

# The Coloring Component of Betacyanin of *Hylocereus Polyrhizus* (Red Dragon Fruit) Flesh Extract as An Alternative Counter Stain for Semen Smear

Shanthel Ira T. Fojas<sup>1</sup>, Sunshine T. Hubahib<sup>1</sup>, Kaycee O. Jaravata<sup>1</sup>, Chellesa Angela R. Mojica<sup>1</sup> and Avril Ley Ann V. Recto<sup>1\*</sup>

<sup>1</sup>Lyceum of the Philippines (LPU), Cavite Campus,  
Governor's Dr, General Trias, Cavite, Philippines.

\*Corresponding author: [avril.recto@lpu.edu.ph](mailto:avril.recto@lpu.edu.ph)

## ABSTRACT

Red dragon fruit (*Hylocereus polyrhizus*) it is an essential source of betacyanin which is responsible for its red purple pigment. The purpose of this study was to determine the betacyanin content of alcoholic *H. polyrhizus* (red dragon fruit) flesh extract as an alternative counter stain for semen smear. This study utilized an experimental comparative design method. Samples were subjected with 20ml of 100% methanol in every 100 ml of *H. polyrhizus* flesh extract and to a series of temperatures: 30°C, 60°C, and 100°C for 5 minutes in the water bath to determine its color stability. This is followed by storing it at low temperature (4°C) without light exposure for 48 hours. The results of this study showed that the pigments were stable at 100°C heat treatment for 5 minutes in water bath and it stained excellently with a rate of 2.48 this signified that the standard eosin Y-nigrosin and alcoholic *H. polyrhizus* flesh extract have the same color affinity in staining sperm cells. Results also showed that the betacyanin obtained from the *H. polyrhizus* flesh extract heated at 100°C was effective as an alternative counterstain for semen smear.

## Keywords

*Hylocereus polyrhizus* (red dragon fruit); betacyanin; alcoholic extract; methanol; eosin Y-nigrosin; bioactive component; seminal smear.

## 1. INTRODUCTION

Dragon fruit is the fruit of several cactus species, of the genus *Hylocereus*. This fruit is widely planted in tropical countries and consumed as crop fruit. There are three main types of dragon fruit which are *Hylocereus undatus* (white flesh with pink skin), *Hylocereus polyrhizus* (red flesh with pink skin), and *Selenicereus megalanthus* (white flesh with yellow skin). Among these three, *Hylocereus polyrhizus* or also known as pitaya has gained interest by many due to its benefits.

Taheraet al. [1], reported that dragon fruit extracts with ethanol showed antibacterial activity against *Bacillus* spp. and *E. coli* (inhibition zone 7.50 and 14.42 mm, respectively). Similarly, study by Yaemchuen, Wichaphon and Klangpetch [2] proved that red dragon fruit peel with 60% ethanol exhibited the highest antibacterial activities against *E. scherichia coli*, *Salmonella* Typhimurium and *Bacillus cereus*, giving the minimum inhibitory concentration from 0.78 to 1.56 mg/mL. Furthermore, the phytochemical properties present in *H. polyrhizus* contribute significantly to the antioxidant capacity. According to Tsai et al. [3], stem, peel, and flower of *H. polyrhizus* are sources of antioxidant polyphenolics. Castro-Enríquez et

al. [4] proposed that ultrafiltration process could be a viable option to improve the biological activity of the natural extracts of this fruit.

Red dragon fruit is also often consumed fresh or make into juices, cordial, jams and ice cream. The responsible component for the red hue of the juice is group of molecules called betalains. One of these is the red purple pigment known as betacyanin. Research revealed that betacyanin is one of the active compounds in the dragon fruit [5]. This betacyanin obtained from the peel of *H. polyrhizus* can be used as natural dye[6] and food colorant [7-8]. Afandi et al. [9] have developed a natural formulated lipstick using essential oils and pigment of *H. polyrhizus* as natural colorant. Apart from cosmetic, betacyanin of *H. polyrhizus* was also utilized as sensitizers for dye-sensitized solar cells [10].

Semen analysis is an important part of a male infertility diagnosis. According to Aksoy et al. [11], sperm morphology evaluation is the most important criterion for determining the quality of a semen sample since this analysis reveals the sperm count, which reflects the number of spermatozoa in the semen sample; and the volume of the semen, which reflects the amount of seminal fluid produced. Evaluation can be done by staining the sperm cells with a variety of techniques to ease the visualization of cells and to provide better identification of abnormalities under the microscope.

Since *H. polyrhizus* has been used as replacement of synthesized dye in various industry, this study aimed to scrutinize the staining property of alcoholic *H. polyrhizus* flesh extract as alternative counter stain for semen smear. This would provide environment friendly and effective substitute to commercially prepared Eosin Y as a naturally derived stain for semen smear. The application of staining is used to distinguish the morphology of the cell such as sperm cell. This would also help in evaluating the percentage of normal and abnormal spermatozoa in human semen to identify their diagnostic significance.

## **2. METHODOLOGY**

### **2.1 Research Design**

This study utilized an experimental comparative design. The betacyanin of *H. polyrhizus* (red dragon fruit) flesh used as an alternative counterstain for semen smear were evaluated as to color uptake by sperm cells and to test at which temperature the extracts will yield the highest content of betacyanin. Experimental comparative research design aimed to know the relationship of two variables. Colour uptakes of alcoholic *H. polyrhizus* were observed in semen smear. A comparative study was done to differentiate the color of alcoholic *H. polyrhizus* extract and Eosin Y- nigrosin when applied for sperm cells.

### **2.2 Subject of Study**

Convenience sampling technique was applied in selecting participants for the study. This technique is a specific type of non- probability sampling method that would allow the researchers to choose participants and to collect sample from the people who were immediately accessible and conveniently available to participate in the study.

Three (3) students from Lyceum of the Philippines University, Cavite were the participants; the three male students underwent 3-5 days abstinence for semen collection and for the preparation of seminal smear.

### **2.3 Instrumentation**

The researcher made use of several laboratory instruments as a research tool in the study. The compound microscope was used to examine the betacyanin of *H. polyrhizus* flesh extract used in staining the sperm cell. The extracted *Hylocereus polyrhizus* flesh were placed into water bath at different degrees of temperature for 5 minutes. After each extraction procedure,

extracts were purified through double filtration process. Whereas, primary filtration was done using cheesecloth. Next, the filtrates were centrifuged for 10 minutes. Lastly, the filtrate was then filtered using Whatmann filter paper no. 1. The pH of *H. polyrhizus* extract was determined using microprocessor pH meter.

The laboratory reagents used were methanol as fixative, nigrosin as primary stain and Eosin Y as counterstain to visualize the morphology of semen smear. Analyses of the data obtained in the experiment were encoded in the computer to aid the interpretation and analysis of the study.

#### 2.4 Materials and Equipment

Materials used for extraction were cheesecloth, water bath, centrifuge, filter paper, and laboratory glassware. pH meter was also used to check the acidity of *H. polyrhizus*. Semen collections were placed in sterile cup. In staining semen smear, methanol was used as a fixative, nigrosin as primary stain and commercially prepared Eosin Y and alcoholic *H. polyrhizus* extract as a counterstain. Lastly, stained sperm cells were observed under a light microscope in HPO.

#### 2.5 Red Dragon Fruit Extraction

The *H. polyrhizus* were washed and peeled to remove the skin. The fruit flesh was cut into cubes, placed in cheesecloth, and squeezed to get the extract. The extracted *H. polyrhizus* flesh were placed into water bath at different degrees of temperature 30°C, 60°C and 100°C for 5 minutes to preserve the betacyanin. The extract obtained was macerated for 48 hours with 20 mL methanol (100%) for every 100 mL dragon fruit (alcoholic). The extract was covered with foil to avoid degradation of betacyanin pigment. Refrigeration storage (4°C) condition avoiding light exposure was used to preserve the color of fruit juice up to three weeks [12].

### 3. RESULTS AND DISCUSSION

This section presented the results, analysis, and discussion of the staining ability of betacyanin in *Hylocereus polyrhizus* extract at varying temperatures at 30°C, 60°C, and 100°C respectively and was then compared with the commercially prepared Eosin Y.

Table 1 tabulated the physical properties of the *H. polyrhizus* extract. Five physical properties such as colour, transparency, odour, consistency, pH and stability.

**Table 1.** Physical Properties of Alcoholic *H. polyrhizus* flesh extract

Physical properties	Observation
Colour	Dark purple to pink
Transparency	Clear
Odour	Mild sweet like
Consistency	Less viscous, water-like
pH	5.5
Stability	Unstable

Shown in Table 2 were the staining results of *H. polyrhizus* flesh extract as an alternative counterstain for sperm cell morphology. Comparison was established through the uptake of the sperm cells with stain heated at 30°C, 60°C, and 100°C. In order to further establish the comparison, the staining results were compared to those smears stained with Eosin nigrosin. The staining result of the extract was evaluated based on the color intensity of sperm cells

following the criteria of Bjorndahl et al. [13]. When heated at 30°C, 60°C, and 100°C, the grand mean were 0.39, 1.54 and 2.48 respectively.

**Table 2.** Staining Result of Alcoholic *Hylocereuspolyrhizus* Flesh Extract upon Exposure at Different Temperatures

Semen smear	Grand mean	Interpretation
Head – tail 30°C	0.39	Poor (pale/poorly stained)
Head – tail 60°C	1.54	Good (stained pink)
Head – tail 100°C	2.48	Excellent (stained dark pink)

Legend: Adapted from Bjorndal L., Soderlund I., Kvist U., (2003)

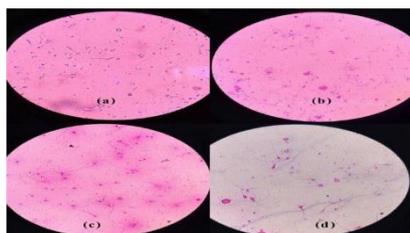
2.26 – 3.00 - Dark pink (Excellent)

1.51 – 2.25 - Pink (Good)

1.76 – 1.50 - Light pink (Fair)

0.00 – 1.75 - Pale/Poorly stained (Poor)

Figure 1 depicted the stained of the sperm smear of Eosin Y-Nigrosin and alcoholic *Hylocereuspolyrhizus* flesh extract-Nigrosin observed under light microscope HPO (400x). The observed stained colour corresponded to the interpretation of the grand mean as presented in Table 2.



**Figure 1.** Photograph of sperm cells stained in Eosin Y-Nigrosin and Alcoholic *Hylocereuspolyrhizus* flesh extract-Nigrosin observed under light microscope HPO (400x) (a) Eosin YNigrosin as Control (b) Alcoholic *H. polyrhizus* flesh extract heated at 30°C Nigrosin (c) Alcoholic *H. polyrhizus* flesh extract heated at 60°C -Nigrosin (d) Alcoholic *H. polyrhizus* flesh extract heated at 100°C –Nigrosin

Based on Table 3, all the extracts have a significant difference with Eosin Y-nigrosin which was the control, as all the generated p-values are <0.05. However, the alcoholic *H. polyrhizus* extract heated at 100°C got the nearest value of 2.48 to the perfect rate of standard Eosin Y-nigrosin, which was 3.

Based on the interpretation of results adapted from the study of Björndahl et al. [13], this extract belonged to the range of 2.26 – 3.0 which reflected that it excellently stained the sperm cells dark pink. This signified the standard

Eosin Y-nigrosin and alcoholic *H. polyrhizus* flesh extract heated at 100°C for 5 minutes can have the same staining reaction result in staining sperm cells since at this temperature, the optimum betacyanin concentration was reached following the study of Harivaindaran et al. [6].

**Table 3.** T- Test results between the staining properties of Betacyanin in *H. polyrhizus* flesh extract at three different temperature compared to Eosin Y as to sperm morphology

Semen smear	Grand mean	p-value	Interpretation
-------------	------------	---------	----------------

30°C Head-Tail	0.39	0.001	Significant
60°C Head-Tail	1.54	0.000	Significant
100°C Head-Tail	2.48	0.000	Significant

#### 4. CONCLUSION

Based on the results gathered through evaluation of the property of betacyanin of *H. polyrhizus* flesh extract as alternative counterstain for semen smear preparation, the researchers concluded the following:

1. The staining property of alcoholic *H. polyrhizus* flesh extract in semen smear was due to its bioactive component, particularly the betacyanin. It was also responsible for its acidity and red purple color. It has a pH of 5.5 which was close to the pH of Eosin Y and with mild sweet like odor. It has a clear transparency and a water-like consistency. *H. polyrhizus* flesh extract were unstable because the betacyanin can be affected by exposure to light and heat.
2. At 30°C, the alcoholic *H. polyrhizus* flesh extract was pale and at 60°C, the extract was pink. Then the extract at 100°C was dark pink. The researchers therefore deduced that the staining process of the alcoholic *H. polyrhizus* flesh extract heated at 100°C was more effective in staining the sperm cells head-tail compared to 30°C and 60°C *H. polyrhizus* flesh extract, because the sufficient amount of betacyanin needed for staining the sperm cells were extracted at 100°C.
3. The staining affinity was at its best at 100°C, because its staining result was the same as the control Eosin Y-nigrosin.
4. There was a significant difference between the staining property of the alcoholic *H. polyrhizus* flesh extract heated at three different temperatures with nigrosin and the control Eosin Y-nigrosin. However, the interpretation of results showed that the 100°C alcoholic *H. polyrhizus* flesh extract and the control can both stain the sperm cells excellently.

Therefore, it was concluded that the alcoholic *H. polyrhizus* flesh extract heated at 100°C was an effective alternative counterstain for semen smear.

#### 5. REFERENCES

- [1] Tahera, J., Feroz, F., Senjuti, J. D., Das, K. K., & Noor, R. (2014). Demonstration of anti-bacterial activity of commonly available fruit extracts in Dhaka, Bangladesh. *American J. Microb. Res*, 2, 68-73.
- [2] Yaemchuen, N., J. Wichaphon, and W. Klangpetch. (2018). Antioxidant and Antibacterial Activities of Natural Red Colorants from RedDragon Fruit Peel and Roselle. *The International Conference on Food and Applied Bioscience*: 163-167.
- [3] Tsai, Y., Lin, C. G., Chen, W. L., Huang, Y. C., Chen, C. Y., Huang, K. F., & Yang, C. H. (2019). Evaluation of the Antioxidant and Wound-Healing Properties of Extracts from Different Parts of *Hylocereus polyrhizus*. *Agronomy*, 9(1), 27.
- [4] Castro-Enríquez, D. D., Montaña-Leyva, B., Toro-Sánchez, D., Carmen, L., Juárez-Onofre, J. E., Carvajal-Millán, E., Guadalupe A. López-Ahumada, G. A., Barreras-Urbina, C. G., Tapia-Hernández, J. A., & Rodríguez-Félix, F. (2020). Effect of Ultrafiltration of Pitaya Extract (*Stenocereus thurberi*) on Its Phytochemical Content, Antioxidant Capacity, and UPLC-DAD-MS Profile. *Molecules*, 25(2), 281.

- [5] Manchali, S., Murthy, K. N. C., Nagaraju, S., & Neelwarne, B. (2013). Stability of betalain pigments of red beet. In *Red beet biotechnology* (pp. 55-74). Springer, Boston, MA.
- [6] Harivaindaran, K. V., Rebecca, O. P. S., & Chandran, S. (2008). Study of optimal temperature, pH and stability of dragon fruit (*Hylocereus polyrhizus*) peel for use as potential natural colorant. *Pakistan Journal of Biological Sciences*, 11(18), 2259-2263.
- [7] Gengatharan, A., Dykes, G. A., & Choo, W. S. (2016). Stability of betacyanin from red pitahaya (*Hylocereus polyrhizus*) and its potential application as a natural colourant in milk. *International journal of food science & technology*, 51(2), 427-434
- [8] Faridah, A., Holinesti, R., & Syukri, D. (2015). Betalains from Red Pitaya Peel (*Hylocereus polyrhizus*): Extraction, Spectro-photometric & HPLC-DAD Identification, Bioactivity & Toxicity Screening. *Pakistan Journal of Nutrition*, 14(12), 976-982.
- [9] Afandi, A., LAZIM, A. M., Azwanida, N. N., Bakar, M. A., AIRIANAH, O. B., & FAZRY, S. (2017). Antibacterial properties of crude aqueous *Hylocereus polyrhizus* peel extracts in lipstick formulation against gram-positive and negative bacteria. *Malaysian Applied Biology*, 46(2), 29-34.
- [10] Al-Alwani, M. A., Ludin, N. A., Mohamad, A. B., Kadhum, A. A. H., & Sopian, K. (2017). Extraction, preparation and application of pigments from *Cordyline fruticosa* and *Hylocereus polyrhizus* as sensitizers for dye-sensitized solar cells. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 179, 23-31.
- [11] Aksoy, E., Aktan, T. M., Duman, S., & Cuce, G. (2012). Assessment of spermatozoa morphology under light microscopy with different histologic stains and comparison of morphometric measurements. *Int J Morphol*, 30(4), 1544-1550.
- [12] Woo, K. K., Ngou, F. H., Ngo, L. S., Soong, W. K., & Tang, P. Y. (2011). Stability of betalain pigment from red dragon fruit (*Hylocereus polyrhizus*). *American Journal of Food Technology*, 6(2), 140-148.
- [13] Björndahl, L., Söderlund, I., & Kvist, U. (2003). Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Human reproduction*, 18(4), 813-816.