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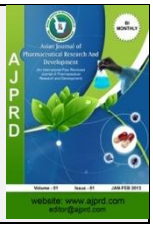

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Research Article

Application of Red Dragon Fruit Dyes as Staining in Histological Study

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A B S T R A C T

Background: The betacyanin pigment found in dragon fruit flesh (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) has many advantages, for instance as a tissue histology dye.
Objectives: determining the properties of organs stained with betacyanin red dye and finding alternative histology colors to replace synthetic hematoxylin-eosin dyes.
Summary of contents of the article: The flesh of the red dragon fruit (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) was extracted using the Ultrasonic Assisted Extraction (UAE) method. The study employed six male white mice divided into two treatment groups: a placebo group induced with 1% Na.CMC carrier solution and a positive control group generated a toxic dose of paracetamol. The results were visually compared to hematoxylin-eosin with standard betacyanin and betacyanin extract of red dragon fruit flesh concentrations of 50% (v/v), 80% (v/v), and 100% (v/v).
Conclusion: Betacyanin from the flesh of the red dragon fruit can be applied as a histological dye in the liver of male white mice and as a counterstaining hematoxylin as a substitute for eosin dye at a concentration of 100% (v/v).
Keywords: Red dragon fruit, Betacyanin, Histology

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INTRODUCTION

Red dragon fruit (*Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose) is one of the plants that contain betacyanin pigment. When ripe, the flesh of the dragon fruit turns purple-red and is surrounded by black seeds[1]. Vitamin B1, vitamin B2, vitamin B3, and vitamin C, as well as protein, fat, carbs, fiber, flavonoids, thiamine, niacin, pyridoxine, cobalamin, phenolic, betacyanin, polyphenols, carotene, phosphorus, iron, and phytoalbumin, are all found in the flesh of dragon fruits[2]. Betacyanin, a red pigment used as a natural dye, is a significant component of dragon fruit[3].

Betacyanin (6-O-3-hydroxy-3-methyl-glutaryl-betanin) with N-heterocyclic is a red pigment with numerous advantages, one of which being its antioxidant properties[4]. According to previous research[5], the superiority of dragon fruit as an antioxidant is because dragon fruit is rich in polyphenolic compounds. Betacyanin is a dye that belongs to the betalain class, which is soluble in water and non-water-soluble organic solvents but not in organic solvents[6]. Betacyanin can be used as a natural food dye or substitute for synthetic dyes. It has pleasing color, is easily soluble in water, and has significant antioxidant activity, making it safer to take[7].

The results of previous studies that betacyanin dyes can be used as natural food colorings are stable at pH 4.5 at room temperature and under acidic conditions[8]. Wider use of betacyanin pigments is as a natural dye in pharmaceutical

preparations in tablet printing[4]. Natural dyes are less stable to light, heat, and at a particular pH value when compared to synthetic dyes; this is a factor in the lack of use of natural dyes in pharmaceuticals[9].

Dragon fruit flesh contains betacyanin pigment, which can be used as a natural dye. So far, betacyanin pigments are widely used as food coloring, so it is necessary to use them more widely, namely as tissue histology dyes.

Histology is the knowledge or science of tissues, plants, and animals[10]. The stages of making animal histology preparations include fixation, dehydration, clearing, paraffinization, embedding (naming), deparaffinization, with the final stage of making histology preparations in the form of staining. Staining is a tissue staining process that aims to observe using a microscope and distinguish the parts of the tissue to be observed, such as the cell nucleus, cytoplasm, and others. The stain used is Hematoxylin-Eosin[11].

Hematoxylin-Eosin is a tissue stain commonly used to stain liver tissue[11]. Hematoxylin-Eosin has more disadvantages than benefits. Disadvantages of Hematoxylin-Eosin are commercial samples that vary from group to group, do not specifically stain the nucleus and cytoplasm of proteins, cause pollution (hematin, the active reagent in hematoxylin solution is oxidized to oxyhematin), and the combination of hematoxylin and metal is difficult to control[12]. This is reinforced by the previous research[13]. In terms of the length of the process, it takes a relatively long time because it has a fairly complex protocol, so that it requires precision and perseverance and requires rather expensive costs. Due to the deficiency of Hematoxylin-Eosin earlier, natural dyes can be rewritten. The standard requirements for ideal dyes are cheap, durable, not tricky to clean, and not damaging the environment. In studies, hematoxylin has been proven expensive and can damage the environment[12].

Researchers are investigating using dye from red dragon fruit flesh (Hylocereus costaricensis (F.A.C.Weber) Britton & Rose) as a histology dye in male white mice and as a substitute for synthetic dyes for the reasons stated above.

METHODS

Materials and Equipments

Red dragon fruits were purchased from farmers in West Sumatera Indonesia. Standard betacyanin was purchased from Sigma-Aldrich. Hematoxylin dye and eosin were obtained from The Science Company. All chemical reagents used in this study were of reagent grade and used as received.

The extraction process was perfomed by using Sonicator (Branson) and centrifuge (Hettich EBA 20), and analyzed by using spectrophotometer UV-Vis (PG-Instrument). Histological study was performed using vacuum infiltration tissue processor (Tissue-Tek*VIP*5 Jr), paraffin embedding center (Myr EC 350), and microtome (Leits).

Experimental Animals

The experimental animals used six healthy male white mice weighing 24-30 grams. Before being treated, the mice were acclimatized for seven days by being given adequate food and water.

Extraction Method with *Ultrasonic Assisted Extraction* (UAE)

At room temperature, a fine sample of red dragon fruit flesh was placed in an ultrasonic bath and sonicated at 50 kHz for 30 minutes (25^{o}C). To make a colored solution, separate the pulp using a funnel and filter it through Whatman filter paper No. 1. The red dragon fruit flesh extract solution was centrifuged at 6000 rpm for 15 minutes at room temperature to obtain and store the supernatant. The supernatant was then used for further investigation.

The levels of betacyanin in dragon fruit flesh extract

By dissolving 250 mg in a 50 mL volumetric flask, adding aquadest to the mark, and shaking homogeneously, standard betacyanin mother liquor with a concentration of 5000 g/mL was created. The 5000 g/mL mother liquor was diluted by 5.0 mL and placed in a 10 mL flask, then filled to the mark with distilled water and homogenized to obtain a 2500 µg/mL). A spectrophotometer UV-Vis measured the absorbance at the maximum wavelength of standard betacyanin (531 nm) three times. After determining the sample's absorbance, the concentration can be calculated using the regression equation: $y = a + bx$.

Preparation of histology preparations for the liver

The stage of making histology preparations was carried out according to the Kiernan method. The livers of healthy and damaged male white mice were dissected and washed with a physiological NaCl solution (0.9% NaCl). The tissues were fixed by immersing them in 10% phosphate buffer formalin for 24 hours, then trimmed so that they could be inserted into the embedding—cassette for processing in the Vacuum Infiltration Tissue Processor. In the next stage, the embedding cassette that already contains the tissue is inserted into the tissue basket and immersed in the paraffin bath reservoir, close, lock, and click start. The embedding cassette containing tissue will be sprayed automatically by absolute alcohol I, absolute alcohol II, absolute alcohol III, absolute alcohol IV, absolute alcohol V, absolute alcohol VI, absolute alcohol VII, xylol I, xylol II, xylol III, cleaning xylol, cleaning alcohol, water, and activated carbon for 24 hours until the alarm sounds and shows tissue basket writing on the monitor. The next stage is unlocking and lifting the tissue basket and draining it. Then the tissue was put into liquid paraffin at 56°C for 2 hours for two times. The tissue was

then taken with tweezers, followed by blocking using a paraffin block. Cutting is done using a microtome thickness of 4-5 µm. The excised tissue was developed over water in a water bath, then dried at room temperature. The preparations were ready to be stained with Hematoxylin-Eosin (HE), standard betacyanin, and extract of red dragon fruit flesh (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) with a concentration of 50% (v/v), 80% (v/v), 100% (v/v).

Staining with hematoxylin and eosin

Hematoxylin-Eosin (HE) staining stages using the Mayer method. (Deparaffinization) by inserting the preparations into xylol I, xylol II, and xylol III for 7 minutes each, that clean the edges of the tissue with gauze. (Rehydration) The preparations were immersed in 100%, 95%, 80%, 70% alcohol for 7 minutes each. The preparations were flowed with running water for 3 minutes in the next stage, followed by painting. The preparations were entered into Mayer hematoxylin for 15 minutes, after which the preparations were flooded with running water for ± 3 minutes. The preparations were put into eosin solution for 5 minutes in the next stage. The next step is (Dehydration) by adding 70%, 80%, 95%, 100% alcohol for 3 minutes, followed by (Clearing) using xylol I and xylol II for 3 minutes each. The next stage is (Mounting) by dripping the preparation using entelan, then covering it with a cover glass. Furthermore, the preparations can be observed under a microscope with a magnification of 20X and 40X.

Standard Betacyanin Staining Process

The standard betacyanin staining stage weighed 250 mg. Betacyanin standard weighed 250 mg and dissolved with 1 mL of distilled water. (Deparaffinization) by inserting the preparations into xylol I, xylol II, and xylol III for 7 minutes each, that clean the edges of the tissue with gauze. (Rehydration) The preparations were immersed in 100%, 95%, 80%, 70% alcohol for 7 minutes each. The preparations were flowed with running water for 3 minutes in the next stage, followed by painting. The preparations were stained with standard betacyanin for 15 minutes, after which the preparations were poured with running water for ± 1 minute. The next step (Dehydration) is to put the preparation into 70%, 80%, 95%, 100% alcohol for 1 minute, followed by (Clearing) using xylol I and xylol II for 1 minute each. The next stage is (Mounting) by dripping the preparation using entelan, then covering it with a cover glass. Furthermore, the preparations can be under a microscope with a magnification of 20X and 40X.

Coloring Process of Red Dragon Fruit Extract (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose)

Staining stages of red dragon fruit flesh extract (Hylocereus costaricensis (F.A.C.Weber) Britton & Rose) with a concentration of 50% (v/v). (Deparaffinization) by inserting the preparations into xylol I, xylol II, and xylol III for 7 minutes each. After that, clean the edges of the tissue with gauze. (Rehydration) The preparations were immersed in 100%, 95%, 80%, 70% alcohol for 7 minutes each. The trials were flowed with running water for 3 minutes in the next stage, followed by painting. The preparations were colored with red dragon fruit extract at a concentration of 50% (v/v) for 15 minutes, after which the trials were poured with running water for ± 1 minute. The next step (Dehydration) is to put the preparation into 70%, 80%, 95%, 100% alcohol for 1 minute, followed by (Clearing) using xylol I and xylol II for 1 minute each. The next stage is (Mounting) by dripping the preparation using entelan, then covering it with a cover glass. Furthermore, the practices can be under a microscope with a magnification of 20X and 40X. Perform the above experiment for staining the flesh extract of red dragon fruit (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) with a concentration of 80% (v/v) and 100% (v/v).

Histological Staining Data Analysis

Analysis was carried out visually on the comparison between hematoxylin-eosin with standard betacyanin and betacyanin extract of red dragon fruit flesh (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) at concentrations of 50% (v/v), 80% (v/v), and 100%. (v/v). If the control and sample stains have the same preparations, the betacyanin stain from the study can be used as a histological stain.

RESULT AND DISCUSSION

Fresh samples of red dragon fruits were extracted with a sonicator at 50 kHz for 30 minutes at room temperature (25°C). The use of ultrasonic causes a cavitation effect that can break down the cell walls of the material so that the active substances can come out quickly and obtain maximum extract results with a much shorter extraction[13]. Then the sample was filtered using Whatman filter paper No. 1 to obtain a red filtrate. The obtained filtrate was centrifuged at 6000 rpm for 15 minutes at room temperature (25°C) to get the supernatant. This centrifuge process aims to bring a red supernatant that is dense, clear, and free of impurities that can still pass during the filtering process so that we get a thick extract in the form of looking for the flesh of red dragon fruit. The yield of red dragon fruit extract was 49.3495%. The average percentage of betacyanin content in the flesh extract of red dragon fruit was 27.8681%. The betacyanin content in the red dragon fruit flesh extract is quite good considering the betacyanin stability disturbed in extreme environments where the betacyanin content can be lost.

Histological observations in the placebo group (given Na. CMC 1%) at 20X and 40X magnification using Hematoxylin-Eosin dye showed sinusoids, central veins, cytoplasm, one hepatocyte cell, cell nucleus, and no damage to hepatocytes (Figure 1).

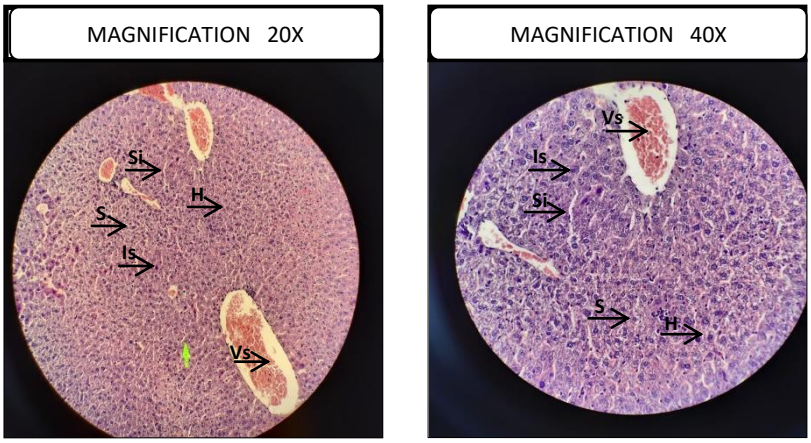

Figure 1; Placebo Group Hepatocytes Cells (administered 1% Na. CMC) with Hematoxylin-Eosin staining (Si: Sinusoid, Vs: Central Vena, S: Cytoplasm, H: 1 hepatocyte cell, Is: Cell nucleus)

The placebo group's standard betacyanin dye contained sinusoids and central veins (Figure 2).

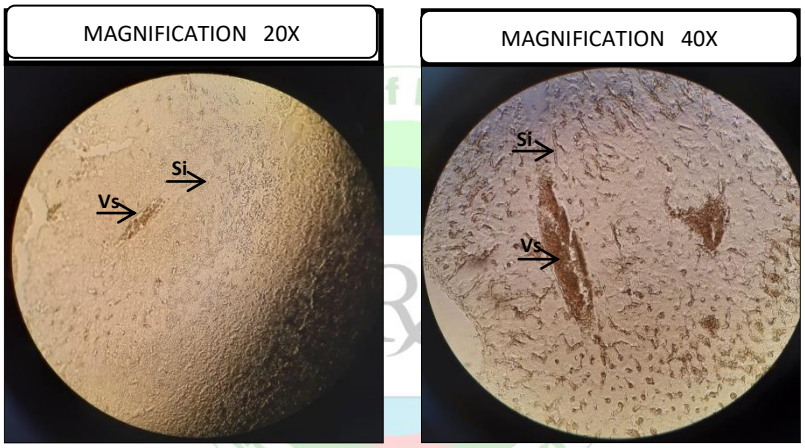

Figure 2: Placebo Group Hepatocytes Cells (administered 1% Na. CMC) with Standard Betacyanin Staining (Si: Sinusoid, Vs: Central Vena)

The stain of red dragon fruit flesh extract with a concentration of 50% (v/v) contained sinusoids, central veins (Figure 3).

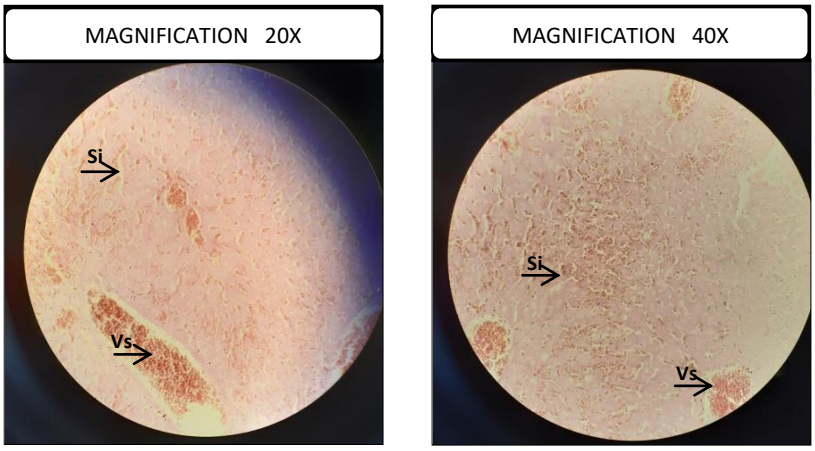

Figure 3: Placebo Group Hepatocytes Cells (administered 1% Na. CMC) with Betacyanin Staining Dragon Fruit Flesh Extract 50% (v/v) (Si: Sinusoid, Vs: Vena Centralis)

The dye extract of red dragon fruit flesh with a concentration of 80% (v/v) contained sinusoids, central veins (Figure 4).

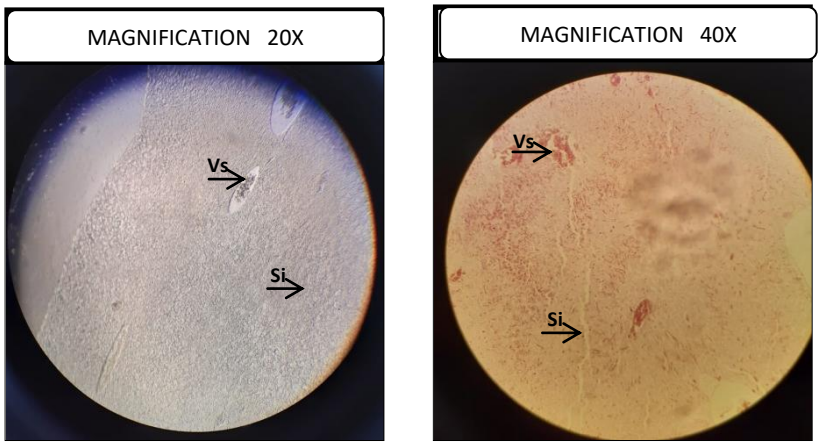

Figure 4: Placebo Group Hepatocytes Cells (administered 1% Na. CMC) with Betacyanin Staining Dragon Fruit Flesh Extract 80% concentration (Si: Sinusoid, Vs: Vena centralis)

The dye of red dragon fruit flesh extract with a concentration of 100% (v/v) contained sinusoids, central vein, cytoplasm, and one hepatocyte cell (Figure 5). This is due to the low levels of betacyanin to color the preparations clearly, and the time the staining intensity needs to be increased. Suabjakyong (2011) states that the length of the process in coloring can determine the intensity of staining.

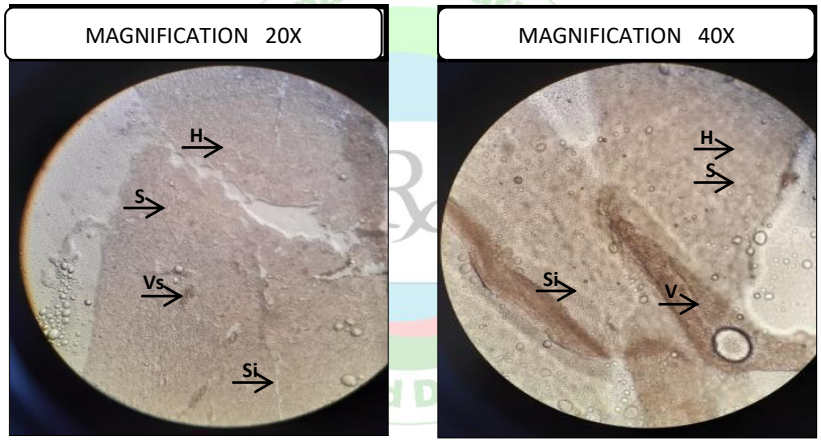

Figure 5: Placebo Group Hepatocytes Cells (administered 1% Na CMC) with Betacyanin Staining Dragon Fruit Flesh Extract 100% concentration (Si: Sinusoid, Vs: Central Vena, S: Cytoplasm, H: 1 hepatocyte cell)

Histological observation of the positive control group (given Paracetamol Toxic Dosage) at 20X and 40X magnification using Hematoxylin-Eosin dye showed sinusoids, central veins, cytoplasm, one hepatocyte cell, cell nucleus, and damage to hepatocyte cells occurred, namely necrosis, degeneration, inflammation, bleeding (Figure 6).

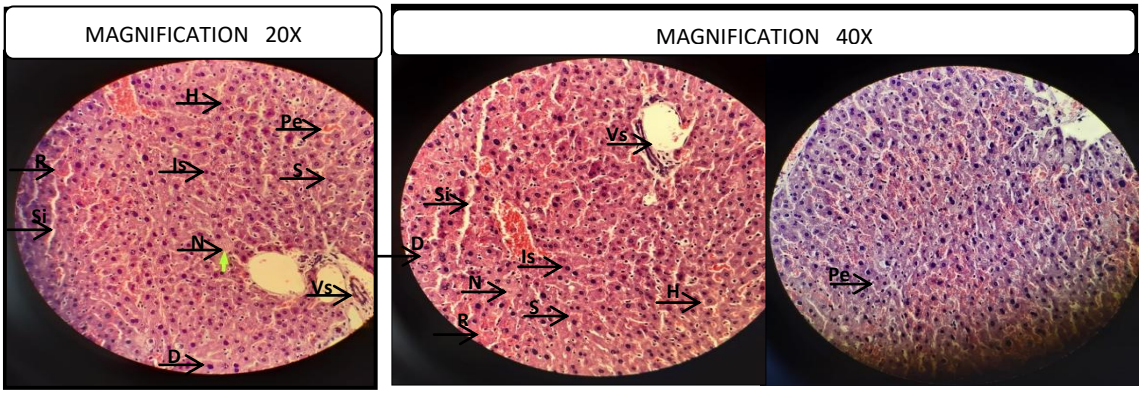

Figure 6: Positive Control Group Hepatocytes Cells (given Paracetamol Toxic Doses) with Hematoxylin-Eosin staining (Si: Sinusoid, Vs.: Central Vena, S: Cytoplasm, H: 1 hepatocyte cell, Is: Cell nucleus, N: Necrosis, D: Degeneration R: Inflammation, Pe: Bleeding)

The standard betacyanin dye contained sinusoids, central veins, and hepatocyte damage could not be seen (Figure 7).

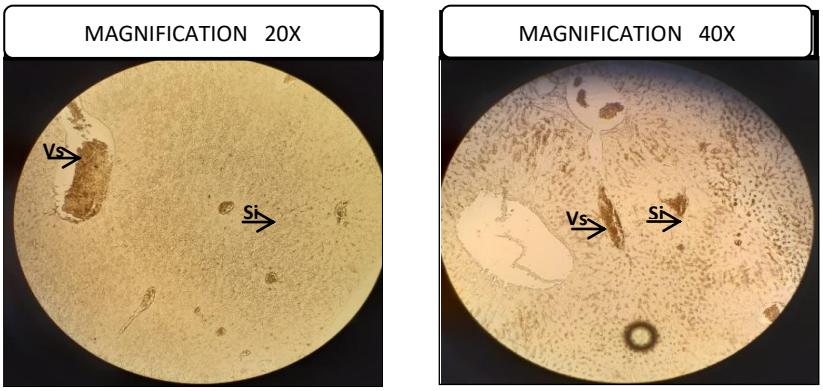

Figure 7: Positive Control Group Hepatocytes Cells (given Paracetamol Toxic Dosage) with Standard Betacyanin Staining (Si: Sinusoid, Vs.: Central Vena)

The stain of red dragon fruit flesh extract (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) with a concentration of 50% (v/v) contained sinusoids, central veins, and damage to hepatocyte cells could not be seen (Figure 8).

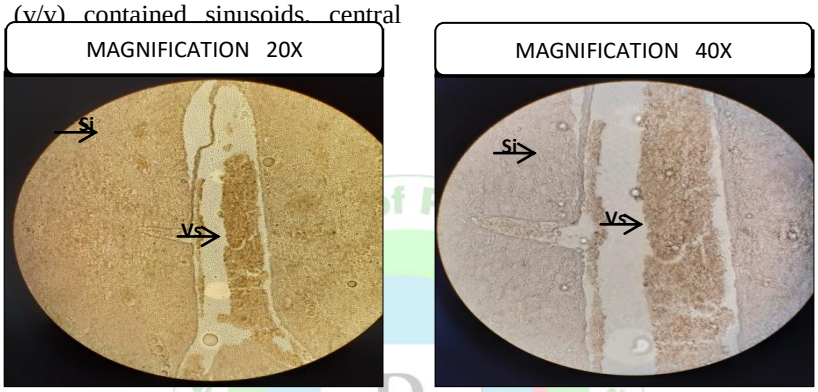

Figure 8: Hepatocyte Cells in Positive Control Group (given Paracetamol Toxic Dose) with Betacyanin Staining Dragon Fruit Flesh Extract at 50% concentration (Si: Sinusoid, Vs.: Vena Sentralis)

The dye of red dragon fruit flesh extract (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) with a concentration of 80% (v/v) contained sinusoids, central veins, and damage to hepatocyte cells could not be seen (Figure 9).

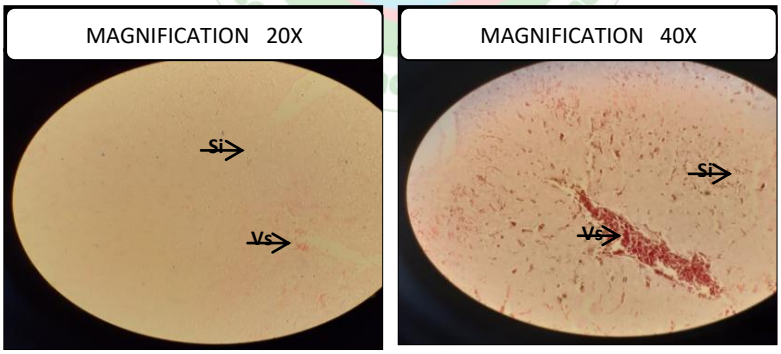

Figure 9: Hepatocyte Cells in Positive Control Group (given Paracetamol Toxic Dose) with Betacyanin Staining Dragon Fruit Flesh Extract at 80% concentration (Si: Sinusoid, Vs.: Vena Sentralis)

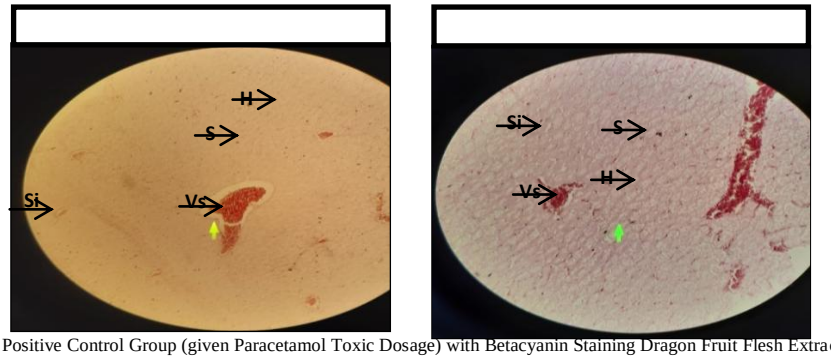

Figure 10: Hepatocyte Cells in Positive Control Group (given Paracetamol Toxic Dosage) with Betacyanin Staining Dragon Fruit Flesh Extract 100% concentration (Si: Sinusoid, Vs.: Central Vena, S: Cytoplasm, H: 1 hepatocyte cell)

The dye of red dragon fruit flesh extract (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) with a concentration of 100% (v/v) contained sinusoids, central veins, cytoplasm, one hepatocyte cell, and the damage to hepatocyte cells could not be seen (Figure 10) This is because the cell nucleus cannot be stained by betacyanin, so the damage cannot be seen. The extraction treatment significantly affects the direction and color density[14]. This is due to chemical compounds with properties that can change and react at certain temperature conditions in an acid-base environment. Under these conditions, chemical compounds can break down or decompose into other compounds, or new compounds appear that give different colors in their initial conditions. The higher the extraction temperature, the redder it gets.

Based on the results of previous studies[15], the stability of betacyanin pigments is also influenced by pH, and betacyanin is stable at pH 4 to pH seven and the most stable at pH four and pH 5. The storage temperature of 10 °C for ten days in red dragon fruit is best for retaining betacyanins[16]. Betacyanin pigments are stable at 25 °C and 40 °C[15]. This can be seen from the absorbance value of the extract at a temperature of 40°C after heating for 30 minutes, which is 0.472. The results obtained were not much different from the absorbance of the extract at a temperature of 25°C, which was 0.474. Low storage temperature can inactivate the enzyme to maintain stability and slow down the degradation of betacyanin; an increase in temperature can cause the color to fade.

The pH of red dragon fruit betacyanin is 4.5 at room temperature and under acidic conditions[8]. This indicates that the betacyanin dye has an acidic pH. Certain factors determine the ability of a dye to color specific tissue structures. One of them is the acidity of the stain. The basic structure will be colored by acid because dragon fruit has a strong affinity for the cytoplasm[17]. Dyes with an acidic pH can stain the cytoplasm as a basic structure[18]. Eosin is acidic and degrades acidophilic components in tissues[19]. Eosin, a counterstaining hematoxylin, is used to stain the cytoplasm of cells connective tissue and gives a pink color with different shades, while hematoxylin works like a dye. Base contains the dye hematin, responsible for the color properties[20-21]. Hematin is anionic, has a poor affinity for tissues, and cannot impart color to the nucleus without the mordant. The mordant imparts a positive charge to the dye-mordant complex. For binding to anionic network sites such as nuclear chromatin, the type of mordant used dramatically influences the tissue component stained and the final color result[21]. Mordant is an essential part of the dyeing process with natural dyes because it will determine the success or failure of the staining process[22].

The color of the preparation can be determined from several factors, namely the process of slicing the thinner tissue, the more the tissue will be seen, the ability of the tissue to bind to the dye, and taking the right photo on a microscope. Based on the results of research on liver tissue of male white mice with red dragon fruit (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose) extract dye with a concentration of 100% (v/v) resulted in better clarity and color contrast of the preparations than the 50% concentration (v/v) and 80% (v/v), this is likely to be different if used on other networks. According to Suntoro (1983), staining will make it easier to observe cells or tissues under a microscope because the dye has a selective affinity for cell organelles. Not all cell organelles can react with the same coloring. This is due to differences in each organelle's constituent components and properties.

CONCLUSION

Histological observation of the placebo group using Hematoxylin-Eosin dye showed sinusoids, central vein, cytoplasm, one hepatocyte cell, cell nucleus, and there was no damage to the cells. The standard betacyanin dye for the placebo group contained sinusoids, central veins. The dye extract of red dragon fruit flesh with a concentration of 100% (v/v) contained sinusoids, central vein, cytoplasm, and one hepatocyte cell. Histological observations of the Positive control group using Hematoxylin-Eosin dye had sinusoids, main veins, cytoplasm, one hepatocyte cell, cell nucleus, and damage to hepatocyte cells occurred, namely necrosis, degeneration, inflammation, bleeding. The standard betacyanin dye contained sinusoids, central veins, and hepatocyte damage could not be seen. The stain of red dragon fruit flesh extract with a concentration of 100% (v/v) contained sinusoids, central veins, cytoplasm, one hepatocyte cell, and damage to hepatocyte cells could not be seen.

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