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Histopathology liver of the *Mus musculus* based on the intensity of induction time of ciu alcoholic beverages

Magdalena Budi Verena Putri, Fitria Diniah Janah Sayekti
Medical Laboratory Technology, Sekolah Tinggi Teknologi Kesehatan Nasional, Sukoharjo, Indonesia

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ABSTRACT

The ciu alcoholic drink is a drink made from molasses that is fermented and undergoes a distillation process to obtain 25% ethanol that can cause liver's damage. The purpose of this study was to determine the difference in the histopathological effect of the liver of *Mus musculus* treated with different periods. This study was experimental with 5 treatment groups, including negative control and 4 treatments of ciu, a traditional alcoholic drink by administering 0.6 ml/25 g for 3, 7, 14, and 30 days. The results of macroscopic observations showed that there was a difference between the control group and the ciu treatment, namely, the liver was brownish red when given the ciu alcoholic drink for 3 days and the liver organs were blackish red when given for 7 days, and on the liver weight, the longer the ciu traditional alcoholic drink is given, the greater the liver weight. The results of the significant value of the analysis of variance (ANOVA) test were 0.000, so it can be seen that the administration of the traditional alcoholic drink ciu can affect the histopathological of the mice liver, where the organ that is given ciu alcoholic beverages progressively causes tissue damage such as degeneration and necrosis.

Corresponding Author:

Fitria Diniah Janah Sayekti
Medical Laboratory Technology, Sekolah Tinggi Teknologi Kesehatan Nasional
Solo Baki Kwarasan Street, Sukoharjo, Central Java, Indonesia
Email: fitria.diniah@stikesnas.ac.id

1. INTRODUCTION

Alcoholic beverages have long been known among the people. Based on the Global Status Report on alcohol and health in 2014, as many as 1,928,000 Indonesians experienced health problems due to excessive consumption of alcohol, and as many as 1,180,900 Indonesians experienced alcohol dependence. The dangers of consuming alcohol are included in the top five risk factors for disease, disability, and death worldwide. Alcohol can cause physical, mental, and social effects, which are determined by quantity and pattern of alcohol drinking [1]. According to the World Health Organization (WHO) [2], there has been an increase in the prevalence of alcohol consumers by 33% since 2007. Alcoholic beverages are obtained from fermentation by microorganisms in sugar, fruit juice, seeds, honey, tubers, and certain cactus sap. Traditional alcoholic drinks that are often found in Indonesia are palm wine, brem wine, lapen, sopi, and ciu. Definition of one alcohol drink as per the Centers for Disease Control and Prevention (CDC) [3] is a half-ounce or 13.7 g pure alcohol which is the amount of alcohol present in 12 oz beer (5% alcohol), 8 oz malt liquor (7% alcohol), 5 oz wine (12% alcohol), 1.5 oz 80-proof "hard-liquor" (40% alcohol) [4].

Ciu is a nickname for a typical alcoholic drink from the Bekonang area (an area on the outskirts of Solo, Indonesia). Apart from being consumed as liquor by the people, "Ciu Bekonang" can also be used as a means of massage. By using whipped param powder mixed with liquid "Ciu Bekonang," then rubbed all over

the body so that it can give a warm effect to the body. Ciu produced in Bekonang uses the basic ingredients of molasses, which is fermented and undergoes a distillation process to obtain 25% ethanol [5]. The resulting compound has the main content of ethanol, while the other compounds produced are citric acid with an average level of 6.82% [6]. The Central Statistics Agency (BPS) proved that in 2018, alcohol consumers in rural areas were 0.72 liters per capita more than in urban areas, which were only around 0.28 liters per capita. Traditional home-distilled ciu typically contains high levels of ethanol, often ranging around 35%–45% alcohol by volume, categorizing it among strong spirits [7], [8].

Consumption of alcoholic beverages such as Ciu can interfere with the body's metabolic processes because ethanol that can be distributed by blood, is only 0.5–0.7l per kg of adult body weight (BW) without causing side effects. If it exceeds these levels, the liver is unable to produce enzymes alcohol dehydrogenase (ADH) to convert ethanol into in acetylaldehyde [9], [10]. The liver is the main organ that metabolizes alcohol, so consuming too much alcohol can cause major tissue injury [11]. This situation can cause the regeneration capacity of the liver to decrease so that liver cell death can occur and therefore it tends to various functional changes and cell damage. Ethanol also undergoes oxidation in the hepatic cell microsomes by microsomal ethanol oxidizing system (MEOS), which produces acetaldehyde. MEOS is part of the P450. The formation of MEOS is induced by ethanol and other substrates belonging to the cytochrome 450 family. Thus, in someone who consumes high doses of ethanol chronically, MEOS will carry out ethanol oxidation in the body around 30%. Catalase in peroxisomes is an enzyme that is responsible for oxidative reactions in ethanol metabolism in the liver. Catalase will also oxidize ethanol to become acetaldehyde [12]. Excessive consumption of alcoholic beverages can cause liver damage, heart damage, stroke, high blood pressure, digestive tract cancer, memory loss, and confusion [13], [14].

Chronic alcohol consumption impairs lipid metabolism by increasing liposis in adipose tissue and causing ectopic fat deposition in the liver. The main acute effect of ethanol consumption is an increase in the amount of nicotinamide adenine dinucleotide hydrogen (NADH), which is a consequence of the reaction of ADH to produce acetaldehyde and ALDH to convert acetaldehyde to acetate [15], [16]. 90% of ethanol consumed will be metabolized by the body, especially in the liver. Ethanol metabolism in the liver results in an increase in the amount of NADH cytosolic and mitochondria, which causes interference with normal metabolic processes in the liver [17]. Based on research showed that giving alcohol for 14 days to rats did not cause an increase in serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT), but giving ethanol would decrease antioxidant enzymes, such as decreased activity of glutathione peroxidase. Increased SGOT and SGPT are signs of liver damage due to various causes, including the result of oxidative stress. This study aims to see the difference in damage to the liver of mice (*Mus musculus*) after alcohol induction with different timeframes that might cause different histopathological changes.

2. METHOD

2.1. Grouping and treatment of animal test

This study uses an experimental research type, using a research design posttest only control group design using 5 groups, including 4 groups that were treated using ciu traditional alcoholic drink. The group consisted of a negative control group (K0), a treatment group giving traditional alcohol ciu 0.6 ml/20 g BW for 3 days (K1), 7 days (K2), 14 days (K3), 30 days (K4). This research was conducted in the Laboratory of Cytohistotechnology, Sekolah Tinggi Ilmu Kesehatan Nasional. The sampling technique for the liver of mice is simple random sampling. The research was preceded by terminating mice test animals adapted for 7 days to room conditions or laboratory environment, such as air, temperature, and humidity. Mice were fed daily with ratbio and drank Aquadest in a bottle, which was replaced and refilled every day. The bedding used for mice is husks or grains because they can absorb mouse droppings and are easy to clean. The mice cages were cleaned and replaced with new mats 3 times a week.

2.2. Histology preparations

Mice were dissected and preparations were made using the hematoxylin-eosin staining method, starting from fixation, washing, dehydration, clearing, infiltration, embedding, sectioning, affixing, deparaffinization, staining, mounting, and labeling [18]–[20]. The source of data used in this study is primary data obtained by researchers directly from microscopic observation data on mice. The results of macroscopic and microscopic observations were then analyzed using statistical package for the social sciences (SPSS).

3. RESULTS AND DISCUSSION

This study aims to look at the histological picture of the liver of mice (*Mus musculus*) after the treatment of traditional ciu drinks based on the length of time, 3, 7, 14, and 30 days. The observations

analyzed were macroscopic and microscopic appearances on the liver histology of mice (*Mus musculus*). Then do the analysis using SPSS.

3.1. Results

Based on Table 1, descriptive data from microscopic observations of liver organs from 5 groups. The group consisted of a negative control group (K0), the treatment group giving traditional alcoholic drinks ciu 0.6 ml/20 g BW/3 days (K1), the group giving traditional alcoholic drinks ciu 0.6 ml/20 g BW/7 days (K2), the group given the traditional alcoholic drink ciu 0.6 ml/20g BW/14 days (K3), the group given the traditional alcoholic drink ciu 0.6 ml/20g BW/30 days (K4) (Source: Primary Data February, 2023).

Table 1. Results of macroscopic observations of mice livers (*Mus musculus*)

Group	Treatment	Macroscopic				Microscopic
		Color	Heavy (grams)	Length (cm)	Width (cm)	Scoring
K0	Normal Control	Red Brick	1.6	2.2	2.2	1.3
K1	Ciu Treatment for 3 days	Red Brick	1.7	2.3	2.4	2.0
K2	Ciu Treatment for 7 days	Red Brick	1.8	2.4	2.4	2.0
K3	Ciu Treatment for 14 days	Red Brick	2.16	2.5	2.5	2.4
K4	Ciu Treatment for 30 days	Blackish Red	2.24	2.5	2.6	2.6

In Figure 1, the liver macroscopic view of mice (*Mus musculus*), K0 = negative control group. KI = Ciu 0.6 ml/20 g traditional alcoholic drink group. K2, K3, and K4. A = injury to the liver, which means there are physiological signs of liver damage. B = white spots, which are fatty liver (Source: Primary Data February, 2023). In Figure 2, a histopathological picture of the liver of mice (*Mus musculus*) in the normal control group (K0). Coloring hematoxylin-eosin with normal 400× magnification without any damage (Source: Primary Data February, 2023).

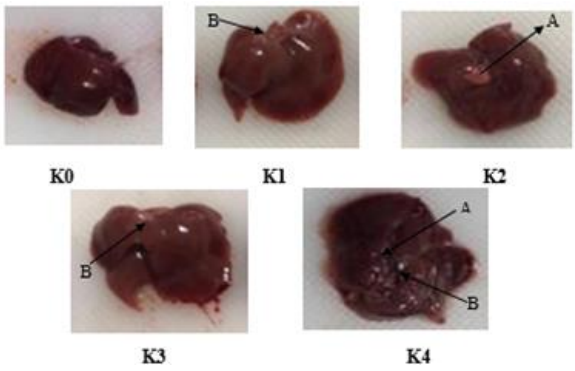

Figure 1. Macroscopic observation of mice livers (*Mus musculus*)

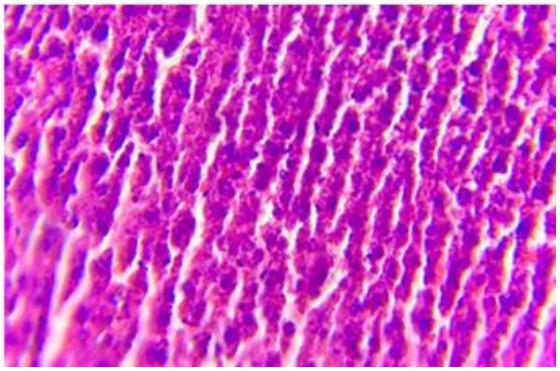

Figure 2. Microscopic observation of mice livers (*Mus musculus*)

Figure 3 shows the histopathological picture of the liver of mice (*Mus musculus*) by staining with hematoxylin and eosin, and 400× magnification. K1 = treatment group with ciu traditional alcoholic drink 0.6 ml/20 g/3 days. K2 = treatment group with ciu traditional alcoholic drink 6.0 ml/20g/7 days. K3 = treatment group with traditional alcoholic beverages ciu 0.6 ml/20 g/14 days. K4 = treatment group with ciu traditional alcoholic drink 0.6 ml/20 g/30 days. Information: A = degeneration, B = fatty degeneration, C = hydropic degeneration, D = picnotic necrosis, E = caryorexic necrosis, F = karyolytic necrosis, and P = bleeding (Source: Primary Data February, 2023).

In Table 2, the result of the significance value of the analysis of variance (ANOVA) test is 0.000 (≤0.005), so that it can be seen that the traditional alcohol drink ciu has a difference in the histopathological picture of the liver of mice (*Mus musculus*) for different lengths of time (Source: Primary Data February, 2023). In Table 3 from the data, after the Duncan test was carried out. The results obtained were p >0.05, which can be concluded that there was a significant difference between the groups (Source: Primary Data February, 2023).

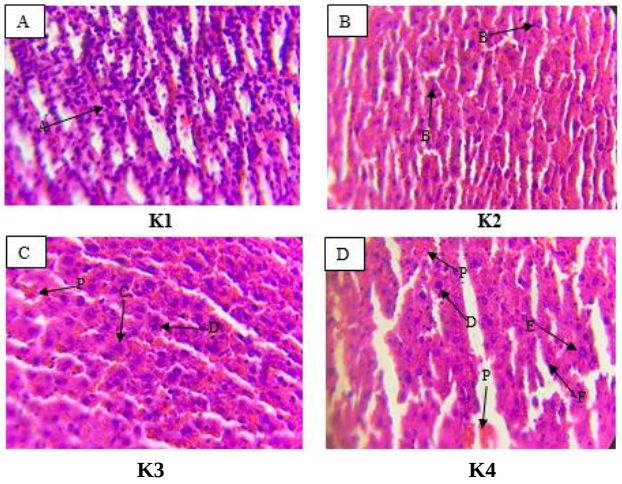

Figure 3. Microscopic observation of mice livers (mouse muscle)

Table 2. Results of ANOVA test analysis

Treatment test results Statement	Sum of squares	Df	Mean square	F	Sig.
Between groups	6.786	4	1.697	13.444	.000

Table 3. Results of post hoc test analysis

Preparate	N	Subset for alpha =0.05 1	2	3	4
Control	25	1.320			
K1	25		1.860		
K2	25		1.120	2.120	
K3	25			2.520	2.520
K4	25				2.820
Sig		1.000	.261	.090	.197

3.2. Discussion

This study is an experimental study that aims to determine differences in liver histopathological damage in mice (*Mus musculus*) based on the difference in the length of time for giving ciu traditional alcoholic drinks. The treatment was divided into 5 groups, namely the control group (K0), namely mice without any treatment, the treatment group was given ciu traditional alcoholic drink as much as 0.6 ml/20 g BW of mice for 3 days (K1), 7 days (K2), 14 days (K3), and 30 days (K4). The results of the study were observed macroscopically based on color and size of the liver, and microscopically observed that the condition of normal cells or the presence of damage in the form of degeneration and necrosis. This study uses male mice because they avoid the mating period, hormones are more stable and homogeneous, and they have reproductive characteristics similar to other mammals, anatomical, physiological, and genetic structures similar to humans [21].

Mus musculus, which had been treated with the traditional alcoholic drink ciu at the end of the study, were terminated using cotton that had been treated with ether. Termination is carried out by placing a cotton swab soaked in ether solution, then putting it in a jar and closing it tightly. To make sure the mice are dead, you can see from their movements, then monitor their heart rate, and can-do surgery to get the liver organs. After the liver organs were made preparations and observed under a microscope with 400× magnification, 5 visual fields were seen in each preparation. In observations, when giving treatment to mice, the longer the ciu traditional alcoholic drink was given (K3), (K4), the mice were thinner compared to (K1) and (K2), but the longer they were given the treatment of the mice's abdomen. The growing belly is due to the ability of ethanol to inhibit gluconeogenesis, so to meet energy needs, the body has to break down fat or protein, which has an impact on weight loss. Enlargement of the abdomen caused by the consumption of alcoholic beverages has various effects on plasma lipid levels, especially on increasing triglyceride levels. Alcohol consumption can stimulate the liver to secrete very low-density lipoprotein (VLDL), due to inhibition of free fatty acid oxidation in the liver, which will trigger triglyceride synthesis and VLDL

secretion [22]. In general, drinking alcohol is associated with a growing belly and waist. Because when you drink alcohol, the liver will burn alcohol and not fat.

The results of the study were seen macroscopically based on the color and size of the liver, as well as microscopic observations of the condition of normal cells or the presence of damage in the form of degeneration and necrosis. The results of macroscopic observations showed changes in the color and weight of the liver in each treatment group. The control group (K0) obtained brownish-red results. This shows that the control group, which was not treated with the traditional alcoholic drink ciu and was only given drinking water and feed, had no effect on liver morphology because it was not a toxic chemical, so that the liver conditions in the control group could be said to be normal. Based on research [23], [24], a normal liver has a reddish-brown color. On macroscopic examination of the traditional alcoholic drink ciu 0.6 ml for 7 days (K2), 14 days (K3), 30 days (K4), it was found that there was injury to the organ, which was marked by a change in color (white). Cell injury is a cell that is experiencing physiological stress or pathological stimulation. Injury to an organ is an early symptom of organ damage before inflammation caused by damage to hepatocytes and macrophages will engulf dead cells to form inflammatory cell clumps. The causes of injury are oxygen deficiency, chemical exposure, and physical imbalance [25].

In the treatment group (K1), (K2), (K3), and (K4), white spots were found, which were fatty liver. The accumulation of fat is caused by inhibition of the tricarboxylic acid cycle and inhibition of fat oxidation caused by the formation of too much NADH. In the treatment group (K4), it was observed that the liver was dark red or blackish red, which was caused by toxic substances that caused blockage in the blood vessels, so that the pressure in the blood vessels was higher than the pressure in the tissue, so that the cells found in the liver would be deposited, so that it will experience changes [26]. Macroscopic observation data on the liver weight of mice show that the effects caused by exposure to the traditional alcoholic drink ciu for different periods of time, namely 3, 7, 14, and 30 days, resulted in significant differences in liver weight. At (K0), the liver weighs 1.6 grams, which is normal. According to [27]. The normal mouse liver weight ranges from 1.2-1.6 grams. In (K1), the liver weighs 1.7 grams, (K2) has a liver weight of 1.8 grams, (K3) has a liver weight of 2.16 grams, and (K4) has a liver weight of 2.24 grams, which takes a longer time to give traditional alcoholic drinks ciu can affect the weight of the liver of mice (mouse muscle). The increase in liver weight occurs due to the process of cell adaptation. Liver cells will adapt due to external attacks, namely, ethanol. The adaptation made was to increase the number of endoplasmic reticulum and cytochrome P450 for incoming ethanol metabolism, resulting in an increase in cell mass. Ethanol is a type of alcoholic compound that can have a negative effect on the body, namely causing damage to biological membranes due to a decrease in lipid viscosity and the emergence of fatty liver so that it can increase liver weight.

Based on the statistical parametric ANOVA test, as shown in Table 2, the results obtained were $p < 0.005$, which was a significant difference between the treatment group and the control group, so that it was continued with further tests. Post Hoc Duncan, the negative control value (K0) was 1.320, (K1) was 1.860, (K2) was 2.120, (K3) was 2.520, (K4) was 2.820. Based on the statistical test results obtained, it can be concluded that there are significant differences between groups so that it can be seen that the administration of ciu traditional alcoholic beverages in different periods of time, namely 3, 7, 14, and 30 days can affect the histopathological picture of the liver of mice (*Mus musculus*), and the heaviest damage was indicated from the 14th day, which can be seen in the follow-up test Duncan.

Microscopic observation of liver organs in each treatment group was given 1 if the preparations were normal. For preparations with degenerative cell damage, a score of 2 was given, while for preparations with necrotic cell damage (pyknotic, karyoexis, and karyolysis), a score of 3 was given. Cell degeneration or cell decline is a cell abnormality that usually occurs due to minor injuries. Mild injury to structures in cells, such as mitochondria and cytoplasm, where it can disrupt the cell's metabolic processes. Degenerative damage is reversible, where the injury can be repaired if the cause is immediately stopped or eliminated. If the cause of degeneration is not stopped, the damage will get worse so that it will become irreversible damage, and the injured cells will die. Parenchymal degeneration is a mild degeneration characterized by swollen cytoplasm and granular cytoplasm. This is because cells are unable to eliminate water, so cell organelles also absorb and swell. This condition can be seen in the alcohol treatment for 3 days (K1).

In treatment (K2), it was found that there were normal nuclear cells and fatty degeneration, which was marked by the accumulation of cytoplasmic fat that pushed the nucleus towards the edges. These histopathological changes occurred after 7 days of treatment. Fatty degeneration describes the abnormal accumulation of triglycerides in parenchyma cells. As a result of fatty changes, the amount of fat deposits increases. If there is not too much fat deposition, there will be no disturbance of cell function, but if there is excessive fat deposition, it will cause fatty changes in the cells and can cause necrosis [28]. Hydropic degeneration is the level of damage to the hepatocytes, which is characterized by the cytoplasm experiencing vacuolization, the vacuoles appear clear and there is an increase in the influx of water into the cells, then enters the vacuoles. The cells turn pale and bleed. Hydropic degeneration has a more severe intensity of pathological stimuli and a longer period of exposure to pathological stimuli. This condition was observed in

the treatment group of ciu traditional alcoholic beverages for 14 days (K3). Necrosis can be said as a morphological change resulting from progressive degradation by enzymes in cells that experience lethal injury, which is generally characterized by destructive changes in the cell nucleus. Necrosis is cell death or autolysis of a tissue that can occur in the body, where the release of cell contents occurs and induces an inflammatory response in the tissue [28]. Necrosis is divided into three picture patterns or stages, including picnotic with a shrinking cell nucleus, cariorexis in which the cell nucleus disintegrates and leaves scattered chromatin fragments, and karyolysis, which shows the nucleus disappears or lysis. In the treatment group (K4) found fatty degeneration and pyknotic necrosis, karyolytic necrosis, cariorexis necrosis, and bleeding were found. Microscopically, necrosis is characterized by morphological changes in the core, namely loss of chromatin appearance, the nucleus becomes wrinkled, no longer vascular, the nucleus looks denser, the color is dark black (pyknosis), the nucleus is divided into fragments, torn (karyoexis), and the nucleus is unremarkably pale (karyolysis). Damage to the sinusoids can also occur due to severe fat degeneration, resulting in the formation of fat vacuoles, which will create empty space in the sinusoids and cause the sinusoids to widen. Another possible cause is due to pressure on the sinusoid wall due to a dam in the blood-stained vein caused by a toxic substance. In general, damming starts from the central vein, which then goes to the middle of the lobule. According to Rani and Singh [1], liver damage due to toxic substances is influenced by several factors, such as the type of chemical substance, the dose given, and the duration of exposure to the substance, such as acute, subchronic, or chronic. The higher the concentration of a given compound, the greater the toxic response generated. Liver damage can occur immediately or after several months. Damage can take the form of hepatocyte necrosis, cholestasis, or gradual onset of hepatic dysfunction.

Based on research, the results of observations made on microscopic images of livers treated with n-hexane extract of Andaliman fruit during the pre-implantation period of 0-3 days and the post-implantation period of 6-14 days found normal hepatocytes and hepatocytes that underwent changes in the form of parenchymatous degeneration, degeneration hydropic, and necrotic. Liver damage due to chemical compounds is characterized by biochemical lesions that provide a series of changes in function and structure. Some changes in liver structure due to chemical compounds can be seen in microscopic observations, such as inflammation, fibrosis, degeneration, and necrosis.

In this study, it can be seen that the administration of traditional ciu alcoholic beverages affects the histopathological picture of the liver of mice where the longer the traditional ciu alcoholic beverages are given the more damaging the liver organs are with marked physiological damage such as white spots and lesions as early symptoms of organ damage and in the administration of alcoholic beverages for 30 days (K4) the color of the liver becomes blackish red. In microscopic observations, the longer the administration of alcoholic beverages, the more damaging the cells are, starting from minor damage such as parenchymal degeneration to severe damage such as pycnotic necrosis, karyolysis, and karyoexis. The present study clearly establishes that alcohol has a direct effect on the physiological functioning of the liver, which is proven by alterations in liver function tests [29].

The constraints in this study were the limitations of the tools used, which were still manual. The knife used was not sharp enough, so that the preparations looked still overlapping (cells appear to accumulate), a lack of maximum light from the microscope. A good preparation should only have one layer of tissue pieces, unlike the pictures in the microscopic images in this study, where cells still appear to be piling up.

4. CONCLUSION

The liver is the main organ that metabolizes alcohol, so consuming too much alcohol can cause major tissue injury. Giving ciu traditional alcoholic drink to *Mus musculus* at a dose of 0.6 ml/20 g BW of mice for 3, 7, 14, and 30 days can affect the histopathological picture of the liver in the form of hepatic cell degeneration and necrosis. The longer the ciu traditional alcoholic beverage is given, the more it affects the histopathological picture of the mice's liver damage in the form of parenchymal degeneration to necrosis.

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AUTHOR CONTRIBUTIONS STATEMENT

This journal uses the Contributor Roles Taxonomy (CRediT) to recognize individual author contributions, reduce authorship disputes, and facilitate collaboration.

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Magdalena Budi	✓	✓	✓	✓	✓		✓	✓	✓		✓		✓	✓
Verena Putri														
Fitria Diniah Janah	✓	✓		✓		✓				✓		✓		✓
Sayekti														

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

This study was approved by the ethics committee of Universitas Muhammadiyah Purwokerto with registration number KEPK/UMP/36/XII/2022.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, [FDJS], upon reasonable request.

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BIOGRAPHIES OF AUTHORS

Magdalena Budi Verena Putri is a student in the undergraduate Medical Laboratory Technology at Sekolah Tinggi Ilmu Kesehatan Nasional Surakarta. She researches cytohistotechnology. She can be contacted at email: putrimagdalena238@gmail.com.

Fitria Diniah Janah Sayekti is a lecturer in the undergraduate Medical Laboratory Technology at Sekolah Tinggi Ilmu Kesehatan Nasional. She teaches courses on cytohistotechnology, molecular biology, and parasitology. She can be contacted at email: fitria.diniah@stikesnas.ac.id.