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RESEARCH ARTICLE

HISTOLOGY OF NEW ZEALAND MALE RABBIT LIVER AFTER TREATMENT WITH SEVERAL TYPES OF BELUNTAS LEAF TANNIN EXTRACT

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Abstract

This study examined *New zealand* rabbits' organ damage and liver histology after administering blunt leaf tannin extract. The type of research is experimental research. The research was conducted at the Chemical Engineering Laboratory of the Malang State Polytechnic, the Kesimma Medica Laboratory of Malang, the Integrated Laboratory, and the Biology Laboratory of the University of Muhammadiyah Malang. The research began in August to September 2020. The research design used a Completely Randomized Design (CRD) with four treatments and four replications. Treatment of hydrolyzed, condensed blunts leaf tannin extract, pure tannin, and the control group, namely distilled water, was given by oral probe at a dose of 3 ml/KGB. Histology preparations were made using the paraffin method and Hemotoxylin-Eosin (HE) staining. Liver damage scoring was observed in 3 fields around the central vein; each preparation was observed in 3 fields of view, each of which was killed by 100 hepatocyte cells. Data analysis used average analysis, one-way ANOVA, and Duncan's further test. The results showed that treating several tannins affected the damage to New Zealand rabbit hepatocyte cells. The results of Duncan's analysis showed that the treatment of pure tannins was not significantly different from hydrolyzed tannins and substantially different from the control group and condensed tannins; the control group was not significantly different from condensed tannins. This study found that pure tannins and hydrolyzed tannins had the highest effect on hepatocyte cell damage; condensed tannins were recommended for further research. Suggestions for further research are the potential of steroid tannin derivative compounds, namely single stigmasterol compounds from blunt leaves, which can be antifertility in vivo, quality, and safety studies.

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Introduction:-

Pluchea indica is one of herbal plants with considerably high number of consumption rates, both as foodstuffs and natural medicine. *Pluchea indica* is rich in benefits as alternative medicine (Fitriansyah, 2018; Susetyarini, 2009a) due to chemical compounds it possesses (Susetyarini *et al*, 2009b). The compounds contain alkaloid, flavonoid, olive oil, chlorogenic acid, natrium, kalium, magnesium, and phosphor. The leaves of *Pluchea indica* are rich in tannin (Agoes, 2010; Susetyarini, 2009b; Febrianta *et al.*, 2015). The use and consumption of active tannin compound must be structured and not be in an excessive dose for it is toxic and potential to cause damage on body organ (Doostar *et al*., 2000; Kunaepah, 2008).

Generally, tannin is classified into two groups, hydrolyzed tannin (gallic polymer and ellagic acid with an ester bond through a glucose molecule) and condensed tannin (flavonoid compound polymer with a carbonic bond in the forms of catechin and gallocatechol (Patra & Saxena, 2010). Tannin that is stemmed from plants generates condensed tannin with a complex bond completed by stronger protein than that of hydrolyzed tannin (Fahey & Berger, 1988).

Tannin extracted from *Pluchea indica* can affect several body organs within the physiological process, including the liver.

Liver is vital since it is responsible for facilitating the production and secretion of bile (Ozougwu, 2017; Ramakkrishnan, 2009; Chen *et al.*, 2012). Physiologically, liver contributes to metabolism process (Surasa *et al.*, 2014), metabolism of foodstuffs and nutrition, such as carbohydrate, fat, and protein, excreting and secreting hormones, excreting medicines (Maulina, 2018; Rosida, 2016), regulating heat production, regulating protein and blood sugar levels (Thakur & Puranik, 1981; Leach, 1961), and neutralizing poisonous substances (Nugraha *et al.*, 2018).

In view of pharmacology, every chemical compound inflowing into the body will be absorbed by the intestine, passing through hepatic portal vein to go inside the blood vessels, and thus, metabolism and excretion take place in the liver (Setiawati, 2007). Liver is one of the body organs that is the most susceptible to damage (Gibson & Skett, 1991; Maulina, 2018) after being exposed to chemical compounds and substances (Gibson & Skett, 1991). The damage, furthermore, is due to excessive dose of chemical compounds (Setiawati, 2007) and duration of inbound medicine exposure (Mahmudah *et al.*, 2018). Histologically, necrosis is the most common damage that occurs at the organ's tissue due to the exposure to chemical compounds and substances (Fitmawati *et al.*, 2018).

New zealand rabbit is a result of crossbreeding between *Flemish giant* and *Belgian hare* native to America (Masanto & Agus, 2013). Ideally, rabbit is the most representative animal for laboratory experiments since it belongs to the class of above guinea pig, rat, and mice, and is found to have similar structure and physiology to that of humans (Hristov, 2006; Mapara *et al.*, 2012; Harmasyanto, 2013; Kastawi, 2003). In addition, anatomical structure of rabbit's liver is majorly coincident to that of humans. Based on the liver anatomy, liver is the biggest gland existing in the body, and is classified into 5 lobes by a slit circuit (Abdullah *et al.*, 2017). In addition, liver is susceptible to damage due to its main function for neutralizing poisons (Nugraha *et al.*, 2018).

This current research aimed at studying liver damage and investigating the damage on hepatocyte cells and the liver histology of male *New zealand* rabbit after the treatment of tannin extraction of *Pluchea indica*. Expectedly, the results of this research can be used as referral access to basic information about the effects of some treatments of tannin extraction such as the pure, hydrolyzed, and condensed tannin extractions of *Pluchea indica*, primarily on the damage of hepatocyte cells and the liver histology of rabbit.

Methodology:-

Research Procedures and Stages

This current research employed experimental design. It was conducted at Chemical Engineering Laboratory of State Polytechnic of Malang for the making of tannin extraction of *Pluchea indica*, Kesimma Medica Laboratory of Malang for the making of preparations and microscopic examination on *New zealand* rabbit liver preparations, Integrated Laboratory for maintenance and provision of tannin extraction treatments, and Biology Laboratory of University Muhammadiyah Malang for surgery, microscopic examination, and observation. This research was initiated from August to September 2019. A Completely Randomized Design with 4 treatments in 4-time repetition was applied. There were three kinds of tannin treatments, namely hydrolyzed, condensed, and pure tannin extractions of *Pluchea indica*. As many as 24 male 14-week-old *New zealand* rabbits were divided into 4 main units of treatments; they were the hydrolyzed tannin extraction of *Pluchea indica*, the condensed tannin extraction of *Pluchea indica*, the pure tannin extraction of *Pluchea indica*, and the control (with the intake of aquades through oral nasogastric by the dose of 3 ml/kgbw).

Data Collection Techniques and Assessment Instruments

After 3 months of the research period, a surgery procedure was undergone, and the liver organ was collected to observe the organ damage. The histological preparations were created by means of block method using paraffin and Hematoxylin-Eosin (HE) coloration. The evaluation score parameter on the liver organ was referred to five fields of

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vision around centralis vein according to Baldatina (2008) as shown in Table 1. Meanwhile, the investigation of the histological damage on the liver cells was referred to criteria proposed by Nazarudin *et al.* (2017) as illustrated in Table 2. Further, each of preparations for histological data was observed based on five fields of vision, primarily by observing 100 hepatocyte cells in order to measure the percentage of necrotic cells (Nazarudin *et al.*, 2017). Data analysis was conducted through the description of average, one-way ANOVA, and Duncan's Multiple Range test with the confidence level of 95% by using a statistical package of *SPSS 21* software program.

Table 1. Evaluation Score Parameter on the Liver Organ by five Fields of Vision around the Centralis Vein

Scores	HP Changes (Histopathology)
0 If, S < 25%	Liver undergoes hydropic degeneration, parenchymal degeneration, and apoptosis around the centrilobular (centralis vein).
1 If, S = 25-50%	Liver undergoes hydropic degeneration, parenchymal degeneration, and apoptosis, expanding to the midzone.
2 If, S= 50-75%	Liver undergoes hydropic degeneration, parenchymal degeneration, and apoptosis, approaching the periportal zone (peri-lobular).
3 If, S > 75%	Liver undergoes hydropic degeneration, parenchymal degeneration, and apoptosis, expanding to the periportal zone (peri-lobular).

Note: 0: normal, 1: low, 2: medium, 3: high

Source: (Baldatina, 2008)

Table 2. The criteria of Histological Damage in the Liver Organ

Normal Cells	Necrotic Cells
1. Cells are in medium size (not too big, nor too small), 2. Spots are found in the nucleus. 3. Cytoplasm's color is all-inclusive.	1. Cytoplasm's diameter is relatively smaller. 2. Cytoplasm is more reddish. 3. Nucleus can be somehow inexistent, thickening (assembling spots), and spreading (no boundary found on the nucleus)

Source: (Nazarudin *et al.*, 2017)

Results:-

The results of liver damage observations (Table 3) showed that the pure tannin and hydrolyzed tannin treatments gave the most significant damage (75% and 80%) with a damage score of 3, which means there are Histopathological Changes (HP) with the criteria of the liver organ experiencing hydropic degeneration, parenchymal degeneration and apoptosis that extends to the periportal zone (per lobular) compared to the control treatment group (15%), and condensed tannin treatment (25%).

Table 3 presents the results of histological observations used to evaluate liver organ damage after administering blunt leaf tannin extract.

Table 3. Liver Evaluation Scoring in 5 Fields of View Around the Central Vein

Treatment	Average Percentage	Damage Scoring
C	15%	0
CT	25%	1
HT	75%	3
PT	80%	3

Description: C: Control; CT: Condensed tannins; HT: Hydrolysable tannins; PT: Pure tannins

Histological observation of the liver is essential to see the damage to the hepatocyte cells. The results of histological observations of the liver on the hepatocyte plate that experienced necrosis are presented in Figure 1.

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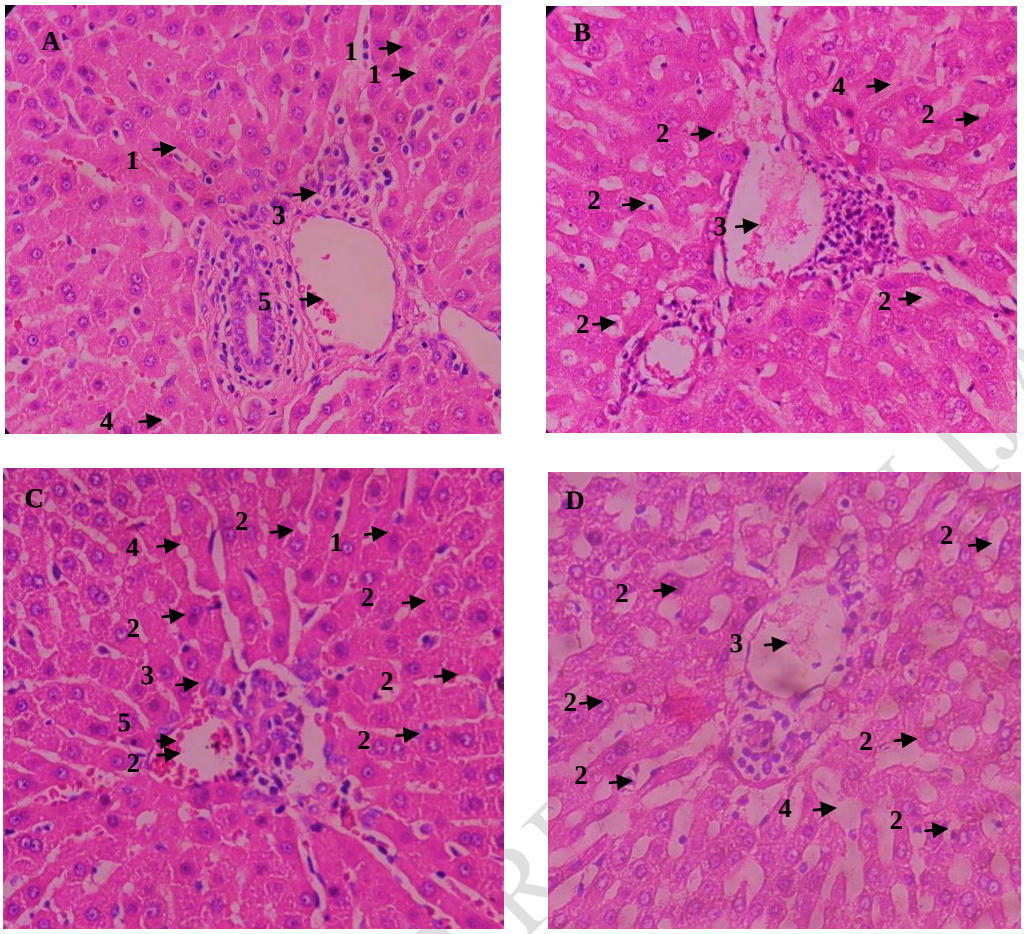

Figure 1. Liver Histology After Administration of Beluntas Leaf Tannin Extract

A. Control Group, B. Hydrolyzed. C. Condensed. D. Pure Tannin

Observation using Olympus CX 2305 binocular microscope

Taken using a cellphone camera, Magnification 400x

Description: 1. Normal Hepatocyte Cells 2. Necrotic Hepatocyte Cells 3. Central vein 4. Sinusoids, 5. Red blood cells

The results of liver organ observations in the control group and condensed tannins were expected, with darker red and slightly brownish characteristics. This was significantly different compared to the tannin treatment, both pure tannins and hydrolyzed tannins, which faded in color more, indicating a small amount of blood flow to the liver because the cells were damaged.

The summary results of the ANOVA test of the effect of several types of tannins on damage to rabbit liver hepatocytes are presented in Table 4.

Table 4. One Way Anova Test Results

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5535.000	3	1845.000	37.782	.000
Within Groups	390.667	8	48.833		
Total	5925.667	11			

The results of the ANOVA test obtained a sig value of 0.00 <0.05, meaning that there is an effect of giving several tannin extracts of blunt leaves on liver cell damage in New Zealand rabbits (Table 4).

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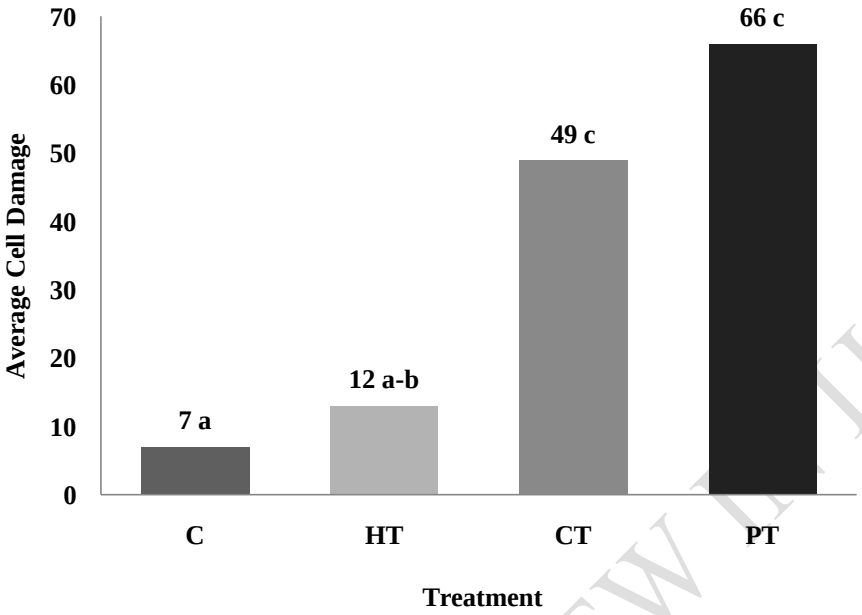

Figure 2. Diagram of Duncan's Test Results for Liver Cell Damage in New Zealand Rabbits
Description: C: Control; HT: Hydrolyzed Tannin; CT: Condensed Tannin; PT: Pure Tannin

The results of the Duncan analysis (Figure 2) showed that the control group had the lowest average hepatocyte cell damage of 7 cells and was not significantly different from condensed tannin with average damage of 12 hepatocyte cells; pure tannin treatment was not significantly different from hydrolyzed tannin giving the most significant effect on liver cell damage compared to other therapies with average damage of 66 cells, hydrolyzed tannin treatment with an average cell damage of 49 cells.

Discussion:-

Liver organ damage in the hepatocyte cells occurs due to chemical influences, including active tannin compounds. This happens because tannins that enter the body undergo a metabolic process; during this process, the liver has an important role, so exposure to these compounds causes liver damage (Underwood, 2000). According to Michael & Cynthia (2006), The liver organ can be damaged due to the influence of chemical compounds through the hepatotoxic mechanism. The liver is very easily damaged, either in the form of damage to the cell structure or impaired liver function due to the influence of chemical compounds (Aisyah, 2015).

A normal liver will be dark red to brownish caused by blood flow entering the liver organ (Fortes, 2017; Helmi, 2007). Research result Fatmawati (2018), The liver of white mice was given a traditional concoction, and the normal liver was brownish red. When the liver is fresh, it has a dark red or brownish color, which is caused by a lot of blood in the liver (Leeson *et al*., 1996). The changes in the color of the liver organ in the condensed tannin, hydrolyzed tannin, and pure tannin treatment groups indicate damage to the organ. Physical liver abnormalities are usually characterized by changes in color (Subronto, 1985).

The damage that occurs to liver cells is characterized by abnormal cells and damage or necrosis (Figure 1). Necrosis is damage to liver cells, also known as tissue death (Robbinson *et al*, 2007), or cell death (Chaville, 1999), due to certain factors, one of which is chemical compounds. Tannin is classified as a chemical compound (Fitriansyah, 2018). Robbins *et al*, (2007) Stated that toxic substances in the liver are characterized by cell degeneration, including hydropic degeneration, fatty degeneration, and necrosis.

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Histology of hepatocyte cells in the control group showed that the number of cells damaged was less than those treated with blunt leaf tannin extract. The tannin extract treatment group indicated that its cells experienced more necrosis, especially in pure tannin and hydrolyzed tannin; cell damage was characterized by the widening of the cytoplasm of cells and cells that did not have cell nuclei; the least damage was in the control and tannin groups (Figure 1). According to Nazarudin *et al,* (2017) The characteristics of cells that experience necrosis are a smaller cell cytoplasm diameter, the cytoplasm's color redder, the cell nucleus loss, the cell nucleus thickening, and the cell nucleus spread (there is no cell nucleus boundary). The results of the Duncan test to determine the most significant treatment in affecting liver cell damage are presented in Figure 2.

The histology of liver cells or hepatocyte cells in the hepatocyte plate around the central vein is very close to and closely related to the part of the liver called the sinusoid. The sinusoid wall, which contains endothelial cells, forms an incomplete layer and limits liver cells and sinusoids. It is called the subendothelial gap, containing microvilli from liver cells (Lesson *et al,* 1996). The observation results showed a widening of the liver sinusoids in the pure tannin, condensed tannin, and hydrolyzed tannin treatment groups compared to the sinusoids in the control group (Figure 1). The widening of the sinusoids in the treatment group occurred due to the influence of tannins, which are toxic to liver cells and quickly come into contact with sinusoids. Toxins in damaged liver cells will soon come into contact with sinusoids, and if the toxicant concentration is high, it will cause the widening of the sinusoids (Junqueira & Carneiro 1998).

These results are because the liver is the main organ of metabolism that often experiences damage due to compounds and the accumulation of metabolites. A compound will undergo metabolism in the liver, and changes in the chemical structure catalyzed by enzymes produced by hepatocyte cell microsomes called biotransformation (Nugraha *et al,* 2018). Liver cells generally function to metabolize food substances and secrete or excrete hormones and drugs (Maulina, 2018), fight and kill bacteria and foreign objects that enter the blood (Snell, 2012), so that liver cells experience more damage (necrosis) (Junquiera & Carneiro, 2012; Fawcett, 2002).

In this study, hepatocyte cell damage was caused by the activity of chemical compounds suspected to also include active tannin compounds. Liver cell damage or necrosis can be caused by ingesting drug compounds or chemicals into the body (Michael & Cynthia, 2006; Chodidjah *et al,* 2007), One of them is the tannin compound. Chemical compound metabolism is not always a detoxification or elimination process of compounds; sometimes, drugs are transformed into intermediate compounds that are reactive and toxic to the liver (Nugraha *et al,* 2018). According to Xuepin (2003) Tannins, mainly hydrolyzed tannins, can be toxic to the body because their components are easily hydrolyzed into gallic acid. Tannins can inhibit iron absorption, interfere with organ function, damage organs at high concentrations, and interfere with the body's metabolism (Clinton, 2009), Because it can precipitate proteins and combine with these proteins (Desmiaty *et al,* 2008). Lipophilic compounds such as tannins can cause damage to body cells, such as hepatocytes in the liver (Arjadi *et al,* 2017).

The results of liver cell observations after blunt leaf tannin extract administration showed that the cell nucleus loss marked necrosis. Cells that experienced necrosis due to disruption of cell function caused liposomes to rupture, thus releasing hydrolytic enzymes into the cells, which then dissolved the chromatin, causing the cell nucleus to rupture and disappear (Sudiono, 2013). The presence of dead cell nuclei characterizes liver necrosis (Robbins *et al,* 2007). Necrosis in liver cells is usually characterized by a liver cell nucleus that appears to shrink, has irregular borders, and is dark in color, a characteristic of a pyknotic nucleus (Price & Wilson 1995). Ressang (1995) states that the pyknosis core is the initial stage of necrosis. Changes in the liver cell nucleus characterize necrosis. The results of the study Irnawati *et al.* (2005) Necrosis causes the cytoplasm and mitochondria to swell, rupturing the plasma membrane. Damage to the liver causes changes in metabolism, resulting in changes in structure and function (Nugraha *et al,* 2018).

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Research result by Susetyarini (2013) mentioned that the active compound tannin given to white mice is thought to be able to affect cell function by damaging the endoplasmic reticulum of the cell. Liver cells or hepatocyte cells have many rough and smooth endoplasmic reticulums (Junquiera & Carneiro, 2012). If the endoplasmic reticulum is damaged, it will affect the cell's work. Another factor that causes liver cell damage and necrosis is tannin, a chemical compound that can damage cell membranes. Chemicals that enter the body can affect chemical changes in the cell membrane, causing the cell membrane to rupture (Robbins *et al*, 2007; Underwood, 2000). The active compound tannin has a toxic potential in liver cells (Purwinta *et al*, 2013; Doostar *et al*, 2000; Kunaepah, 2008).

CONCLUSION:-

This study concludes that the treatment of pure tannin and condensed tannin caused the highest damage (80%) to the liver; there is an effect of giving several tannin extracts of blunt leaves on liver cell damage in New Zealand rabbits; pure tannin treatment is not significantly different from hydrolyzed tannin giving the highest effect of liver cell damage and is substantially different from the control, the control treatment is not significantly different from the condensed tannin treatment damage in the form of cell necrosis and widening of the sinusoids.

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RECOMMENDATION:-

The results of this study are significant for further research because although there are many studies on tannin compounds that have the potential of natural antifertility, tannin is one of the secondary metabolite compounds that are still quite large. This is based on the results of further research using bioinformatics or in silicon research based on the size of the target protein of potential compounds for certain activities; the single compound stigmasterol contained in bluntas leaves, which are still classified as a steroid group and has the potential as a natural medicine ingredient as an antifertility in men. Further, in vivo research is needed on the parameters of quality and safety tests.

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