# MORPHOLOGICAL AND VITALITY ANALYSIS OF SPERMATOZOA USING *Basella alba* (ALUGBATI) FRUIT EXTRACT: A SUPRAVITAL AND POSITIVE STAINING APPROACH

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**ABSTRACT**

*Basella alba* fruits contain betalain pigments like gomphrenin I and betacyanin, which have been used as natural stains for tissues and bacteria. However, their potential as morphological and supravital stains for spermatozoa remains largely unstudied. This experimental study investigated the potential of *B. alba* fruit extracts as a positive stain for sperm morphology and as a supravital stain for sperm vitality assessment. Aqueous extracts of *B. alba* fruits with acetic acid and ethanol were used for morphological analysis, while ethanolic extracts were applied for supravital staining, with vitality results compared to motility. Results identified 100% extract concentration as optimal for both staining applications. A time-dependent effect was observed, where prolonged incubation led to a slight increase in stained non-vital sperm cells, potentially overestimating the percentage of dead sperm cells. Morphologically, the *B. alba* stain performed comparably to Giemsa, with no statistically significant difference. However, in supravital staining, the extract's performance was significantly lower than that of Eosin Y. These findings suggest that *B. alba* fruit extract is a viable alternative for morphological sperm staining but requires further refinement for accurate supravital staining. Future studies should explore broader cellular applications, assess shelf life, test additives to enhance supravital staining, and consider more accurate vitality methods, such as eosin-nigrosine or phase-contrast microscopy.

*Keywords:* betalains, betacyanin, Eosin Y, Giemsa, gomphrenin I

## INTRODUCTION

### Background of the Study

Fertility assessment is crucial for reproductive health, with sperm morphology and vitality analysis playing a vital role in evaluating male fertility or predicting infertility. According to the World Health Organization (2024), infertility affects about 17.5% of adults globally, with male infertility linked to issues like abnormal sperm function and semen ejection dysfunction. However, in the Philippines, male infertility cases remain underreported, with fewer than five cases per month (Unas & Martinez, 2022).

Spermatozoa exhibit significant diversity in form and function, which affects fertility (Roldan, 2020). A single ejaculate contains both normal and abnormal sperm cells (Andraszek *et al*., 2014). Morphological and vitality assessments using stains help determine sperm abnormalities. Common morphological stains like Giemsa, Papanicolaou, and Shorr allow clear visualization of sperm structure, while supravital stains assess live-dead sperm ratios, aiding in fertility prediction. However, synthetic stains often pose environmental and health risks due to their chemical composition (Ebrahimi & Parham, 2020; Bordoloi *et al*., 2017). Concerns about toxicity, sustainability, and the lack of standardized staining methods (Mohandas *et al*., 2018; Xu *et al*., 2022) have prompted the search for natural, safer alternatives.

The use of natural stains for sperm morphology and vitality analysis remains underexplored, particularly with *Basella alba* fruit extract as a potential sperm stain. Although *B. alba*'s betalain pigments have shown staining potential in histology and bacteriology, their ability to highlight sperm structural details for morphological assessment and distinguish live from dead sperm for supravital staining has not been thoroughly investigated. This study evaluates *B. alba* fruit extract as a sustainable alternative to synthetic stains like Giemsa and Eosin Y, aiming to enhance fertility diagnostics in resource-limited settings while promoting eco-friendly laboratory practices. This study addresses this gap by evaluating *B. alba* fruit extract as a sustainable, eco-friendly alternative to synthetic stains like Giemsa and Eosin Y, aiming to enhance fertility diagnostics in resource-limited settings

This research addresses the environmental and health concerns associated with synthetic dyes in laboratory settings by investigating eco-friendly, natural alternatives such as *B. alba* fruit extracts. These natural stains offer a safer, more sustainable option that reduces chemical exposure risks for laboratory personnel and lessens environmental impact. Additionally, the study contributes to male fertility testing methodologies by introducing innovative staining techniques that enhance the accessibility and effectiveness of sperm vitality and morphology assessments, thereby aligning ecological sustainability with advancements in reproductive health diagnostics.

**Statement of the Objectives**

The study focused on the potential of *B. alba* fruit aqueous extracts as a supravital and positive stain for spermatozoa. Specifically, it achieved the following objectives from March to April 2025:

1. To identify the optimal concentration of *B. alba* prepared staining solution for the morphological staining of spermatozoa head, midpiece, and tail, at the following concentrations:
    a. 25%;
    b. 50%;
    c. 75%; and
    d. 100%;
2. To determine the optimal concentration of *B. alba* staining solution for vitality analysis of spermatozoa using supravital staining, consistent with results from the motility test, at the following concentrations:
    a. 25%;
    b. 50%;
    c. 75%; and
    d. 100%;
3. To determine the time-bound effects of *B. alba* extracts on the vitality of spermatozoa, determined using the supravital staining technique; and
4. To determine whether there is a significant difference in staining quality between the optimal concentration of *B. alba* prepared staining solution and controls for both sperm morphological and supravital stains.

## METHODOLOGY

**Research Design**

The study used an experimental approach to determine the staining potential of extracts from B. alba ripe fruits in human spermatozoa as a morphological and supravital stain for assessing sperm cell vitality, using Giemsa stain as a control for morphological staining and Eosin Y for supravital staining.

**Study Site and Sample Collection**

The study was conducted at the CNS Research Laboratory of Saint Mary's University in Bayombong, Nueva Vizcaya, which was chosen for its comprehensive research facilities and support. *B. alba* fruits were collected from various local municipalities due to their abundance. Semen samples were obtained from male donors who abstained for 2–7 days and were screened through routine semen analysis to ensure quality, with validation by registered medical technologists.

## Plant Identification

*B. alba* fruits were identified and certified by the College of Forestry, Environment, and Resources Management of Nueva Vizcaya State University, Bayombong, Nueva Vizcaya.

## Data Gathering Procedure

*Basella alba Fruit Collection and Preparation*

The fruits of *B. alba* were collected from various sources across Nueva Vizcaya. The ripe fruits were carefully selected and thoroughly washed to remove dirt and debris, then manually deseeded for extraction.

*Semen Collection and Processing*

Semen samples were obtained from five volunteers according to WHO (2021) protocols, and registered medical technologists evaluated standard parameters, including volume, pH, liquefaction time, and appearance. Two samples with optimal characteristics were chosen for staining. For aqueous extraction, deseeded *Basella alba* fruits were homogenized in distilled water (1:5 w/v), extracted for 24 hours, filtered, centrifuged at 8,000 rpm for 15 minutes, and the supernatant was stored at 4 °C.

For ethanolic extraction, fruits were processed similarly with 95% ethanol. The resulting extracts were stored at 4 °C and used for supravital staining, following the formulation principles of alcoholic eosin Y to enhance staining performance.

*Preparation of Morphological Stain*

Following the method of Mamay et al. (2023), the Basella alba fruit extracts were used to prepare staining solutions at concentrations of 25%, 50%, 75%, and 100%. Potassium alum was omitted to prevent crystal formation on slides. Each 100 mL solution contained the specified proportion of extract and distilled water, with 5 mL of 70% ethanol and 4 mL of 5% acetic acid added as mordants.

*Preparation of Supravital Stain*

The *Basella alba* supravital stains were prepared by mixing specific volumes of fruit extract with 95% ethanol to make 100 mL solutions per concentration, following the formulation of alcoholic eosin Y (CHS Protocols, 2014). Using 95% ethanol ensured pigment solubility, preservation, and consistency with standard staining methods for accurate comparison with the control stain.

*Morphological Analysis AND Vitality Analysis*

For morphological analysis, semen smears were prepared using the wedge smear technique, air-dried, fixed in 95% alcohol for 5 minutes, and stained with Giemsa for 15 minutes. After rinsing and drying, at least 200 sperm cells were examined microscopically to evaluate staining quality and morphological characteristics of the head, midpiece, and tail (WHO, 2021).

For vitality analysis, sperm motility was first graded according to WHO criteria. Equal volumes (0.2 mL each) of semen and alcoholic Eosin Y (pH 5.7) were mixed, smeared, air-dried, and examined microscopically. Dead sperm stained red, while live sperm remained unstained, with at least 200 cells evaluated to determine the percentage of nonviable spermatozoa.

*Staining with B. alba Fruit Extracts*

Standard procedures for morphological and supravital staining of spermatozoa were followed during the application of the prepared *B. alba* fruit extract staining solutions in the respective tests. The procedures for both stains were carried out in three trials per concentration to ensure the accuracy of the results. Additionally, the time-dependent effects of the extracts on spermatozoa vitality were evaluated by varying the incubation time of the semen samples in the optimal staining solution. The semen-supravital stain mixture was examined after 2, 5, 10, 15, 20, 25, and 30 minutes (Jager *et al*., 1984).

*Microscopic Evaluation of Staining Quality*

The stained smears were examined under a light microscope at high-power objective (40x) for supravital analysis and oil immersion objective (100x) for morphology analysis to clearly visualize the sperm's head, midpiece, and tail. Each smear was systematically evaluated to determine the effectiveness of staining and the accuracy of detail. The collected data were statistically analyzed using descriptive statistics and an independent t-test.

**Treatment of Data**

The study used descriptive statistics and a one-tailed independent t-test to evaluate the staining capacity of Basella alba fruit extracts and compare their effectiveness with that of control stains. A scoring system adapted from Mamay et al. (2023) was used to assess color quality of sperm components (head, midpiece, tail), with scores ranging from 0 (unstained) to 4 (very clear color). Criteria from Agarwal et al. (2022) and the World Health Organization were also used to evaluate sperm viability, morphology, and motility.

**Ethical Consideration**

The study's ethics review was approved by Saint Mary's University Institution Biosafety Committee of University Research Ethics Board (SMU-REB), located on the 2nd floor of Rev. John Van Bauwell, SMU Main Campus, Ponce Street, Don Mariano Marcos, Bayombong, Nueva Vizcaya, Philippines.

## RESULTS AND DISCUSSIONS

**Section 1. Morphological Staining of Spermatozoa**

**Table 1**

*Descriptive Analysis of Morphological Staining Quality*

| Component | Stain | N | Mean Score | Qualitative Interpretation |
|---|---|---|---|---|
| Head | 25% | 3 | 1.6 | Unclear color |
| | 50% | 3 | 2.3 | Unclear color |
| | 75% | 3 | 2.6 | Clear color |
| | 100% | 3 | 3.6 | Very clear color |
| | Control | 3 | 4 | Very clear color |
| Midpiece | 25% | 3 | 1.6 | Unclear color |
| | 50% | 3 | 2.3 | Unclear color |
| | 75% | 3 | 2.6 | Clear color |
| | 100% | 3 | 3.6 | Very clear color |

| | | | | |
|---|---|---|---|---|
| | Control | 3 | 4 | Very clear color |
| Tail | 25% | 3 | 1.6 | Unclear color |
| | 50% | 3 | 2.3 | Unclear color |
| | 75% | 3 | 2.6 | Clear color |
| | 100% | 3 | 3.6 | Very clear color |
| | Control | 3 | 4 | Very clear color |

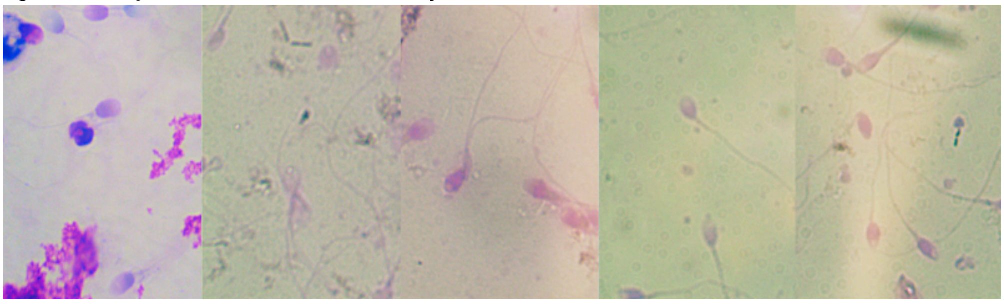
Table 1 shows that the staining quality of *Basella alba* fruit extracts improved with increasing concentration. Scores ranged from 1.6 at 25% ("unclear color") to 3.6 at 100% ("very clear color"), with the control achieving the highest score of 4, indicating that the 100% extract provided strong but slightly lower staining effectiveness than the control.

The staining performance of *B. alba* extracts aligns with findings from studies on other betalain-rich plants, such as beetroot, which have shown effective biological staining (Singh *et al*., 2017; Udonkang *et al*., 2018a; 2021b; Mohamad *et al*., 2023). Previous research also supports *B. alba*'s staining potential in blood samples (De Leon *et al*., 2010) and sperm cells (Mamay *et al*., 2023). Similar to beetroot, which produced results comparable to safranin at 5–12.5% concentrations (Oktari *et al*., 2023), *B. alba* showed stronger staining at higher concentrations without affecting sperm morphology, meeting WHO (2021) standards. These findings support its use as a natural alternative for sperm cell staining.

The 100% concentration of *Basella alba* fruit extract produced the best staining results, though slightly below the control. At full strength, the extract contained the highest level of betalain pigment, enabling stronger binding to sperm components and improved contrast for clearer observation of morphological details.

**Figure 1**

*Morphological Analysis at Oil Immersion Objective*



*a) Giemsa Control* *b) 25% B. alba fruit staining solution* *c) 50 % B. alba fruit staining solution* *d) 75% B. alba fruit staining solution* *e) 100% B. alba fruit staining solution*

Figure 1 shows the morphological staining of spermatozoa using the B. *alba* fruit extract in various concentrations from 25% to 100% and the Giemsa control stain. This accurately evaluates its staining quality based on how clear and observable the color is at different parts of the sperm, namely, its head, midpiece, and tail, relative to the control stain used as a comparison.

**Section 2. Supravital Staining of Spermatozoa**

**Table 2**

*Descriptive Analysis of Sperm Vitality*

| Mean %Immotile sperm | N | Stain | N | Mean %Dead Sperm |
|---|---|---|---|---|
| 66.6% | 3 | 25% | 3 | 52% |

| | | |
|---|---|---|
| 50% | 3 | 52.66% |
| 75% | 3 | 52.5% |
| 100% | 3 | 54% |
| Control | 3 | 56.33% |

Table 2 indicates that sperm vitality improved with higher *Basella alba* extract concentrations. The 100% concentration showed a mean of 54% dead sperm, closely matching the control (56.33%) and aligning with motility analysis results. Thus, the 100% extract demonstrated the best performance and was identified as the optimal concentration for supravital staining.

In a comparison of sperm motility and supravital staining results, Dougherty *et al.* (1975) reported that the eosin staining technique closely correlated with motility analysis. However, the percentage of stained (dead) sperm cells was consistently lower than the estimated proportion of immotile sperm, which parallels the results of the present study. This discrepancy may be explained by Chen *et al.* (2017), who noted that some immotile sperm cells retain the capacity to fertilize in vitro.

The findings from this study, along with previous research, underscore important considerations for interpreting sperm vitality using supravital staining methods. The observation that the 100% B. alba extract yielded results closest to both the control stain Eosin Y and motility analysis suggests its potential as a natural alternative for assessing sperm viability, albeit with limitations.

**Table 3**

*Descriptive Analysis of Supravital Staining Quality*

| Stain | N | Mean Score | Qualitative interpretation |
|---|---|---|---|
| 25% | 3 | 1 | Pale or poor color |
| 50% | 3 | 1 | Pale or poor color |
| 75% | 3 | 1.3 | Pale or poor color |
| 100% | 3 | 2 | Unclear color |
| Control | 3 | 4 | Very clear color |

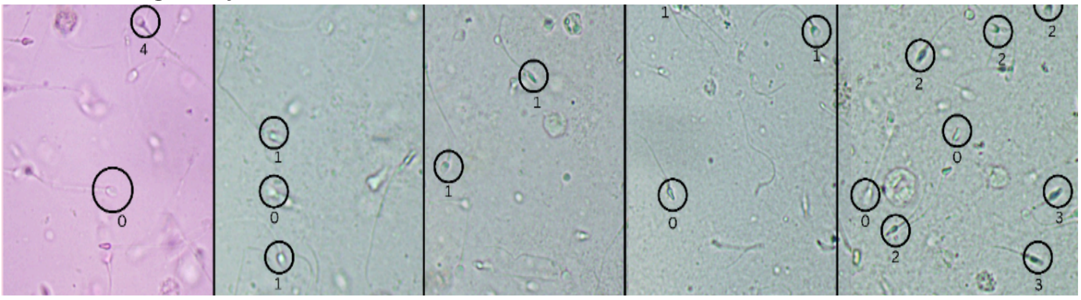
Table 3 evaluates the quality of supravital staining for spermatozoa at various concentrations: 25%, 50%, 75%, and 100%, and a control group, as demonstrated in Figure 5. Staining quality was assessed using a scoring system with a qualitative interpretation ranging from "Unstained" to "Very Clear Color". It highly shows that the control group demonstrates very clear staining and serves as a standard for comparison. The 100% exhibit an unclear color with a mean score of 2 (SD = 1), which is slightly comparable to the control group. On the other hand, the 25% to 75% yielded low average scores (1.0 to 1.3; SD = 0 to 0.57735), which suggests pale or poor color.

Poor supravital staining is often caused by overly diluted dyes (Lone et al., 2022), which explains why lower concentrations of *B. alba* extracts were less effective. Similar studies showed that natural dyes, such as black mulberry and B. alba, stain weakly compared with synthetic stains (Ebrahimi & Parham, 2020; Alfornon et al., 2015). Synthetic stains such as eosin Y perform better, likely because plant pigments in *B. alba* have glycosylated or charged structures that limit their ability to penetrate cell membranes and bind effectively (Alshamar et al., 2022).

These findings imply that natural dyes, such as *B. alba* extracts, have the potential to serve as alternative supravital stains for spermatozoa, given their ability to produce some staining. However, the observed limitations in staining intensity and specificity highlight the need for further optimization. This may involve refining extract concentrations, modifying extraction protocols, or incorporating mordants and other chemical enhancers to improve cellular affinity and staining accuracy for reliable assessment of sperm cell viability.

**Figure 2**

*Supravital staining analysis*



*a) Eosin Y Control* | *b) 25% B. alba fruit staining solution* | *c) 50 % B. alba fruit staining solution* | *d) 75% B. alba fruit staining solution* | *e) 100% B. alba fruit staining solution*

*Note. Spermatozoa encircled showing staining quality. 0 = unstained; 1 = pale or poor color; 2 = unclear color; 3=clear color; 4 = very clear color*

Figure 2 shows the supravital staining of spermatozoa at various concentrations from 25% to 100% of B. *alba* fruit extract and compared with Eosin Y, which was used as the standard control. It assessed how effectively the extract could stain dead sperm cells and differentiate them from live ones.

**Section 3. Time-bound Effects of *B. alba* Extract on Vitality of Spermatozoa**

**Table 4**

*Summary of Time-Bound Effects on Sperm Vitality*

| Incubation time (minutes) | % Dead sperm |
|---|---|
| 2 | 54 |
| 5 | 54.5 |
| 10 | 58 |
| 15 | 59 |
| 20 | 59.5 |
| 25 | 59.5 |
| 30 | 60.5 |

Table 4 shows that the percentage of dead sperm cells increased slightly with longer incubation times using *B. alba* fruit extracts, from 54% at 2 minutes to 60.5% at 30 minutes. This trend suggests that prolonged exposure may lead to overestimation of sperm mortality.

Studies by Nur *et al*. (2023), Haridas *et al*. (2022), and Khanom *et al*. (2020) confirm that *B. alba* is non-toxic. However, Cooper and Hellenkemper (2013) and WHO (2021) emphasize that extended incubation with supravital stains can cause viable sperm to absorb dye, leading to false positives. Similar findings by Ploskonos (2014) and García-Molina *et al*. (2023) support that prolonged exposure affects membrane integrity and sperm vitality.

Thus, the increase in stained (non-viable) sperm may be due more to extended incubation than to *B. alba*'s toxicity, highlighting the need for standardized exposure times.

**Section 4. Significant Difference Between *B. alba* Extracts and Controls**

**Table 5**

*Significant Difference Between B. Alba Extract and Control Stain on Supravital Staining Application*

| Component | n | B. *alba* 100% | | Control | | p-value |
|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | |
| Head | 3 | 3.6 | 0.6 | 4.0 | 0.0 | 0.374 |
| Midpiece | 3 | 3.6 | 0.6 | 4.0 | 0.0 | 0.374 |
| Tail | 3 | 3.6 | 0.6 | 4.0 | 0.0 | 0.374 |

*Note. Significant at* $\alpha \leq 0.05$

Table 5 shows no significant difference (p = 0.374) between *B. alba* extract and Giemsa stain in sperm morphology assessment, indicating comparable staining quality for the head, midpiece, and tail.

Studies by Mamay *et al*. (2023) and Chomean *et al*. (2019) also support the effectiveness of plant-based stains like beetroot and black rice, which performed similarly to synthetic stains in sperm morphology analysis.

These findings suggest *B. alba* is a viable, eco-friendly alternative to Giemsa for morphological staining, offering comparable accuracy while minimizing health and environmental risks.

## CONCLUSIONS AND RECOMMENDATIONS

### Conclusions

This study evaluated *B. alba* fruit extract as a natural stain for spermatozoa, aiming to offer an eco-friendly alternative to chemical stains. Results showed that the 100% concentration was most effective for both morphological and supravital staining. Morphological staining with 100% *B. alba* was comparable to the standard Giemsa stain, while supravital staining was less effective than alcoholic Eosin Y. Prolonged incubation led to increased staining of non-viable cells, emphasizing the need for controlled exposure times. Overall, *B. alba* extract proved promising for morphological staining but requires further validation for supravital use.

### Recommendations

1. Explore alternative methods for vitality analysis, such as the use of nigrosine background stain or advanced equipment.
2. Investigate and evaluate more appropriate mordants to improve stain adherence and overall staining quality.
3. Test the *B. alba* extract on the other type of cells to find out if it can be used for other staining applications.
4. Determine the shelf life of natural stains to improve the stability and preservation.
5. Find a solution that can be added to the plant dye so that it will leave a synergistic effect, improving the supravital stain application.

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