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THE HEPATOPROTECTIVE EFFECT OF ETHANOL EXTRACT OF PLANTAIN (*Plantago major L.*) ON DRUG INDUCED HEPATOTOXICITY RAT (*Rattus norvegicus*) MODEL

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ABSTRACT

This study was to evaluate the effect of ethanol extract of *Plantago major L.*on reduction of hepatic transaminases and improvement of histopathologic appearances on Omeprazole and Ciprofibrate induced hepatotoxicity rat (*Rattus norvegicus*) model. By experimental study and post test only with control group design, 20 of rats were divided in to 4 groups. Group I as a negative control was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBBrat/day per oral. Group II was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBB rat/day and *Plantago major L.*50mg/200gBW rat/day per oral. Group III was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBB rat/day and *Plantago major L.* 100mg/200gBW rat/day per oral. Group IV was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBB rat/day and *Plantago major L.*200mg/200gBW rat/day per oral. There were significantly difference of *Aspartate aminotransferase* (p=0,003; α<0,05), *Alanine aminotransferase* (p=0,004; α<0,05) and histopathological appearance between groups (p= 0,001; α<0,05) Dose 50mg and 100mg/200gBW rat/day per oral of ethanol extract of *Plantago major L* are more effective as a hepatoprotective than dose 200mg/200gBW rat/day per oral. This research can be concluded that administration of ethanol extract of *Plantago major L.* can protect the liver damage on drug induced hepatotoxicity rat (*Rattus norvegicus*) model.

KEYWORD

Extract of *Plantago major L*, Hepatoprotective and Drug Induced Hepatotoxicity.

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INTRODUCTION

Drug-induced hepatotoxicity (liver injury) is common consequence of the liver as a metabolizing organ for various drugs and also other chemical substances in the body. Liver is one of the main organ that has highly risk to damage cause by drug administration, especially hepatotoxic drugs[1]. Liver is the important organ which responsible to

metabolism process and a lot of biochemical reaction in cellular level. Liver damage tend to metabolic disorder and to rise dangerous systemic disease[2].

Drug-induced hepatoxicity has been one of the main risk factor of hepatic failure and liver transplantation in the some country such as United State and other west country[3,4]. About 50% of all hepatic failure cases occured by drug induced mechanism[3-7]. Drug induce hepatotoxicity has been one of the frequent reason for removing approved drug from the population (market)[8]. In spite of, every drug has been developed under preclinical and clinical trial for detection the hepatotoxic effect, this toxic effect may occur in some individuals who are more susceptible to drug effects that related to genetic factor[9]. In many clinical trials of new drugs, up to 15% of study patients may demonstrate mild elevations of *alanine aminotransferase* (ALT) or *aspartate aminotransferase* (AST) activities[10]. Elevation of ALT and AST associated with hepatic cellulare injury (inflammation, degeneration or necrosis)[2].

The mechanism of drug-induced hepatotoxicity commonly involves the toxic drug or metabolite that either elicits an immune response or directly affects the biochemistry of the cell. The drug metabolites can be free radicals (reactive metabolite) that promote a variety of chemical reactions, such as the depletion of reduced *glutathione*, covalently binding to proteins, lipids, nucleic acids or inducing lipid peroxidation [6,11,12]. This process can cause direct effects on organelles such as mitochondria, the endoplasmic reticulum, the cytoskeleton, microtubules, or the nucleus and also indirectly influence cellular through the activation and inhibition of signaling kinases, transcription factors, and gene-expression profiles. These process can also stimulate to liver-specific cytokines cause cytokine-induced hepatotoxicity Intracellular stress leads to cell death caused by either cell shrinkage and nuclear disassembly (apoptosis) or swelling and necrosis [11,13].

On the other hand, the reactive metabolite may alter liver proteins, such as *cytochrome P-450* enzymes. The altered protein would be detected as foreign substance by the immune system, resulting in an autoimmune attack on normal hepatocellular constituents and may cause the immune-mediated liver injury [14,15]. The mechanism of the immune-mediated drug liver injury may involve a hapten-like action [16]. One of the hapten like action substant is drug metabolite produced by involving *cytochrome P-450* [17].

Omeprazole and Ciprofibrate are agents which have potency to cause drug induced hepatotoxicity by different mechanism. Some research reported that omeprazole increase the level of liver transaminasse (ALT and AST, while Ciprofibrate stimulate oxydative stress on hepatic cellulare, followed by decreasing of antioxydant level, leads to liver injury [18-20]. Omeprazole is a potent acid inhibition that has widely used in clinical medicine [21,22]. Omeprazole induced hepatotoxicity has been reported by Navarro et al. in a patient with recurrent hepatitis when omeprazole was rechallenged [18]. A case-control study over 108, 981 users of ranitidine, cimetidine, famotidine, and omeprazole found 33 cases of acute liver disease. The adjusted relative risk of developing acute liver injury was 2.1 with omeprazole compared with non use [23]. The mechanism of omeprazole-induced hepatotoxicity is unknown but some hypothesis said that process may through a metabolic idiosyncrasy pathway. Idiosyncratic reactions is immune-mediated hypersensitivity that there is enough supporting evidence for an underlying genetic edisposition in susceptible individuals [24-26] idiosyncratic reaction is occurred unpredictable, dose-independent, host-dependent and has intermediate (1-8 weeks) or late latency periods(up to 12 months) [25,27].

Ciprofibrate is a hypolipidemic drug that was classified into peroxisome proliferators class. Ciprofibrate showed effect of hepatotocicity through *peroxisome proliferators–activated receptor* (PPAR)α. Peroxisome proliferators cause an adverse cellular and molecular changes in liver, including an increase in the number and size of peroxisomes and proliferation of hepatocytes[28]. PPARα activation is required for peroxisome proliferators–induced growth responses and for liver carcinogenesis[29,30].

Early events in liver induced by PPARα activation are including *Kupffer cell* activation, release of *reactive oxygen species* (ROS) and production of mitogenic *cytokines* [31,32]. These responses facilitate the formation and fixation of oxidative DNA lesions and clonal expansion of mutated cells, which could predispose cells to tumor development [29,30].

Nevertheless, the influence of peroxisome proliferators on lipid peroxidation and oxidative DNA damage, increase in enzymes which produce H_2O_2 suggests that peroxisome proliferators may increase hepatic nuclear *NF-KB* levels. *NF-KB* activity is induced in regenerating liver [33,34]. Peroxisome proliferators also increases the activity of the *cytochrome P-450 4A* family, the decrease of cellular antioxidants (such as vitamins C and E) and antioxidant enzymes (such as *glutathione peroxidase*). So, it can increase levels of active oxygen in hepatocytes due to stimulate oxydative stress on hepatic cellulare [35,36].

Plantain (*Plantago major L*) is a weed that grows in many tropical regions, include in Indonesia. *Plantago major L* have used as a traditional medicine for various conditions of health disorder. However, the scientific data for pharmacological effect is still poor. Traditionally, *Plantago major L* used for treatment of many diseases, such as gallstone, renal stone, urinary truct infection, respiratory truct infection (productive cough, bronchitis), conjuctivitis, intestinal disorder (dyspepsia), fluor albus, diabetes mellitus, bleeding disorder, gout arthritis, acute hepatitis and also often used as diuretic [37-39].

In previously research about pharmacological effect of *Plantago major L*., showed that this weed has a lot of potential effect as a hepatoprotector agent[40]. *Plantago major L* has many active substances include *flavonoid*, that showed antioxydant effect or against to free radical such as lipid peroksidase [41-44]. These *flavonoids* are *baicalein, hispidulin*, *scutallarein* and *plantaginin* which can inhibit production of lipid peroksidase from metabolic process [42-44]. The other substances, *Ursolic Acid*, *Apigenine*, *Oleanic Acid* and *Lutheolin* also have antioxydant effect that prevents oxydative stress in hepatic cellulare, while *Aucubin* showed anti inflammatory effect [45]. Previously research reported that extract of *Plantago major L* protected hepatocellulare injury of animal model induced by CCl_4 and also has antiproliferative effect lead to inhibit process of hepatocellulare fibrosis. The other research showed that extract of *Plantago major L* was effective as a prophylactic and anti metastatic agent for some carcino genesis, such as carcinoma mammae, hepatoma and ehrlich ascites tumour [46-51]. However, The mechanism of a prophylactic and anti metastatic effect of *Plantago major L* is still unclear.

This study was to evaluate the effect of ethanol extract of *Plantago major L*.on reduction of hepatic transaminases and improvement of histopathologic appearances on Omepprazole and Ciprofibrate induced hepatotoxicity rat (*Rattus norvegicus*) model.

MATERIALS AND METHODS

This study was conducted by experimental study and post test only with control group design.

Preparation of Plant Extraction

Plantago major L seeds were collected from Slamet mountain, Purwokerto, Central Java, Indonesia and authenticated by Dr. Pudji Widodo MSc, Laboratory of Taxonomy Faculty of Biology, Jenderal Soedirman University. 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 by maceration (48 h). The solvent was removed under vacuum at temperature below 50°C, and then the extracts were freeze-dried [52,53]. Dose of the plaintain extract were devided into 3 groups that are 50mg /200gBW rat/day, 100mg /200gBW rat/day, 200mg /200gBW rat/day per oral.

Drugs

Ciprofibrate (2-[4(-2, 2 *dichlorocyclopropyl) phenoloxyl] 2-methyl propanoic acid*; Modalim, Sanofi Synthelabo Ltd, Newcastle, UK, Omeprazole; SOCID, PT SOHO, Jakarta were used for making drug induced hepatotoxic animal model.

Animal and Experiment Protocol

The rats were housed in wire-bottom cages at 20°C and adaptation for a week at Laboratorium of Pharmacology and Therapy. 20 of rats 2-3 month age, weighing between about 190 -210g was divided in to 4 groups. Group I as a negative control was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBBrat/day per oral. Group II was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBB rat/day and *Plantago major L*.50mg/200gBW rat/day per oral. Group III was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBB rat/day and *Plantago major L.* 100mg/200gBW rat/day per oral. Group IV was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBB rat/day and *Plantago major L*.200mg/200gBW rat/day per oral. They were killed after 2 month intervention under anesthetic. Samples from each liver were collected for histopathology assessment and blood for laboratory examination of AST and ALT. Degree of liver destruction was determined by using *Manja Roenigk Score*. This protocol was reviewed and approved by The Health Research Committee Faculty of Medicine University of Padjadjaran Bandung, Indonesia.

Histopathological techniques

Liver specimens were taken from the distal portion of the left lateral lobe and fixed for at least 48 hours by immersion in 10% buffered formalin. Following dehydration of the specimens in ascending grades of ethanol and cleared in *xylene* and embedding in *paraffin wax*. 5 mm thick sections were cut and then stained with *hematoxylin* and *eosin* (H and E). The histological slides are examined under the light microscope by a pathologist for assesing of hepatocellulare injury.

Liver Enzym Transaminase Examination.

Measurements of *aspartate transaminase* and *L-alanine aminotransferase* serum were determined using commercially laboratory diagnostic service, Diagnostica, located in Bandung, West Java, Indonesia.

Statistics

Statistical analysis conducted with computer program of SPSS version 17. The differences of AST and ALT between groups of the study were tested *Kruskal-Wallis* test followed by *Mann-Whitney* test and *One Way Anova* test followed by *Post Hoc Tukey* for difference analysis of histopathology appearance between groups of the study.

RESULT AND DISCUSION

The effect of *Plantago major L* Extract on alteration of serum AST and ALT levels

The normal serum consentration of AST on biochemistry examination is 55, 8U/L, while ALT consentration is 50, 4U/L. In negative control (Group I) was given Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBBrat/day per oral, there are eight times increasing of AST level and four times increasing of ALT compare to normal level. Administration of *Plantago major L* extract can reduce the escalating of these enzymes. Decreasing of liver transaminase in this study depend on dosage of the *Plantago major L* exract. There is no evidence that dosage increasing of the *Plantago major L* extract always equivalent with increasing effect to reduce liver transaminase level. The data in Table No.1 demonstrate a trend of decreased levels of serum AST and ALT among the group of the study. The lower level of AST and ALT were found in Group II followed by Group III but there is inclination to increase AST and ALT consentration in Group IV compare to Group II and III.

According to statistical analysis, there were significantly difference of AST levels (p=0,003; 95%CI) and ALT level (p= 0,004; 95%CI) between groups. Dosage 50mg and 100mg/200gBW rat/day per oral of ethanol extract of *Plantago major L* are more effective to inhibit the increasing of AST and ALT levels than dose 200mg/200gBW rat/day per oral. There were significantly difference of AST and ALT levels between Group I and Group II (p=0,009 and p=0,009; 95%CI), Group I and Group III (p=0,009 and p=0,009; 95%CI), Group II and Group IV (p=0,009 and p=0,016; 95%CI) and also between

Group III and Group IV (p=0,016 and p=0,028; 95%CI) but There were not significantly difference of AST and ALT levels between Group I and Group IV (p=0,602 and p=0,602; 95%CI) and Group II and Group III (p=0,602 and p=0,248; 95%CI). The lowest level of AST and ALT were found Group II (262,6±72,4 U/L and 83,4±11,67U/L). It is means that the most effective dosage of *Plantago major L* extract for inhibit escalating liver transaminase in this study is 50mg/200gBW rat/day per oral. However, dose 100mg/200gBW rat/day of *Plantago major L* extract can also inhibit escalating liver transaminase that showed significantly difference with control groups, so the range of effective dosage of plantain extract for inhibit hepatocellulare injury by serum AST and ALT levels indicator are 50-100mg/200gBW rat/day.

The effect of *Plantago major L* Extract on histophatological feature of hepatocellulare changing

There was difference of liver histophatological features of rat medel between groups of this study. Histophatological feature in Group I (negative control) showed remarkable hepatocellulare change. There are a lot of inflammatory infiltrate, necrosis hepatocytes (highly eosinophilic amorphus cytoplasm, organelles swelling specially mitochondria, endoplasmic reticulum and rupture of lysosomes, shrinking and dissolution of nuclei), hydropic (vacuolization of the hepatocytes cytoplasm) and parenchymatous degeneration (ballooned hepatocyte with wispy cleared cytoplasm on H and E staining, nucleus in the centre and pyknotic cause undergoing karyorrhexis) (Figure No.1).

Difference histophatological features found in Groups II, III and IV that treated by plantain extract. Group II treated with 50mg/200gBW rat of plantain extract while Group III treated with 100mg/200gBW rat of *Plantago major L* extract and group IV with 200mg/200gBW rat of plantain extract. However, all of the groups showed diminishing of hepatocellulare change. There are reduction of inflammatory infiltrate, degeneration and necrosis compare to negative control. In Group IV found histophatological feature similar with negative control. There are a lot of inflammatory infiltrate, hydropic degeneration, parenchymatous degeneration and necrosis.

Manja Roenigk Score method used to evaluate degree of hepatocellulare injury between groups of treated rat by *Plantago major L* extract compare to negative control. As it were showed on histophatological feature, the remarkable hepatocellulare change found in group I (negative control). According to *Manja Roenigk score*, the highest score found in group I (358, 4±24, 16) indicated the most severe injury occured in this group compare to the others that treated by *Plantago major L* extract. While the lowest score found in group III (213,8±5,43) followed by score on group IV (228,5±9,64) and group II (275,6±10,06) (Table No.2).

One Way Anova Test used to statistical analysis and the result showed that there were significantly difference of *Manja Roenigk Score* between groups (p-value=0,01;95%CI). It mean that there were significantly difference of hepatocellulare change at least two groups of the study. *Post Hoc Tukey* test used to determinate significance of hepatocellulare change difference between two groups of this study. The result showed that there were significantly difference of hepatocellulare change between group I and II (p-value=0,009; 95%CI), group I and III (p-value=0,009; 95%CI), group I and IV (p-value=0,009; 95%CI), group II and III (p-value=0,009; 95%CI), group II and IV (p-value=0,009; 95%CI) and also between group III and IV (p-value=0,009; 95%CI).

Administration Omeprazole and Ciprofibrate can cause drug induced hepatotoxicity. In this study, administration Omeprazole 10mg/200gBW rat and Ciprofibrat dose 16 mg/200gBBrat/day per oral, able to cause eight times increasing of AST level (473,4±138,9) and four times increasing of ALT (198,6±148,9) compare to normal level. Omeprazole can induced hepatotoxicity occur through immune mediated reaction. Omeprazole metabolized by *cytochrome P-450* produce an reactive metabolite (*sulphonamide tetracyclic*) that more reactive and

able to act as a hapten. This metabolite would covalently bind to a liver protein and subsequently, alter that protein. The altered protein would be perceived as a foreign substance that would stimulate immune system to against it in an autoimmune reaction on hepatocytes. Immunologic mechanism of liver injury marked by activation of *kuffer cells* and other immune cells lead to infammatory reaction and cell lesion impact from *cytokine* release. In this pathway also occur induction of TNF-α, apoptotic stimulation, inhibition of mitochondrial function and neoantigenic synthesis [3,4]. It is possible that reactive metabolite may also cause oxidative stress on liver cells to make hepatocellulare injury by reducing anti oxydant activity of GSH and cause directly necrosis on mitochondria[17].

Ciprofibrate can decrease liver antioxydant and induce to hepatocellular injury. Ciprofibrate is one of peroxisome proliferator induce to alteration of liver morphology and even some of previously research report that this drug can induce hepatocellular carcinoma. The mechanism of Ciprofibrate-induced hepatomegaly also related to peroxisome proliferation, stimulation of endoplasmic reticulum and *cytosolic* enzyme. There are secondary hyperplasia and hypertrophy of hepatocytes cells lead to increase in weight and volume of liver or hepatomegaly. The increasing of become permanent after second weeks administration. If the drug administration is continued after second weeks, the hepatocellulare injury will develop to carcinogenesis (hepatocellulare carcinoma)[20]. As a peroxisome proliferator, ciprofibrate also inhibit *glutathione peroxydase*, decrease liver antioxydant and induce peroxisome proliferation. Persistently peroxisome proliferation related to increase in H_2O_2 production and decreasing of free radical scavenger lead to oxydative stress on hepatocytes cells. This process cause occur lipid peroxydation and DNA oxydation lead to DNA destruction. H_2O_2 is inductor of *NF-kappa B* as an antiapoptotic agent and tumor promoter[20,54,55].

Plantago major L is one of Indonesian weed that have used as a traditional medicine for various conditions of health disorder in Indonesian society. Plantain has a lot of active substances which have various pharmacological effects, include as hepatoprotector. Hepatoprotective effect of *Plantago major L* supported by antioxydant, anti inflammatory, antiproliferation and antiapoptotic effect of active substances inside [40, 56-58]. In this study, the plantain extract can reduce AST and ALT levels of rat model omeprazole and Ciprofibrate induced hepatotoxicity. Increasing of AST and ALT levels in group I rat showed that there are hepatocellulare injury caused by omeprazole and ciprofibrate administration. Administration of *Plantago major L* extract can stimulate hepatocellulare improvement and inhibit continous injury on hepatocyte cells. *Plantago major L* extract can reduce remarkable hepatocellulare change as showed on liver histopatological feature of rats treated by *Plantago major L* extract (Group II, III and IV) compared to negative control treated by aquadest (Group I). Reducing of hepatocellulare alteration on rats treated by plantain extract indicated that there improved in hepatocytes of these rats.

Antioxydant within plaintain extract inhibit oxydative sress lead to liver injury caused by Ciprofibrate and increasing antioxydant levels that was restrained by omeprazole. So, there will stimulate hepatocellulare regeneration to improve hepatocyte cells damage. By inflammatory effect, the *Plantago major L* extract inhibit inflammatory reaction induced by ciprofibrate while antiproliferative effect against proliferative effect of ciprofibrate lead to occur hyperproliferation of hepatocyte cells. This antiproliferative effect indicated to inhibit hepatomegaly and hepatocellulare alteration caused by ciprofibrate and omeprazole. According to *Manja Roenigk score*, among rats in the group treated by *Plantago major L* extract have lower score than negative control group and there were significantly difference of *Manja Roenigk score* among the groups of study (p-*value*=0,000;95%CI). The result of this study similar and can strength of previously study by Turel at al about hepatoprotective effect of plantain on CCL_4 induced hepatotoxicity. The result showed that

plantain can improve hepatic damage on rats which The pharmacological effect of *Plantago major L* especially carried out by *Ursolic Acid, Apigenine, Lutheoline, Baicalein, Scutellarin* and *Aucubine*. *Ursolic acid* inhibits *Cyclooxygenase-2* (COX-2) and prostaglandine (PG) synthesis. Both of substances play role in inflammatory reaction and inhibition of transduction pathway of *proteinkinase* C to mediate immune response [59,60]. *Apigenine* inhibit biosynthesis of COX-2, to regulate *prostaglandine* and *Nitrit oxyde* release through regulation of ^{NF}-kappa B, blockade of IL-1β, TNF and IL-8 synthesis on *Lipopolysaccharide* [61]. *Apigenine* also induce synyhesis of pro-inflammatory cytokines on monocytes and macrophage cells through NF-kappa B inactivation with suppress to phosphorilation sub unit p65 [62].

Lutheoline within *Plantago major L* extract can suppress NF-kappaB pathway and inhibition of pro inflammatory mediators. *Lutheoline* demonstrated to inhibit production of serum TNF-α and also *arachidonic acid* synthesis [63,64]. *Baicalein* showed inhibition effect in mast cells to produce IL-1β and TNF-α through activation of NF-kappa B pathway, phosphorilation and degradation [65]. Even as Aucubin have been induced by CCL4 [40].

demonstrated to inhibit expression of COX-2 gene, TNF-α synthesis and block l-kappa B degradation. *Aucubi*n also suppress NF-kappa B activity and reduce liver transaminase (AST and ALT) [65].

Baicaleine is a substance that demonstrated antiproliferative effect through inhibit collagen accumulation and PDGF-β receptor synthesis. *Baicaleine* also inhibit *stellate cells* activation, down regulation of PDGF-β receptor and suppress activation cells to produce fibrotic tissue lead to inhibit hepatomegaly[66-68]. In the other mechanism, *baicalein* can reduce *reactive oxygene species* (ROS) lead to antioxydant activity [69].

However, antioxydant effect of *Plantago major L* extract is very important to inhibit hepatocellulare damage cause oxydative stress process. This antioxydant effect especially carried out by Hispiduline, *Baicaleine, Oleanic Acid* and *Lutheoline*. *Lutheoline* play role in increasing antioxydant level, include vitamine A, C and *ß-Carotene* [63]. As *baicaleine* and *hispiduline* decrease *glutathione*, inhibit iNOS gene expression and protect hepatocytes from various toxic agents [70-72].

Table No.1: The means of serum AST and ALT levels among the groups of this study

S.No	Group of Study	Means of	
		AST levels (U/L)	**ALT levels (U/L)**
1	I ; Negative Control II; 50mg of extract	473,4±138,9 262,6±72,4	198,6±148,9 83,4±11,67
2	III; 100mg of extract IV; 200mg of extract	296,6±28,4 433,2±86,9	93,0±4,3 180,6±110,55

Table No.2: Mean, Standard Deviation and Normality Test of Manja Roenigk Score Among the groups of Study

S.No	Groups of study	Mean ± SD	*Shapiro-Wilk* (p); 95%CI
1	I ; Negative Control II; 50mg of extract	358,4±24,16 275,6±10,06	0,032 0,024
2	III; 100mg of extract IV; 200mg of extract	213,8±5,43 228,5±9,64	0,943 0,265

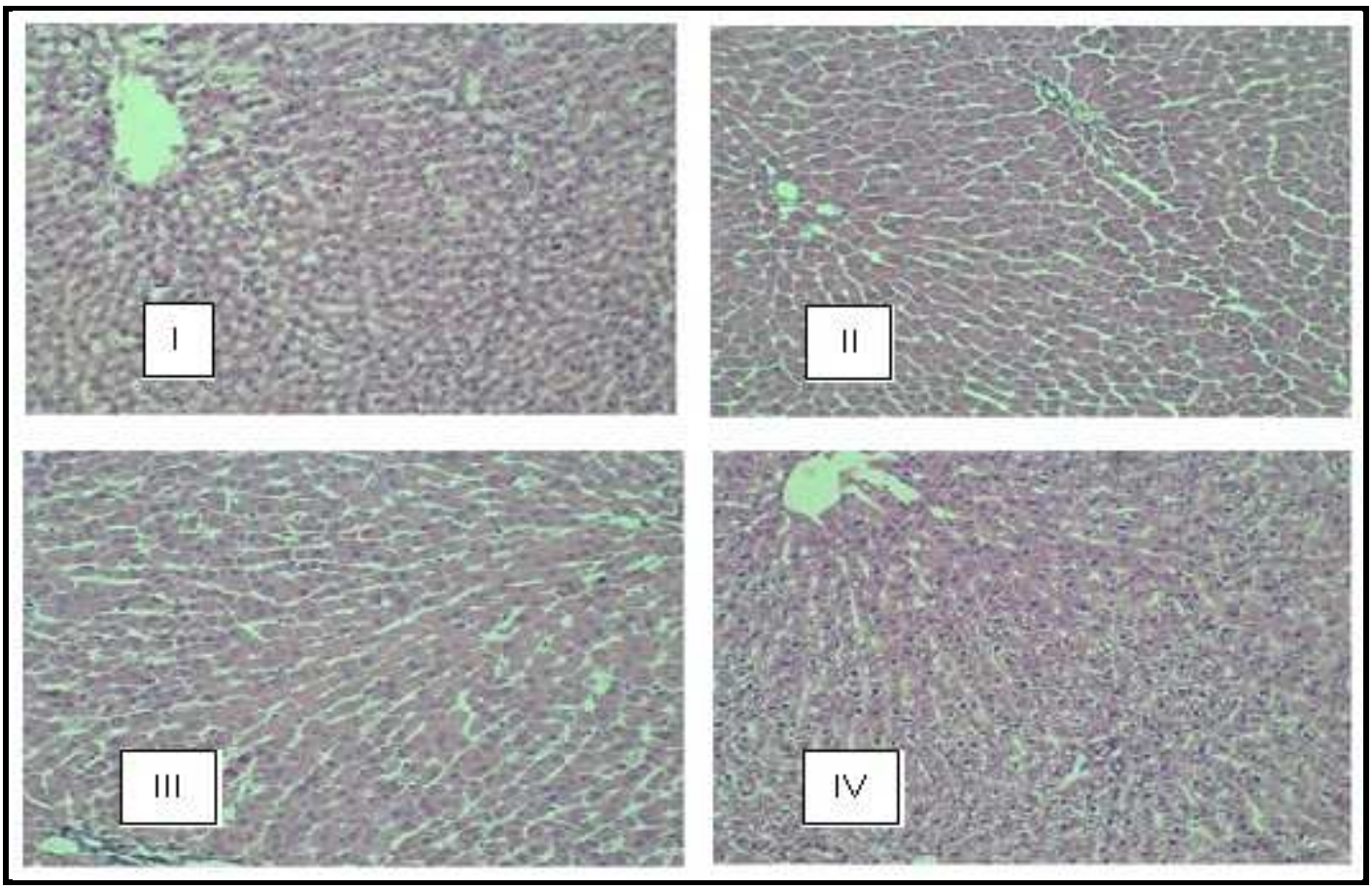

Figure No.1: Comparison of Liver Histophatological Alteration among Groups of the Study on Rat. H and E staining.x100.

CONCLUSION

Administration of ethanol extract of *Plantago major L.* reduce hepatic transaminases (AST and ALT) and can improve histopathologic appearances of the liver indicated that this plant extract can protect the liver damage on drug induced hepatotoxicity rat (*Rattus norvegicus*) model.

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