This value can indicate that the chance of a type I error, rejecting a correct H0 is too high: 0.7865 (78.65%). Given these, it can be inferred

Table 6: Results of Two-way ANOVA in comparison of the staining effect of *S. cumini* extract using 1:1 formulation of acetone.

Source	DF	Sum of Square (SS)	Mean Square (MS)	F Statistic (df_1, df_2)	*p*-value
Factor A - rows (A)	1	0.1302	0.1302	0.5682 (1,8)	0.4726
Factor B - columns (B)	1	0.04688	0.04688	0.2045 (1,8)	0.6631
Interaction AB	1	0.04687	0.04687	0.2045 (1,8)	0.6631
Error	8	1.8333	0.2292		
Total	11	2.0573	0.187		

Table 7: Results of Two-way ANOVA in comparison of the staining effect of *S. cumini* extract using 1:2 formulation of acetone.

Source	DF	Sum of Square (SS)	Mean Square (MS)	F Statistic (df_1, df_2)	*p*-value
Factor A - rows (A)	1	0.1302	0.1302	0.1953 (1,8)	0.6702
Factor B - columns (B)	1	0.04688	0.04688	0.07031 (1,8)	0.7976
Interaction AB	1	0.005208	0.005208	0.007812 (1,8)	0.9317
Error	8	5.3333	0.6667		
Total	11	5.5156	0.5014		

that the sample averages of all groups do not statistically differ enough from one another.

Table 7 shows the results of Two-way ANOVA in comparison of the staining effect of *S. cumini* extract using a 1:2 formulation of acetone. By utilizing two-way ANOVA, the *S. cumini* extracts with a 1:2 formulation of acetone showed no significant difference. Since the *p*-value is greater than α, the null hypothesis cannot be rejected. All group averages were equal. In other words, when analyzing the mean values of all groups, researchers do not observe a significantly large difference. An insignificant result indicates only that the null hypothesis cannot be rejected; it does not imply that H_0 must be true. The computed p-value for Factor A equals 0.6702, [P(x≤0.1953) = 0.3298]. Moreover, the computed value for Factor B equals 0.7976 [P(x≤0.07031) = 0.2024]. With these given values, it fails to reject the null. It means that the chance of a type I error, rejecting a correct H_0, is too high: 0.6702 (67.02%). The more significant the *p*-value, the more it supports H_0. In addition, the results from Interaction AB equals 0.9317, [P(x≤0.007812) = 0.06826]. All group averages are equal. With such, it can be established that there are minimal statistical differences between the sample averages of all classifications.

Significant variation in the staining effect of *S. cumini* extract concentrations when stored at room temperature (20-25°C) and refrigerator temperature at 4°C

Storage is an essential factor for stains used in the laboratory. Factors like stability, shelf-life, and preservations are affected by storage temperature. Certain stains have specified storage requirements to achieve optimal staining capability. In this instance, the *S. cumini* leaf extract was subjected to different storage temperatures, room temperature and refrigerated temperature (4°C) and different storage intervals or time, to see if the length of storage over time affects its staining capability.

Table 8 shows the staining effect of distilled water in storage time. The *p*-value of Factor A equals 0.5793, [P(x≤0.3189) = 0.4207] and Factor B *p*-value equals 0.1496, [P(x≤2.115) = 0.8504] which indicates that Ho cannot be rejected. Moreover, Interaction AB's p-value is 0.2133, [P(x≤1.6855) = 0.7867]. It suggests there is no statistically significant difference amongst each group's sample averages.

The staining effects of *S. cumini* leaf extract through acetone regarding its storage time through Two-way ANOVA are shown in the table 9. Factor A has a *p*-value of 0.08135, (P(x≤3.4091) = 0.9187). While Factor B has a p-value of 0.1721, (P(x≤1.9432) = 0.8279). Because of this, the Hocannot be rejected. It has been assumed that all group averages are equal. In other words, the difference between the sample averages of all groups is not big enough to be statistically significant. Also, the p-value for Interaction of AB is equal to 0.3682, (P(x≤1.0568) = 0.6318). All group averages are equal. As a result, the difference between the sample averages of all groups is not statistically significant.

Table 8: Two-way ANOVA results for the staining effect of distilled water regarding storage time.

Source	DF	Sum of Square (SS)	Mean Square (MS)	F Statistic (df_1, df_2)	*p*-value
Factor A - rows (A)	1	0.1276	0.1276	0.3189 (1,18)	0.5793
Factor B - columns (B)	2	1.6927	0.8464	2.115 (2,18)	0.1496
Interaction AB	2	1.349	0.6745	1.6855 (2,18)	0.2133
Error	18	7.2031	0.4002		
Total	23	10.3724	0.451		

Table 9: Two-way ANOVA results for acetone's staining effect in storage time.

Source	DF	Sum of Square (SS)	Mean Square (MS)	F Statistic (df_1, df_2)	*p*-value
Factor A - rows (A)	1	1.0417	1.0417	3.4091 (1,18)	0.08135
Factor B - columns (B)	2	1.1875	0.5938	1.9432 (2,18)	0.1721
Interaction AB	2	0.6458	0.3229	1.0568 (2,18)	0.3682
Error	18	5.5	0.3056		
Total	23	8.375	0.3641		

DISCUSSION

The primary objective of this study was to develop a natural stain from the leaf extract of *S. cumini* and to ascertain if the extract could be an alternative stain for Eosin in staining cytological tissues such as buccal and urine. The dried *S. cumini* leaves were blended with distilled water and 90% acetone at 1:1 (50 g: 50 mL) and 1:2 (50 g: 100 mL) formulations to develop the stain. The solution prepared was then stored at room temperature and at refrigerator temperature of 4°C for 1 hr, 3 days, and 7 days from the date of extraction to further test the stain's viability over time. Solutions were also subjected to spectrophotometric analysis to determine the amount of chlorophyll content present that contributes to the staining capability of the stain.

The dilution that yielded the least total chlorophyll is the one that was diluted 1:2 in distilled water, and the dilution that produced the most is the one that was made 1:2 in acetone which can be supported by the study of Kwartiningsih *et al.*, in which distilled water can yield chlorophyll but in a degraded state because of pH on the extract, turning the extracted color leaning on the brown spectrum, like the result of the study, in contrast to the green color of the acetone as solvent.[13] This study results signify that water and acetone can be used as solvents in extracting chlorophyll by crushing and grinding the leaves with a mortar and pestle.

Moreover, the amount of chlorophyll from the leaf extract is the contributor to the ability to stain cytological smears, and the dilution that yielded the most total chlorophyll is ascertained that have the strongest affinity to cellular components, specifically epithelial cells, of buccal and urine specimens. This is because distilled water was also used as a solvent for extracting the chlorophyll content and although distilled water is a potent solvent, the solubility properties of the chlorophyll with water affected its efficiency as a stain.[12] Both chlorophyll and distilled water are polar molecules hence, they follow the principle of "*like dissolving like*".

On the other hand, the statistical data obtained showed a favorable response leaning towards the *S. cumini* extract prepared with 90% acetone. The results can be further supported and strengthened by the study conducted by Sasadara *et al.*, wherein among the solvents utilized in the study, acetone demonstrated the most promising result in extracting the chlorophyll content.[14] The use of acetone yielded a significantly higher amount of chlorophyll as compared to methanol and ethanol.

The results also showed that with the use of distilled water as a solvent with 1:1 and 1:2 concentrations showed none to slightest statistical difference compared to the existing study of Hu *et al.* wherein they boiled their natural dyes for 5 to 10 sec to get the extracts.[7] The method the research used in this study to get the extract of Duhat is mortar and pestle, although this method is longer, there is no damage done to the leaf while in the study of Hu *et al*, pheophytin was altered, but this pigment did not alter the results of the staining capability of natural dyes.

Furthermore, in comparing the absorbances of the two (2) concentrations of the distilled water, 1:2 concentrations yielded the most chlorophyll content than the 1:1 concentration. Although the results of the existing studies made by Kamel *et al.* and Krampah *et al.* had minimal statistical differences with this research results, using boiled water, and mortar and pestle in extracting the dye both can stain cellular cytoplasm, and urine and buccal specimen, respectively.[9,10]

For the staining effect of acetone regarding 1:1 formulation, it can be inferred that the sample averages of all groups do not statistically differ enough from one another. In fact, the study of Singnarpi *et al.* used alcohol to fix 20 buccal smears, splitting them in half for H&E and beetroot staining.[15] The duration of fixation in alcohol was 30 min. The alcohol was used to fix the buccal smears, and the smears were moved to a beetroot solution, where they were kept for a period. The alcohol also helped the samples have a well-defined structure when they were dyed with red beet extract and viewed under the microscope.[14] The staining intensity was like 30% beetroot and 30% H&E. Hence, it can be concluded that acetone can be used to extract plant constituents without altering the staining efficacy of the extract.

In comparison of the staining effect of *S. cumini* extract using a 1:2 formulation of acetone, it can be established that there are minimal statistical differences between the sample averages of all classifications. The stability of chlorophyll in 100% acetone was studied here. At normal

temperature, acetone extraction of WT leaves revealed chlorophyll production after only 6 min. After extracts were kept for a day in the dark at room temperature, HPLC was used to measure chlorophyll and chlorophyll levels. One day old extracts showed no change in chlorophyll concentration. The high concentration of chlorophyll in the extracts resulted from its own production during extraction and homogenization.[3] Thus, it is possible to use acetone to extract chlorophyll without changing any quality, including the leaf extract's volume, viability, and staining effectiveness.

Using acetone as a solvent with 1:1 and 1:2 formulations for chlorophyll extraction inferred that the sample averages of the groups have only very minimal statistical differences. The study also proved that 100% acetone does not alter the chlorophyll extracts' quality, yield volume, viability, and staining capability. The outcomes have aligned with the results of the existing study by Singnarpi *et al*.,[15] which utilized beetroot extract and alcohol fixation compared with H&E to buccal smears. These further strengthened acetone's consistent staining efficacy without alterations while offering a more accessible, cost-effective, and safer approach in comparison with traditional synthetic stains.

Furthermore, the study's results using the 1:2 formulations that demonstrated minimal statistical differences were in line with Singnarpi *et al*.'s method that reported a 30% staining intensity with beetroot and H&E. Moreover, the extracted chlorophyll's stability using 100% acetone supported the conclusion that acetone as a solvent yielded faster for only about 6 min without compromising the quality of the extracted components.

Acetone has the potential to be a dependable staining solvent for many different biological specimens, as demonstrated by the positive results of this investigation and the successful comparison with the methodology used in the prior study. As a result, acetone-based staining techniques can be applied and tested in various staining applications in medical laboratories.

Meanwhile, regarding the staining capability of distilled water during storage the results showed that the sample averages of each group are not statistically different. Extracts, whether freshly prepared or stored for 4 weeks at room temperature, did not exhibit any contrast, and staining variations in the days that followed, lending credence to the findings of Kuiper and Giepmans' study of a compound that is insoluble in water at high concentrations.[12] Moreover, it was reported that when chlorophyll extracts were kept at ambient temperature and in the refrigerator for a day, it was noticed that there were no significant changes in the amounts of chlorophyll after that time[3] which is also shown in this study's outcome. This result will help future researchers to store their distilled water-chlorophyll extract in a longer period and utilize it without degrading the chlorophyll content in the sample and limiting other harmful chemicals in mixing to the sample which may lead to minimising possible extraneous variables.

Meanwhile, the staining effects of *S. cumini* leaf extract through acetone regarding its storage time shown to have no significant changes in chlorophyll levels when treated at different storage times while extracting chlorophyll using acetone.[3] Furthermore, when compared to methanol and ethanol, acetone is the most effective solvent because it produces distinct absorption peaks.[13] The results can be compared and supported by Kumar *et al*., and Goli *et al*. study both mentioned that acetone is an excellent solvent for chlorophyll extraction. As a result, the researchers determined that the storage time of acetone-extracted chlorophyll has no effect on *S. cumini* staining capabilities. These results can be used as basis for future studies that aim to extract chlorophyll with the use of acetone, and this may also be used to prove that acetone does not degrade chlorophyll vitality.

CONCLUSION AND RECOMMENDATION

In conclusion, the quantity of chlorophyll extracted contributes significantly to the staining capacity of the extract and demonstrates a greater affinity for cellular components found in buccal and urine specimens. Data gathered from the spectrophotometric analysis revealed that the 1:2 dilution ratios in acetone yielded the greatest amount of chlorophyll, whereas the lowest was obtained in the 1:2 dilution ratios in distilled water. The staining efficiency of *S. cumini* extract made with 90% acetone was not significantly different from that of the control Eosin, as determined by a paired *t*-test. Spectrophotometric analysis revealed that the acetone-prepared extract had the most chlorophyll, and the results of the paired *t*-test corroborated the idea that chlorophyll concentration has a major impact on staining intensity. Although acetone is considered as the more potent solvent in comparison with distilled water when both compared with Eosin, the two-way ANOVA results demonstrated that there are no significant differences between the staining capabilities of extracts prepared with 90% acetone and distilled water. Furthermore, despite being stored in room temperature and at 4°C for 1 hr, 3 days, and 7 days, the *S. cumini* extract remained viable and the staining intensity remained constant in staining buccal and urine specimens.

Researchers recommend labeling additional cells present in the urine and buccal swab samples based on observations and conclusions. Staining additional specimens including bacteria, blood smears, and tissue samples is another way to investigate the potential of the *S. cumini* extract. Since it was found that Hematoxylin's staining capacity is improved in the presence of *S. cumini* extract, this potential cannot be ignored, and the researchers highly advise using the leaf extract as a mordant. Moreover, anthocyanin is not present in *S. cumini* leaves but nonetheless demonstrates excellent potential as a stain hence, the researchers suggest utilizing other leaf extracts without anthocyanin. Lastly, aside from chlorophyll, it is highly recommended to look for other components of the extract and consider their contribution to the staining potential of the developed stain.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

***S. cumini*:** *Syzgium cumini*; **DE:** Distilled Extract; **AE:** Acetone Extract; **H&E:** Hematoxylin and Eosin; **OSHA:** Occupational Safety and Health Administration; **WT:** Weighted; **HPLC:** High-performance liquid chromatography; **DNA:** Deoxyribonucleic acid.

SUMMARY

The primary objective of this study was to develop a natural stain from the leaf extract of *Syzigium cumini* and to ascertain if the extract has the potential to be an alternative stain for Eosin in staining cytological tissues such as buccal and urine. In developing the stain, the dried *S. cumini* leaves were blended with both distilled water and 90% acetone at 1:1 (50 g: 50 mL) and 1:2 (50 g: 100 mL) formulations. The solution prepared was then stored at room temperature and at refrigerator temperature of 4°C for 1 hr, 3 days, and 7 days from the date of extraction to further test the stain's viability over time. Solutions were also subjected to spectrophotometric analysis to determine the amount of chlorophyll content present that contributes to the staining capability of the stain.

The study findings revealed that the quantity of chlorophyll extracted from the leaves is crucial in its role in staining cytological smears and has a great affinity for cellular components in buccal and urine specimens. The results showed that *S. cumini leaves* with acetone in the concentration of 1:2 yield the most amount of chlorophyll; meanwhile, *S. cumini* leaves with distilled water in the concentration of 1:2 yield the lowest amount of chlorophyll.

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