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The Protective Effect of *Artocarpus altilis* Leaf Extract on Rat Models of Diethylene Glycol-Induced Hepatotoxicity

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ABSTRACT

Diethylene glycol (DEG), an alcohol derivative, is metabolized into toxic products and is one of the main causes of liver disease. The compounds contained in *Artocarpus altilis* (AA) leaves include sapogenin, cycloartenone, cycloartenol, β-sitosterol, and tannins. These compounds show estrogenic activity and flavonoids have antioxidant, anticancer, and neuroprotective roles. This study aims to determine the hepatoprotective effect of AA by analyzing the levels of malondialdehyde (MDA), aspartate aminotransferase (AST), alanine transaminase (ALT), and liver hypertrophy in the DEG-induced rat model. Twenty-five male rats were divided into 5 groups: normal, DEG (0.75% v/v), AA1 (DEG + AA 100 mg/kg BW), AA2 (DEG + AA 200 mg/kg BW), and AA3 (DEG + AA 400 mg/kg BW). Rats were given DEG for 28 days ad libitum, and AA was administered from days 14 to 28 orally. Twenty-eight days later, serum levels of MDA, ALT, AST, and liver hypertrophy were determined. DEG induction increased body weight and liver hypertrophy, levels of MDA, ALT, and AST, whereas treatment with AA was shown to reverse to normal conditions which were observed for 28 days depending on the dose of AA ($p < 0.05$). We conclude that treatment with AA is an effective therapeutic option as a hepatoprotector in a rat model induced by DEG in a dose-dependent manner. Future challenges need to be developed as prospective complementary drugs or phytopharmaceuticals.

Keywords: ALT, AST, *Artocarpus altilis*, liver hypertrophy, MDA

INTRODUCTION

Liver damage can be caused by drugs, toxins, or infections. The incidence of drug-induced liver injury is 19 cases per 100,000 people. The most common drug causing drug-induced liver injury is amoxicillin/clavulanate (Leise *et al.,* 2014). Other causes include common bile duct stones and hepatitis E infection (Galvin *et al.,* 2015). Hepatitis E infection can masquerade drug-induced liver injury in 3% to 13% of the cases (Davern *et al.,* 2011). Diethylene glycol (DEG) is an alcohol derivative, which is metabolized into toxic products and is one of the main causes of liver disease due to physiological and psychological

changes such as rapid glycogen consumption, hypoglycemia, and acidosis. Long-term DEG induction leads to the formation of calcium oxalate (CaOx) crystals in the kidney and ureter (Mayans, 2019). High exposure to oxalate (Ox) or CaOx leads to excessive production of cellular reactive oxygen species (ROS), followed by inflammation and cell injury (Zhang *et al.,* 2017), which ultimately leads to chronic kidney disease (CKD) or permanent kidney failure (Mayans, 2019). In the case of CKD patients with or without end-stage renal disease (ESRD), serum aminotransferase levels are low, and their levels become lower with the severity of CKD (Ray *et al.,* 2015). The study shows that alcohol liver injury (ALI) has a relationship with the increase of alcohol-induced CYP2E1 activity (Chen *et al.,* 2014), but it is not found efficacy on therapeutic modalities or drugs to pre-protect livers and slow down the progress of ALI (Wang *et al.,* 2013). DEG poisoning can cause oxidative stress (OS) and cause liver damage due to calcium oxalate deposition, neurotoxic and nephrotoxic.

Liver disease may predispose to renal failure after DEG poisoning because the degree of renal failure is related to the severity of jaundice (Lin *et al.,* 2012). In addition to damaging the kidneys, continued inflammation, destruction, and regeneration of parenchymal cells, results in structural and functional abnormalities in the liver (Heegazy *et al.*, 2015), and leads to fibrosis and cirrhosis (Oh *et al.,* 2017). Oxidative stress increases free radicals which leads to overproduction of MDA. OS and biochemical disturbances caused by DEG resulted in significant changes in several parameters. The most important changes include the activity of liver parameters (AST and ALT) and OS markers; lipid peroxidation product (such as thiobarbituric acid reactive substances (TBARS) or MDA levels (Sommerfeld *et al*., 2022). Oxidative stress and inflammation are linked to a number of chronic diseases including alcoholic liver disease, chronic kidney disease, cancer, and aging (Ambade & Mandekar, 2012). Chronic liver disease is characterized by hepatocyte injury and inflammation leading to the development of cirrhosis and liver cancer. Recent study shows hepatocytes play an important role in liver inflammation (Gong *et al*., 2022).

Herbal medicines take attention for their low toxicity, multi-target action, and other effects (Mathurin & Bataller, 2015), and it has been developed into a billion-dollar industry. It is known for its health-promoting properties, and prophylactic and therapeutic benefits for relieving minor disease symptoms for a long time by people around the world (Suroowan & Mahomoodally, 2019). *Artocarpus altilis* (AA) or breadfruit produces secondary metabolites such as artocarpin. Artocarpin is an isoprenyl flavone from the *Artocarpus species* (Moraceae), has pharmaceutical activities, including cytotoxic, anti-inflammatory, antioxidant, anti-microbial, anti-androgen, anti-tubercular, anti-plasmodial, neuraminidase inhibition, termiticide and wound healing (Chan *et al.,* 2018). In a study on the antioxidant of flavonoids, it is found out that artocarpin has strongest radical scavenging efficiency. Mukesh *et al.,* (2014) reported that the ethanol extract of AA leaves had an EC_{50} value of 140.54 g/mL. Similarly, Adaramoye & Akanni (2014) demonstrated that the methanol extract of AA leaves has antioxidants that are effective against degenerative processes caused by OS in the liver, heart, and serum. N-Acetylcysteine (NAC) and Glutathione (GSH) are conventional drugs as hepatoprotectors. NAC is a mainstay of therapy for acetaminophen toxicity, whereas GSH is a tripeptide that plays an important role in several physiological processes, such as preservation of redox balance, reduction of OS through detoxification of xenobiotics and endogenous compounds, and modulation of the immune system (Pizzorno, 2014). However, the use of GSH supplements can cause cramps and bloating, or allergic reactions (Weschawalit *et al.,* 2017). In addition, inhaled GSH has caused breathing problems (bronchoconstriction) in some people with mild asthma (Marrades *et al.,* 1997). Long-term use of glutathione supplements can reduce zinc levels (Steiger *et al.,* 2017). Herbal medicines have been used in the treatment of liver disease for a long time. The plant-derived phytoconstituents (polysaccharides, proteins and flavanoids, lignans, rotenoids, etc.) stimulate the immune system and maintain hepatic diseases (Ilyas *et al.,* 2016). In that study, AA was not listed in the table of herbal hepatoprotective agents that had been reported by various previous studies. As mentioned above, the use of conventional hepatoprotector drugs has side effects, for this reason, it is necessary to look for natural or herbal medicines that are more effective and safer. Therefore, it is necessary to conduct research on the effect of AA leaf extract as a hepatoprotector by examining liver hypertrophy, MDA, AST, and ALT levels in DEG-induced rat models.

MATERIALS AND METHODS

Commercial kits used for determining the activities of serum ALT, and serum AST, were provided by ALT-AST Liqui-UV® Stanbio Laboratory EKF Diagnostics USA, MDA by Sigma-Aldrich Corporation St. Louis, Missouri, USA, and diethylene glycol purchased from MaxLab Tangerang, Indonesia. The botanical origin of *Artocarpus altilis* from Ungaran, Semarang Regency, Indonesia was authenticated carefully by Ecology and Biosystematics Laboratory, Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia. This study has received ethics approval from the Ethics Committee of the Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia, based on the letter No.0140/EC-FKH/Ex/2019.

Experimental groups:

This study used 25 male Wistar rats in 5 groups: N (normal group), DEG (DEG 0.75%), AA1 (DEG 0.75% + AA 100 mg/kg BW), AA2 (DEG 0.75% + AA 200 mg/kg BW), and AA3 (DEG 0.75% + AA 400 mg/kg BW). The administration of DEG 0.75% v/v started from the first day to the 28th-day ad libitum, while the extract was given from the 14th day to the 28th day. Furthermore, an overdose of anesthetic is administered before blood sampling through the heart, and the liver is rapidly removed and weighed (Susilo *et al.,* 2018).

Extract preparation

Artocarpus altilis leaves are dried in the sun covered with black cloth and powdered. The powder was macerated using the ethanol-water solution: 1:5 (w/v) and left for 6 hours, then extracted. The maceration was repeated with the same amount of solvent and time, filtered, and extracted with the extract from the first maceration. The crude extract obtained was purified with n-hexane using a rotary evaporator, and the ethanol extract was used for further analysis. For the treatment, it is prepared doses of 100, 200, and 400 mg/kg BW.

Preparation of AST Reagent Solutions (STANBIO)

Solution of reagent 1 (R1) and reagent 2 (R2) which were mixed in a ratio of 5:1. Solution R1 consisted of 100 mmol/L, pH buffer 7.8 tris(hydroxymethyl) aminomethane (TRIS), 300 mmol/L L-aspartate, 0.23 mmol/L nicotinamide adenine dinucleotide (NADH), and 0.53 IU/mL malate dehydrogenase (MDH). Solution R2 contained 75 mmol/L α-ketoglutarate. Then the absorbance was measured with a spectrophotometer at a maximum wavelength of 340 nm.

Preparation of ALT reagent solutions

The ALT reagent (STANBIO) also consisted of a solution of R1 and R2 which was mixed in a ratio of 5:1. Solution R1 contained 125 mmol/L, TRIS buffer pH 7.3, 625 mmol/L L-alanine, 0.23 mmol/L NADH, and 1.5 IU/mL lactate dehydrogenase (LDH). Solution R2 contained 94 mmol/L α-ketoglutarate. Then the absorbance was measured with a spectrophotometer at a maximum wavelength of 340 nm.

Preparation of plasma

Blood was collected into a tube filled with an anticoagulant (EDTA). Cells were removed from the plasma by centrifugation for 10 minutes at 1000-2000 x g using a refrigerated centrifuge. The process for 15 minutes at 2000 x g to deplete the platelets in the plasma sample.

Liver function analysis

Blood plasma is taken using a micropipette and then mixed with the AST or ALT Kit reagent, then the absorbance was read at a wavelength of 340 nm.

Liver hypertrophy analysis

The liver was taken in wet condition and immediately weighed. Hypertrophy was calculated by wet liver weight (g) per 100 g body weight at the end of the experiment (Feyissa *et al.,* 2013).

Lipid peroxidation analysis

After a complete blood draw, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15 minutes. Remove the clot by centrifugation at 1000 x g for 10 min in a refrigerated centrifuge. The antioxidant activity of AA was measured based on serum MDA levels using the TBARs spectrophotometric method and the absorbance was read at a maximum wavelength of 532 nm (Zeb & Ullah, 2016). MDA level expressed as mmol/mg.

Statistical analysis

The experimental results were used to establish a database using Excel, and IBM SPSS Statistics 19 software was used for statistical analysis. One-way ANOVA and the LSD test were presented as the mean ± standard deviation and confidant interval of 95%.

RESULTS AND DISCUSSION

Effect of AA on liver weight, body weight, and liver hypertrophy

Data on liver weight, body weight, and liver hypertrophy in the normal, DEG, and AA groups were compared to determine the effect of AA (Figure 1). The results showed that AA reduced liver weight and liver hypertrophy, and increased body weight in a DEG-induced rat model (p < 0.001).

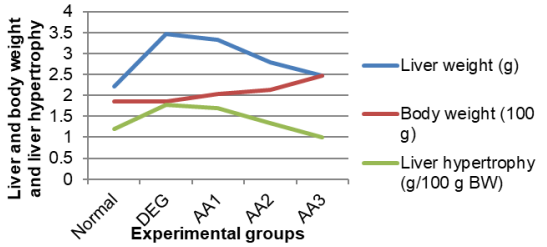

Figure 1. The effect of *Artocarpus altilis* leaf extract on liver weight, body weight, and liver hypertrophy

The term liver or hepatic hypertrophy can have various connotations, including an increase in the weight of the organ (liver hypertrophy), an increase in the average size of the hepatocytes (hepatocellular hypertrophy), and even hepatic enzyme induction (functionally sometimes referred to as "work" hypertrophy). An enlargement of the hepatocyte morphology (hepatocellular hypertrophy), resulting in an increase in organ weight or hypertrophy. Hepatocyte hypertrophy is commonly associated with microsomal enzyme induction secondary to exposure to certain xenobiotics (Yoshida *et al.*, 2015).

Changes in liver weight can be useful for detecting and measuring the effects of hepatotoxins. Decreased liver weight generally reflects loss of functional mass associated with hepatocellular atrophy or injury. Liver weight can also be affected by primary or secondary neoplasia (Russell *et al.*, 2013).

Drug- or chemical-induced hepatic hypertrophy tends to be a combination of all of these parameters in addition to others, including profound changes in the intracellular enzymes involved, not only in phase 1 and 2 drug metabolism (Maronpot *et al.,* 2010), but also in other more fundamental cell processes such as altered oxidative status, fatty acid metabolism, energy production, and utilization, cell turnover and altered hepatocellular cytoplasmic, and nuclear morphology (Grasso *et al.,* 1991).

The routine assessment of liver mass (liver weight) is a commonly applied endpoint in toxicology studies. Liver weights are often characterized as absolute liver weights and as ratios to body weight in individual animals (Russell *et al.,* 2013). Liver enlargement or hepatomegaly is usually associated with hepatocellular hypertrophy and transient hepatocyte hyperplasia in response to hepatic enzyme induction. However, the two terms are quite different, hypertrophy is an increase in the number of organelles (e.g. myofilament) and cell size. Therefore, there is an increase in the size of the organ cell, while hyperplasia is a form of an increase in the number of cells (Russell *et al.,* 2013).

Toxicity and hepatic carcinogenicity may occur when liver responses exceed adaptive changes or induced enzymes generate toxic metabolites (Maronpot *et al.,* 2010). Enlargement of the liver was more common in DEG-associated renal failure than in non-DEG-associated renal failure (53% and 33%, respectively) (Okuonghae, *et al.,* 1992).

As a consequence of hepatocellular hypertrophy without histologic or clinical pathological changes suggestive of liver toxicity, hepatomegaly is considered a non-adverse adaptive change (Hall *et al.,* 2012). The archetypal changes that frequently accompany this phenomenon include elevations of liver-derived enzymes (AST, Alkaline phosphatase (ALP), and gamma glutamyltransferase (GGT) that may appear in plasma after liver enlargement (Ennulat *et al.,* 2010).

The findings of Kawamoto *et al.,* (1990) suggest that DEG poisoning can result in liver damage in patients without underlying liver disease and possible manifestations, including hepatomegaly, jaundice, elevated ALP, and GGT. Based on the data, this study has proven that treatment with AA reduced both parameters (liver weight and liver hypertrophy) significantly in a dose-dependent manner (200 and 400 mg/kg BW). These results align with previous studies analyzing renal hypertrophy in a DEG-induced rat model (Susilo *et al.,* 2021).

Effect of AA on MDA level

As shown in Figure 2, MDA levels in the DEG group increased by 1.55 times compared to the normal group. Compared with the DEG group, MDA levels were significantly decreased by treatment with the AA (p < 0.05) depending on the dose.

Treatment with AA (100, 200, and 400 mg/kg BW) reduced the level of lipid peroxidation by suppressing the effects of free radicals due to DEG induction but was unable to restore it to a normal state.

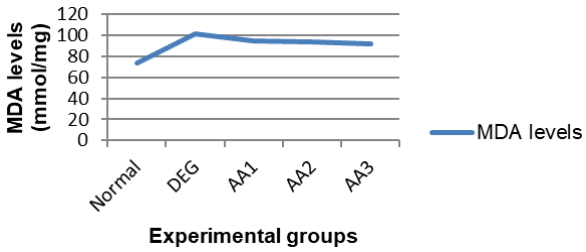

Figure 2. The effect of *Artocarpus altilis* leaf extract on MDA levels

Hepatocellular damage can be triggered by alcohol and carbon tetrachloride through metabolism. The liver metabolizes alcohol using alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) (Jiang *et al.,* 2017). ADH enzymes metabolize alcohol into acetaldehyde, but CYP2E1 activity also results in the production of reactive oxygen species (ROS) that promote OS (Doody *et al.,* 2017). High expression of CYP2E1 is a risk factor for alcoholic liver disease (Lu & Cederbaum, 2018). The free radicals react to proteins or lipids, or abstract hydrogen from polyunsaturated fatty acids, and result in lipid peroxidation such as MDA and Glutathione (GSH) (Ayala *et al.,* 2014). MDA is an end product of lipid peroxidation that can be a marker of lipid peroxidation. GSH, an intracellular thiol-based antioxidant, holds the post of an important line of defense against the OS that reduces H_2O_2, hydroperoxide (ROOH), and xenobiotic toxicity (Ma *et al.*, 2012).

The results showed that the induction of DEG led to a marked decrease in antioxidant enzyme activity and an increase in levels of the biological marker of lipid peroxidation (MDA), but treatment of AA increased antioxidant capacity and inhibited lipid peroxidation. The treatment with AA significantly prevented these changes in a dose-dependent manner (100, 200, and 400 mg/kg BW).

These findings suggested that the hepatic protective effect of AA may be closely related to the reduction of OS. The results are also in accordance with those of the study by Zeng *et al.,* (2017). The study demonstrated that histological deterioration of rat liver disease, such as steatosis and hepatic cell necrosis, accompanied by destruction of cell structure, and changes in the concentration and vitality of serum indicators related to liver function, such as MDA, GSH, and TG, are the main characteristics of in the ALI model.

Effect of AA on liver function

In Figure 3, it is shown that DEG induction increased ALT and AST serum levels by 1.47 and 1.14 times compared to the normal group. Liver function, serum ALT levels, and AST were reduced by AA treatment (200 and 400 mg/kg BW, $p < 0.05$) and returned to normal levels.

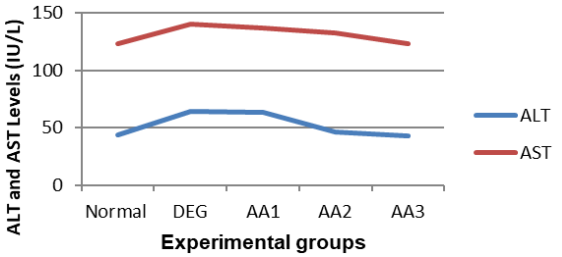

Figure 3. The effect of *Artocarpus altilis* leaf extract on ALT, and AST levels

The reference value for ALT levels for laboratory tests of human serum is 7–41 IU/L (Kratz *et al.,* 2014), while the AST level in normal rats is 18-45 IU/L (Giknis & Cliford, 2008). According to medical education standards, most cases of ALT levels >1,000 IU/L will be caused by acute ischemia, acute drug-induced liver injury (DILI) (usual paracetamol), or acute viral hepatitis (Xu *et al.,* 2012).

Toxin-induced liver damage can be divided into hepatocellular, cholestatic, and mixed types (Francis & Navarro, 2022). Hepatocellular damage is characterized primarily by elevated levels of ALT, AST, GGT, and ALP (Heegazy & Fouad, 2015). Any process that causes loss of hepatocyte membrane integrity or necrosis or damage to liver parenchyma cells results in the release of ALT and AST at higher concentrations in serum (Oh *et al.,* 2017). When the liver, heart, or muscles are compromised or damaged, ALT or AST levels rise (Goorden *et al.,* 2013). Serum ALT and AST activities are very sensitive indices for the diagnosis of liver disease (Zeng *et al.,* 2017). Increased ALT and AST activity may be associated with liver cell membrane injury because these enzymes are generally localized in the cytoplasm and released into the blood after cell injury.

Hepatic transaminases, ALT accumulates only in the hepatocyte cytoplasm (cALT) (Botros &

Sikaris, 2013). ALT is more specific than AST. ALT serves to catalyze the transfer of amino from alanine to α-ketoglutarate, ALT is found ubiquitously throughout the human body, kidney, myocardium, skeletal muscle, brain, pancreas, spleen, and lung. ALT release from damaged hepatocytes increases serum ALT levels in patients with acute or chronic hepatocellular injury (Giannini *et al.,* 2005). https://www.ncbi.nlm.nih.gov/books/NBK559278/In addition to liver tissue, AST is also present in the heart and skeletal muscle as well as in erythrocytes, making ALT the most specific marker for liver damage (Goorden *et al.,* 2013)

The index of ALT and AST were increased by DEG. The degree of toxicity of DEG exposure depends on the activity of endogenous alcohol dehydrogenase (ADH). If ADH activity is high, more DEG metabolites are produced, and the possibility of DEG poisoning is higher (Lin *et al.,* 2012). Elevated ALT and AST activity may be associated with liver cell membrane injury, as these enzymes are usually localized in the cytoplasm and released into the blood after cell injury happened (Gan *et al.,* 2012). Nevertheless, the treatment of AA significantly decreased those damages, regulated liver cells, kept the liver structure intact, and relieved liver damage caused by alcohol. In our study, it was proven that DEG increased ALT levels, whereas treatment with AA was able to reduce ALT levels significantly ($p < 0.05$).

The AST activity was widely distributed in all human tissues with the highest activity found in the heart, liver, skeletal muscle, kidney, and brain. Increased AST activity reflects tissue damage (plasma membrane disruption or apoptosis), plasma membrane bleb formation, and increased tissue and macroenzyme expression (AST complex with plasma proteins) (Ndrepepa, 2020). The AST is located in the hepatocyte cytoplasm (cAST) and mitochondria (mAST). cAST and mAST are immunologically distinct. AST catalyzes transamination reactions and plays a role in changing aspartate and α-ketoglutarate into oxaloacetate and glutamate. AST is more abundant in the myocardium than in liver cells. It is also present in striated muscles, kidneys, and brain (Bastiansyah, 2012).

Elevated mAST is seen in extensive tissue necrosis during myocardial infarction (MI) and chronic lung disease (CLD) like liver tissue degeneration and necrosis. About 80% of AST activity in the liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme (Thapa & Walia, 2007). AST levels are often measured to check overall liver health. However, as mentioned above, AST levels can also be caused by damage to other organs, such as the heart, kidneys, or muscles. Therefore, AST is often paired with other tests to determine the specific location of the problem (Botros & Sikaris, 2013). AST elevations often predominate in patients with cirrhosis and even in liver diseases that typically have an increased ALT (Green & Flamm, 2001).

Liver damage from various liver diseases increases AST. This includes fatty liver disease and viral hepatitis affecting the liver (e.g., mononucleosis), or liver cancer (Wang *et al.,* 2013). In addition, AST also increases when liver damage occurs due to toxins such as lead, mercury, or pesticides (Schmeltzer *et al.,* 2016), but AST may also be low in CKD, unrelated to vitamin B6 levels (Mayans, 2019).

Normal healthy levels of this AST in your blood range from 5 to 40 IU/L (Goorden, *et al.,* 2013), or 12–38 IU/L (Davern *et al.,* 2011). A high AST (above 40 IU/L) can signal a liver, heart, or muscle problem (Botros & Sikaris, 2013). The reference value for AST levels in normal rats is 73-143 IU/L (Giknis & Cliford, 2008). In this study, it was shown that the AST levels of all rats exceeded the normal threshold value (> 143 IU/L). DEG induction was shown to increase AST levels, and treatment with AA decreased AST levels (200 and 400 mg/kg BW, $p = 0.05$).

The utility of De Ritis ratio, AST-to-ALT ratio (AAR), has been highlighted in alcoholic hepatitis, where the AST is largely higher than the ALT. Elevated De Ritis ratio has been widely used as a marker of advanced liver fibrosis (Giacomo *et al,* 2016) Therefore, AAR is >2.0 for alcoholic hepatitis, 1.5 to <2.0 in acute viral hepatitis, and >1.0 in fibrosis and cirrhosis. The ratio is affected by the number of post-exposure days and the severity of the disease. Another important factor is the relatively short half-life of AST (18 hours) compared to ALT (47 hours), the fact that gender requires consideration, and an intra-individual variation of both AST and ALT (Botros & Sikaris, 2013).

In this study, it is found that AA extract (200 mg/kg) caused a significant decrease in serum AST and ALT levels, this result is in line with the study of Mohn Ali *et al.,* (2013) that administration of AA maintains liver cell membrane stability by attenuating the liver cell membrane stability and

ethanol-induced liver damage, as demonstrated by substantial reducing activity in serum ALT, AST, and ALP. Beneficial effects were also shown by administering AA to hypercholesterolemia (HC) mice (Adaramoye & Akanni, 2014), and Mohd Esa *et al.,* (2013) proved that ALT and AST activity increased in HC mice. Elevated serum levels of AST and ALT indicate increased permeability and hepatocyte damage and/or necrosis.

CONCLUSION

The results of this study demonstrated that treatment with AA significantly reduced liver hypertrophy, serum MDA, ALT, and AST levels, and increased body weight in a dose-dependent rat model. AA may act as an effective antioxidant to prevent degenerative processes caused by OS, later it can be developed as a phytopharmaceutical or complementary medicine to reduce the risk of hepatotoxicity.

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CONFLICT OF INTEREST

Authors state no conflict of interest.

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