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Sub-chronic hepatotoxicity test of *Plantago mayor* L. extract

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ABSTRACT. The aim of this study was to examine hepatotoxicity of *Plantago mayor* L. extract on rat by using effective dosage. By experimental study and post test only with control group design. 15 rats (*Rattus norvegicus*) were divided in to 3 group. Group A as a natural control was given aquades. Group B and C were given extract of *Plantago major* L. 50mg, and 100mg/200g BW rat/day per oral. Liver function was determined with measurement of *Aspartate aminotransferase* (AST) level, *Alanine aminotransferase* (ALT), total bilirubin, and histopathological feature of liver. Blood sampling and Liver organ were taken after 28 days of intervention. The average of AST levels, ALT and total bilirubin among groups A, B and C were AST levels (145.40±52.92, 129.00±34.89, and 115.60±13.24 U/l), ALT (76.40±18.87, 83.20±18.71, and 61.00±8.45 U/l) and total bilirubin (0.56±0.03, 0.77±0.22, and 0.58±0.08 mg/dL). Statistical analysis showed that there were not significantly differences of AST levels (p=0,63; CI95%), ALT (p=0,47; CI95%) and total bilirubin (p=0,0,09; CI95%) between the groups. In histopathological features, the average Scheuer score between groups A, B and C is 1.79 ± 0.74, 3.30 ± 0.66 and 2.84 ± 0.77. There is a significant difference in Scheuer scores between the groups (p=0,005; CI95%) that show that there is a difference in the effect of giving extract of *Plantago major* L. to hepatocyte cells leading to a piecemeal necrosis. This study can be concluded that in effective dosage, *Plantago major* L. extract able to induce hepatocytes injury although it cannot cause liver disfunction yet.

Keyword: *Alanie aminotransferase, aspartate aminotransferase, Plantago major* L. *extract, sub-chronic hepatotoxicity test, total bilirubin*

INTRODUCTION

Plantago major L. is a weed that grows in many tropical regions, include Indonesia both in the highlands and in the lowlands. This plant has many names depending on the area where it grows. (Kandou, Marhaenus, & Agustina, 2006) Traditionally, this plant has been widely used as a medicine to heal wounds, bloody urine, gallstones, kidney inflammation, respiratory tract infection (bronchitis, productive cough), diabetes mellitus, vaginal discharge, prostate inflammation, fever and various problems of gastrointestinal tract. (Zubair, Anders, Hilde, Stefan, & Cecilia, 2012; Sugiyarto, Setyawan, & Pitoyo, 2006) *Plantago major* L. is believed to have anti-hypertensive, diuretic, antibiotic, anti-fungal, antiviral, analgesic, anti-inflammatory, antigout arthritis, pro-coagulation, anti-hyperglycemic, antidispepsia, hepatoprotector, antioxidants, immuno-stimulants and antineoplastic. (Zubair at al., 2012)

In several previous studies related to pharmacological effects, *Plantago Major* L. has proven effects such as anti-inflammatory, immuno-modulating, anti-asthma, antiviral (Chiang, Chiang, Chang, & Lin, 2003), antihyperglycemic (Ayu, Fatmawati, & Citraningtyas, 2014), antioxidant, and chemopreventive (Oto, Ekin, Ozdemir, Demir, Yasar, & Levent, 2011). Other studies have also shown that ethanol extract of *Plantago Major* L. can prevent gastric ulceration and inhibit the growth of *Helicobacter pylori in vitro* so that it is potentially developed as a gastrointestinal disorder drug, such as *dyspepsia*, gastritis to *peptic ulcer* (Cogo et al., 2010; Awaad, El-Meligy, & Soliman, 2013). In studies related to chemopreventive effects, ethanol extract of *Plantago Major* L. can also inhibit excessive expression of the *Regenerating-1α* gene responsible for gastric carcinogenesis and increase *Caspase-3* which can increase cancer cell apoptosis. The most effective dosage of *Plantago major* L. extract found in this research were 100 mg/200g BW rat (Sutrisna, Ani, Muchtan, & Herri, 2013).

Ethanol extract of *Plantago major* L. contains many active compounds such as *alkaloids* (*indicain, plantagonin*), *caffeic acid* derivatives, *flavonoids* (*Luteolin7-glucoside, Hispidulin 7-glucuronide, luteolin7-*

diglucoside, apigenin 7-glucoside, plantaginin, homoplantaginin, baicalein, scutallarein), glyside iridoid (aucubin, asperuloside, catapol, gardoside), Triterpenoid (oleanolic acid, ursolic acid, 18b-glycyrrhetinic acid, sitosterol), n-hentriakontan, and plantagluside (methyl D-galactose, L-arabinosa, *methyl D- galakturonat, rhamnosa*) and *tannin*, potassium, vitamin A, B1 and C. In seeds contain *Planterolic, plantaginin, aucubin, ursolic acid, Beta-si-tosterol, n-hentriakontan,* and *plantagluside* which consists of *methyl D-galakturonat, D- galactose, L-arabinose and L-rhammosa* (Taskova, Handjieva, Evstatieva, & Popov, 1999).

The most active compounds contained in the ethanol extract of *Plantago major* L. were *phenolic* compounds (13.05mg/g in leaves and 7.43mg/g in roots), *flavonoids* (6.41mg/g on leaves and 3.03mg/g on roots or 0.69-3.09%) and *tannin* (5.63mg/g on leaves and 2.43mg/g on root (Kobeasy, Abdel-Fatah, El-Salam, & Mohamed, 2011). Another study found that the levels of phenol compounds in *Plantago major* L. were 672mg/100g of leaves , while *tannins* were 0.56-2.26% (Souri, Amin, Farsam, & Barazandeh, 2008).

Triterpenoids and *flavonoids* are active compounds that have cytotoxic effects, inhibit the occurrence of carcinogenesis and increase tumor cell apoptosis (Sutrisna et al., 2013). *Flavonoids* are strong antioxidants and are known to play role as free radical scavengers. *Baicalein, hispidulin, scutallarein* and *plantaginin* are components of *flavonoid*s that function as free radical scavengers and inhibit lipid peroxidation (Samuelsen, 2000). *Aucubin* compounds of *glyside iridoid* have proven efficacy in improving cell function. *Glyside iridoid* also play a role in biosynthesis of mRNA and function as a hepatoregenerator (Lintong & Carla, 2013).

However, according to the physicochemical characteristics of the main active compound *Plantago major* L. besides having a therapeutic effect but also having the potential to cause toxic effects on several organs in the body. The potential toxic effect of *Plantago major L*. extract is closely related to the *lipophilic* properties in group of active compounds it contains, such as *flavonoids, alkaloids* and *tannins*. This characteristic cause the compounds easily binds to cell walls and can induce damage to cell membranes. In addition, tannins can also inhibit to enzymes that play a role in drug metabolism (Purwita, Indah, & Trimulyono, 2013).

One of the main organs in the body most at risk of toxic effects of drugs and other chemicals that enter the body is the liver. The liver is the main organ for metabolism and detoxification of the drug so that has the potential to be damaged. Liver injury that can be fatty liver (*steatosis*), *hepatocyte* necrosis, *cholestasis*, or liver dysfunction both mild, moderate to severe (Sugiyarto et al., 2006; Suhita, Wayan, & Ida, 2013). Until now, it is still difficult to find scientific information regarding the toxic effects of ethanol extract of *Plantago major L*. The study aimed to identify and analyze the effect of ethanol extract of *Plantago major L*. on the liver, especially on long-term administration (*sub-chronic*) with effective doses. whether ethanol extract of *Plantago major L*. able to cause liver damage that is characterized by increased levels of AST, ALT and total bilirubin and also *hepatocyte* cells injury.

EXPERIMENTAL SECTION

This study was conducted by experimental study and posttest only with control group design. The procedure of this study was reviewed and approved by The Health Research Committee Faculty of Medicine University of Padjadjaran Bandung, Indonesia. No. Reg: 0215090759. Ethical approval No.679/UN6.C1.3.2/KEPK/PN/2015.

Material and Instruments

The materials used in this study were solvent of 96% ethanol, 1% *Carboxymethyl Cellulose* (CMC), *Diazotized Sulphanilic Acid* Reagent, T-*Nitrite*, reagent of AST and ALT (Dyasis®), ketamine (Ketalar®), solution of Mayer's *Hematoxylin-Eosine* and xylol. While, instruments used include Spectrophotometers (Optima® Sp 300), centrifuges (Kubota®), portable digital scales (Nagata® Type Lcs 12), multihead microscopes (Nikon® Eclipse Ci-L), Microscopes (Motic® B2 Series), Optilab®, and minor surgical tools.

Plant Extraction

Fresh *Plantago major* L. were collected from Slamet mountain, Purwokerto, Central Java, Indonesia and authenticated at Laboratory of Taxonomy Faculty of Biology, Jenderal Soedirman University. Except for roots, all parts of the plant were extracted with 96% ethanol using maceration techniques. The leaves were collected and dried at room temperature, protected from dust and sunlight. Leaves and seeds were pulverized manually. Fifty grams of each plant powder was extracted in 500 mL of ethanol 96% by maceration (48 h). Then, using a rotary vacuum evaporator to evaporate the remaining ethanol 96% solvent and then the extract is dried with a water bath at a temperature of 60-70 ^{o}C until it thickens into a paste.

Animal and Experimental Protocol

The rats were housed in wire-bottom cages at 25-28 °C and adaptation for a week at Pharmacology laboratory. 15 of healthy rats weighing between about 150 - 200g and 2 - 3 month age were divided in to 3 groups. Group A as a natural control was given aquades. Group B and C as the treatment group were given 50 mg *Plantago major L*. extract, and 100 mg/ 200 g BW rat/ oral day. Liver function was determined by measuring levels of *Aspartate aminotransferase* (AST), *Alanine aminotransferase* (ALT), total bilirubin, and histopathological feature of the liver. Taking blood and liver samples was taken after 28 days of intervention.

Liver Enzyme Transaminase examination

AST and ALT were measured using the Optimized UV-test methods and spectrophotometry, based on the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine). The blood serum was taken as much as 100 µL and mixed with 1 cc of working reagent (consisting of 4 cc enzymes or buffers mixed with 1 cc substrate with a ratio of 4: 1. Then, read the absorbance on a spectrophotometer with a wavelength of 340 nm. (Sihombing & Raflizar, 2010).

Bilirubin Examination

Total bilirubin was measured by spectrophotometry with a wavelength of 546 nm. Reagents of 1000 µL were put into cuvette and added 1 drop of T-*Nitrite* reagent, homogenized and incubated for 5 minutes. Add 100 µL of blood serum into the cuvette containing the reagent and be homogenized and incubated for 15 minutes at 37 ° C. Then, read the absorbance on a spectrophotometer with a wavelength of 546 nm. (Sihombing & Raflizar, 2010)

Histopathological Techniques

The liver organ is fixed with 10% buffered formalin for 48 hours, be continued dehydration of the specimens in multilevel concentration of ethanol, cleared in xylene and the process of embedding into paraffin. After freeze, the paraffin blocks were cut using a microtome with a thickness of 5 µm and put into a water bath with a temperature of 42-45 ºC and dried. Then, stained with *hematoxyline* and *eosin* (H and E). The histological slides are examined under the light microscope for assessing of hepatocellulare injuries. The severity of hepatocellulare injuries was determined using the *Scheuer* score, that is score from liver histology based on indicators inflammatory of porta area and *piecemeal* necrosis which is calculated at 50 porta area with 100x magnification. Score 0: does not occur inflammation; Score 1: inflammation in the surrounding of porta area; Score 2: mild *piecemeal* necrosis up to zone 1 of the liver; Score 3: moderate *piecemeal* necrosis up to zone 2 of the liver; and Score 4: severe *piecemeal* necrosis or widespread up to zone 3 of the liver (Guido, Alessandra, & Gavino, 2011).

Statistical Analysis

The differences of AST, ALT, total bilirubin and *Scheuer* score among groups of the study were tested with *Kruskal-Wallis* test followed by *Mann-Whitney* test.

RESULTS AND DISCUSION

Alteration of Serum ALT and AST Levels

The effect of *Plantago major* L. extract on the levels of liver *transaminase* enzymes causes non-significant changes in enzyme levels. These enzymes were measured using the Optimized UV-test methods and spectrophotometry. The data in **Table 1** shows a tendency to decreasing AST levels between study groups. Group A as a natural control not given *Plantago major* L. extract had higher AST levels (145.40 ± 52.92 U/L) compared to group B (129.00 ± 34.89 U/L)) and Group C (115.60 ± 13.24 U/L). Whereas ALT levels increased in group B (83.20 ± 18.71 U/L) and decreased in group C (61.00 ± 8.45 U/L) compared to the control group (76.40 ± 18.87 U/L).

AST and ALT levels found in this study higher than the normal levels in healthy rat, that are 61.07±5.57 U/L (AST) and 13.87±1.26 U/L (ALT). (Sihombing & Tuminah, 2011) This finding can occur due to several conditions that occur in this study, such as the condition of mice that have experienced elevated levels of AST and ALT since the beginning, but researchers have anticipated by holding healthy controls to illustrate the natural conditions of experimental animals to be used as basic data sources. In addition, all experimental animals, both the control group and the research group, received the same treatment during the study so that the changes that occurred during the study were only caused by the influence of the intervention. The differences in levels of AST and ALT that were not significant between the control group and the study group showed that *Plantago major* L. extract didnot cause severe toxic effects on the liver. Additionally, if it was assumed that AST and ALT levels of experimental animals have incresed since the beginning, the tendency of decreasing levels of both liver enzymes in this study can be strengthen the results of previous studies related to the potential hepatoprotective effect of *Plantago major L*. Extract. (Sutrisna et al., 2013). Statistical analysis by using *Kruskal-Wallis* test showed that there were not significantly differences of AST levels among group of study (p=0,63; CI95%) and also on ALT levels (p=0,47; CI95%).

Table 1. Serum AST and ALT Levels Among Groups of this Study

No	Group of Study	Mean of	
		AST Levels (U/l)	ALT Levels (U/l)
1	A; Natural Control (Health)	145.40±52.92	76.40±18.87
2	B; 50mg of Extract	129.00±34.89	83.20±18.71
3	C; 100mg of Extract	115.60±13.24	61.00±8.45

Table 2. The Mean of Serum Total Bilirubin Levels Among Groups of this Study

No	Group of Study	Total Bilirubin Levels (mg/dL)
1	A; Natural Control (Health)	0.56±0.03
2	B; 50mg of Extract	0.77±0.22
3	C; 100mg of Extract	0.58±0.08

Alteration of Serum Total Bilirubin Levels

Total bilirubin was measured by spectrophotometry with a wavelength of 546 nm. The effect of extract of *Plantago major* L. to the total bilirubin levels of experimental animals showed a non-significant effect. There were a slight increase in groups B and C (0.77 ± 0.22 and 0.58 ± 0.08 mg/ dL) compared to the control group (0.56 ± 0.03) but in group B it was relatively higher than group C, as shown in **Table 2**.

Statistical analysis by using *Kruskal-Wallis* test showed that there were not significantly differences of total bilirubin (p=0.09; CI95%) between the groups. It was means that the administration of *Plantago major* L. extract at doses of 50 mg and 100 mg/ 200g BW rat does not cause liver damage which significantly affects to bilirubin production. When compared with the normal levels of total bilirubin in healthy rats, which are 0.42 ± 0.1 mg / dL (Sihombing & Raflizar, 2010), the total bilirubin levels in the results of the study appear to be slightly higher, although not significant. The increasing of bilirubin levels in this study are very likely caused by several active compounds of *Plantago major* L. extract which can interfere to hepatocyte cell function, such as *flavonoids, alkaloids* and *tannins*.

Flavonoids, alkaloids and *tannins* are the active compound known to be very lipophilic so that it easily binds to cell walls, disrupts cell membrane permeability, and causes cell membrane damage (Purwita, Indah, & Trimulyono, 2013). *Flavonoids* can inhibit the activity of the enzyme *Cytochrome P-450* and enzymes that work on phase II metabolism where a detoxification process occurs that has the potential to increase the toxicity of a xenobiotic (Kyselova, 2011). The lipophilic properties of *flavonoids, alkaloids*, and *iridoids glycosides* that interfere with permeability and cause damage cell walls will interfere with hepatocyte intracellular function and canalicular cell membrane function. Disruption of hepatocyte cells can be a decrease in production of ATP and *actin*, cell swelling which followed by hepatocyte rupture and cause tissue necrosis. Necrosis of liver *parenchymal* cells is the most common cause of intrahepatic cholestasis (Lee & William, 2003; Lindseth & Glenda, 2006). Swelling of hepatocytes and canalicular cells will also suppress and clog the canalicules resulting in blockage of bilirubin flow. This condition can inhibit all phases of bilirubin metabolism that is characterized by increased bilirubin levels (Lindseth & Glenda, 2006)

Changing of Histopathological Features

The liver histopathology examination was found that there were differences in liver histology among study groups. In group A, it is generally normal with a normal in cell nucleus and cytoplasm. However, there are some of periportal inflammation in the form of spreading of *lymphocytes* without or with some *piecemeal* necrosis. Necrosis is characterized by the presence of one of the following features, namely k*aryololysis* (loss of the hepatocyte cell *nucleus*), *karyorexis* (fragmentation of the hepatocyte cell *nucleus*), or *pycnosis* (shrinkage, reduction in cell *nucleus* size) (Robin, Cotran, & Kumar, 2007).

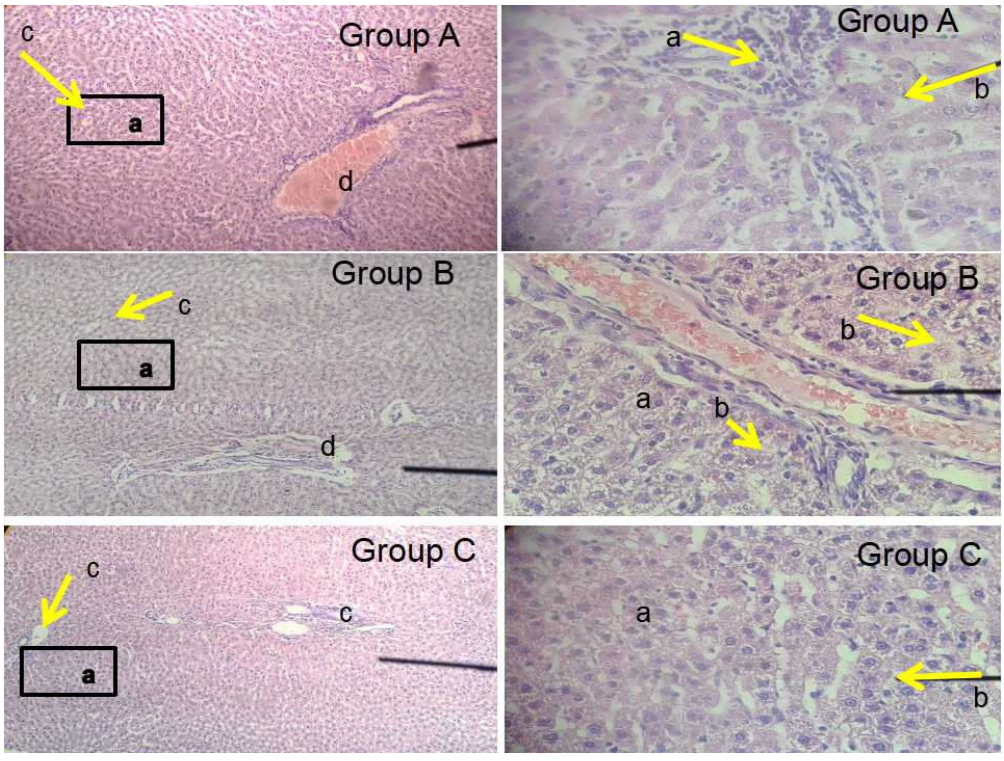

Figure 1. Comparison of Liver Histopathological Appearance among Group of Study with H and E Staining. be detected *Periportal lymphocytes* (a), *piecemeal* necrosis (b), *porta* area (c), central vein (d) The orange box is an area where 400x magnification is shown in the right picture for each study groups.

In group B, a portion of normal hepatocyte cells with normal in cell nuclei and *cytoplasm*. There was a little *periportal* inflammation and necrosis but many *piecemeal* necrosis were found that spread in to the central vein area. Whereas in group C, hepatocyte damage and extensive necrosis were found to be increasingly scattered away in the *porta* area. When compared between the study groups, group B had the worst histological feature compared to the others. The severity of a hepatocellular injury is determined using the Scheuer score by examining the histological slide of the liver under a microscope. The results of the *Scheue*r score calculation showed that the lowest average score was in group A (1.79 ± 0.74) and the highest was in group B (3.30 ± 0.66), while the average of *scheuer* score in group C was 2.84 ± 0.77 as displayed at **Table 3**. Statistical analysis showed that there was a significant difference in *Scheuer* scores between the groups (p=0,005; CI95%). it was showed that there was a difference of the effect of giving ethanol extract of *Plantago major L*. which lead to cause different severity of the *piecemeal* necrosis at hepatocyte cells. Statistical analysis continued by *post hoc* test using the *Mann-Whitney* test. The result of this analysis showed that there were significant differences of *Scheuer* scores between group A with group B (*p*-value = 0.009; CI95%) and group C (*p*-value = 0.028; CI95%). Similarly between groups B and C there were significant differences of *Scheuer* scores between both of the groups with *p*-value = 0.047; CI95%.

Table 3. The Mean of *Scheuer* Scores Among Groups of this Study

No	Group of Study	*Scheuer* Scores
1	A; Natural Control (Health)	1.79 ± 0.74
2	B; 50mg of Extract	3.30 ± 0.66
3	C; 100mg of Extract	2.84 ± 0.77

The Significant difference of *Scheuer* score between the study groups (B and C) which received the ethanol extract of *Plantago major L* with the control group showed a difference in the degree of damage to hepatocyte cells characterized by increasing of inflammatory cell and *piecemeal* necrosis. Group B (dose 50mg/ 200 g BW rat) suffered the most severity of hepatocellular damage with the highest *Scheuer* score. This condition is also consistent with the results of observations on bilirubin and ALT levels which showed that was found the highest levels of bilirubin dan ALT in group B than other groups. The results of this study indicate that the sub-chronic administration of ethanol extract of *Plantago major* L. can potentially cause hapatocellular damage, especially at doses of 50 mg/ 200 g BW rat. While the doses of 100 mg/ 200 g BW rat is relatively safer than doses of 50 mg/ 200 g BW rat.

If analyzed as a whole the results of this study indicate that hepatocellular damage which appears on the histopathological examination has not caused significant liver dysfunction. This was indicated by there were not significant effect of ethanol extract of *Plantago major* L. on changes on AST, ALT and total bilirubin levels, so that the toxic effects that occur can still be compensated. However, this research still has limitations that need to be followed up by conducting further research related to the quality of animal health, the potential toxic effects on other important organs such as the kidneys, hematological systems and other organs involved in the pharmacokinetics of drugs in the body, thus completing the scientific data of toxicology of *Plantago major* L. extract in its development as a herbal medicine and phytopharmaca.

CONCLUSIONS

Sub-chronic administration of ethanol extract of *Plantago major* L. with effective doses can induce hepatocellular damage but has not caused significantly liver disfunction. In this study, the dose of 50 mg/ 200 g BW rat was relatively more potent to cause toxic effects than the dose of 100 mg/ 200 g BW rat, so that in the next use it was recommended to use a dose about 100 mg/ 200 g BW rat and need further research.

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