The Effects of Aqueous Extract of Jamaican Cherry (*Muntingia calabura*) on D-galactose-induced Liver Damage in BALB/c Mice (*Mus musculus*)

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Abstract: D-Galactose induces oxidative stress that will damage the hepatocytes. The aqueous extract of Muntingia calabura leaves (AQMC) is known to have a potential antioxidant to prevent hepatocyte damage caused by oxidative stress. This study aimed to determine the effects of AQMC leaf extract on liver damage induced by D-Galactose in BALB/c Mus musculus. This was experimental research with a completely-randomized design involving 20 hepatic samples (paraffin blocks and HE staining) from 5 groups. The groups included K1 (healthy), K2 (D-galactose-induced), K3 (D-galactose + AQMC 35 mg/kgBW), K4 (D-galactose + AQMC 75mg/kgBW), and K5 (D-galactose + vitamin C 28mg/KgBW). D-galactose was administered for 6 weeks prior to the therapy (vitamin C or AQMC given for 4 weeks). Liver damage was observed in all fields of view and described comprehensively. The degree of hepatocyte damage was calculated using the Manja Roenigk scoring and analyzed using One-way ANOVA with post-hoc Tukey's HSD test (CI = 95%, $\alpha = 0.05$). AQMC leaf extract could reduce D-galactose-induced liver damage in BALB/c Mus musculus. The hepatocyte damage in the groups given AQMC therapy was less than that in the D-galactose negative control group (K1 = 73.5 + 2.39, K2 = 92.00 + 5.24, K3 = 69.00 + 2.79, K4 = 76.25 + 4.42, K5 = 77.25 + 6.48; $p = 0.029$). AQMC at a dose of 35mg/KgBW showed more effective therapeutic potential against D-galactose (K3 = 0.003, K4 = 0.027; post-hoc toward K2). AQMC administration could reduce liver and hepatocyte damage of BALB/c Mus musculus induced by D-Galactose at a potential dose of 35 mg.

1 INTRODUCTION

Aging can raise the levels of free radicals (ROS) in the blood and tissues. Excessive ROS in an aging process is induced by an imbalance between ROS and antioxidant. Studies show that D-galactose induced in animal models can describe the process of oxidative stress in aging. D-galactose increases plasma Malondialdehyde (MDA) levels (Sulistyoningrum et al., 2019), raises MDA levels in the liver, and reduces hepatic antioxidant (SOD) levels (Hadzi-Petrushev et al., 2015). Induced D-galactose will also activate the p-53 pathway, thus leading to cell apoptosis, and stimulate the p-21 pathway that plays a role in the cell cycle (Bo-Htay et al., 2018).

Aging reduces the ability of the liver to regenerate during cellular injury. Research shows that the hepatocytes in aged rats lose the capability of entering mitosis (Biondo-Simões et al., 2006), and the cells' ability to recognize growth factors, such as EGF, also decreases in aging rats (Schmucker & Sanchez, 2011). The reduced regenerative capability increases the likelihood of cell damage and apoptosis. Cell apoptosis in the liver is susceptible not only to oxidative stress but also to genomic instability and lipotoxicity (Zhong et al., 2017).

In addition to increasing cell apoptosis, the aging process in the liver is also histologically marked by

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dilation of blood vessels, increasing number of Kupffer cells, and escalation of cell degeneration (Hashish, 2016). Therapy to reduce aging effects on the liver should therefore be considered in order to prevent age-related liver diseases, including hepatic cirrhosis, liver cancer, or non-alcoholic fatty liver disease (NAFLD)

Muntingia calabura (MC) is among the medicinal plants with highly potential preventive properties against oxidative-stress-induced cell damage. The administration of methanol extract of Muntingia calabura leaves (MMC) can prevent increased uric acid levels and damage to the proximal renal tubules in DM model rats (Safrida & Sabri, 2019). In addition, MMC leaf extract given as premedication for hepatotoxic substances (CCl4) has proved to reduce the levels of parenchymal liver damage (Zakaria et al., 2019). The potential protective role of MC has also been observed in non-methanolic extracts. A number of studies report the administration of MC ethanol extract that can suppress an inflammatory process and prevent gastric ulcers in ethanol-induced rats (Aziz Ibrahim, 2012; Lin et al., 2017; Sarimanah et al., 2017). Combined MC-Ficus carica infusion is able to reduce SGOT and SGPT levels in paracetamol-induced rats (Lalihatu & Sudharmono, 2019). Furthermore, the administration of MC as premedication can prevent carbonated-drink-induced or ethanol-induced liver damage (Murti et al., 2016).

2 MATERIAL AND METHODS

2.1 Research Design and Subjects

This research was purely experimental with a completely-randomized design. The experiment was conducted from April to October 2016 at the Laboratory of Histology and Anatomical Pathology of the Faculty of Medicine, Universitas Islam Indonesia Yogyakarta after passing the ethical review from the ethics committee of the Faculty of Medicine of Universitas Islam Indonesia. This study used 20 livers of BALB/c mice (Mus musculus) obtained from a previous study (Sulistyoningrum et al., 2019). The inclusion criterion was the hepatic tissue obtained from a previous research protocol. If damage to the hepatic tissue was found (microscopically or macroscopically) and thereby resulting in difficulty to interpret the results, the tissue was excluded. The 20 samples were obtained from 5 groups named K1 (healthy normal group), K2 (negative control group, D-galactose-induced), K3 (dose-1 treatment group, D-galactose-induced and 35mg/kgBW AQMC leaf extract), K4 (dose-2 treatment group, D-galactose-induced and 75mg/kgBW AQMC leaf extract), and K5 (positive control group, D-galactose-induced and 28mg/KgBW vitamin C). D-galactose was administered for 6 weeks while the treatment (administration of AQMC or vitamin C) was given daily for 4 weeks after induction. The hepatic tissue was then transversely embedded in a paraffin block and stained with HE staining.

2.2 Observation of Hepatic Histomorphological Changes

The liver damage was observed descriptively and semi-quantitatively. The changes observed in all fields of view consisted of inflammation, degeneration, necrosis, indistinctive cell boundaries, and affected size of the central vein (Zulfi et al., 2013). The levels of liver damage were calculated on the basis of Manja Roenigk scoring on 50 cells (1: normal, 2: inflammation, 3: degeneration, 4: necrosis) in six identical fields of view for each sample (total magnification of 400x). Inflammation was marked by lymphocyte invasion, degeneration was manifested as clear cytoplasm and giant cells in hepatocytes (Sookoian et al., 2016), and necrosis was indicated by changes in hepatocyte nuclei (pyknosis, karyorrhexis, and karyolysis). The final score of liver damage in each sample was obtained by multiplying the number of cells by the Manja Roenigk scoring (Zulfi et al., 2013). In addition, cell boundaries were classified into distinctive, indistinctive, and fairly distinctive. The size of central vein was then compared with that of the healthy control group to obtain three categories of size change named normal, slightly enlarged (>1.5-2-fold), and enlarged (>3-fold).

2.3 Statistical Analysis

An analysis was performed on the semi-quantitative data of liver damage scores. The normality was examined using the Saphiro Wilk test while the significance test involved One-way ANOVA followed by Post-Hoc Tukey's HSD test. All of the statistical tests were done at a confidence level of 95% ($\alpha = 0.05$)

3 RESULTS

The hepatic histomorphological changes during the aging process (D-galactose-induced) include

inflammation, degeneration, necrosis and a change in the central vein size (Figure 1). The hepatic histological changes were evident in the negative control group (K2), and inflammation was found in all of the study groups. The administration of AQMC leaf extract improved the histopathological features of D-galactose-induced liver damage in mice. Meanwhile, the administration of vitamin C resulted in histopathological features resembling those of AQMC leaf extract at a dose of 75mg/kgBW (Table 1). The hepatocyte damage found in this study showed a significant difference ($p = 0.029$, Table 2), which was mainly found between the AQMC leaf extract treatment group and the K2 group (dose of 35mg/KgBW: 0.003, dose of 75mg/KgBW: 0.027, Table 3).

Table 1: Histological features of liver damage in different groups.

	Inflammation	Degeneration	Necrosis	Cell boundaries	Central vein size
K1	+	-	-	distinctive	normal
K2	++	+++	++	indistinctive	enlarged
K3	+	+	+	distinctive	normal
K4	+	++	+	fairly distinctive	slightly enlarged
K5	++	++	+	fairly distinctive	slightly enlarged

Note: - normal, + mild, ++ moderate, +++ severe. K1: healthy normal group, K2: negative control group (D-galactose-induced), K3: dose-1 treatment group (D-galactose-induced and 35 mg/kgBW AQMC leaf extract), K4: dose-2 treatment group (D-galactose-induced and 75 mg/kgBW AQMC leaf extract), K5: positive control group (D-galactose-induced and 28 mg/kgBW vitamin C).

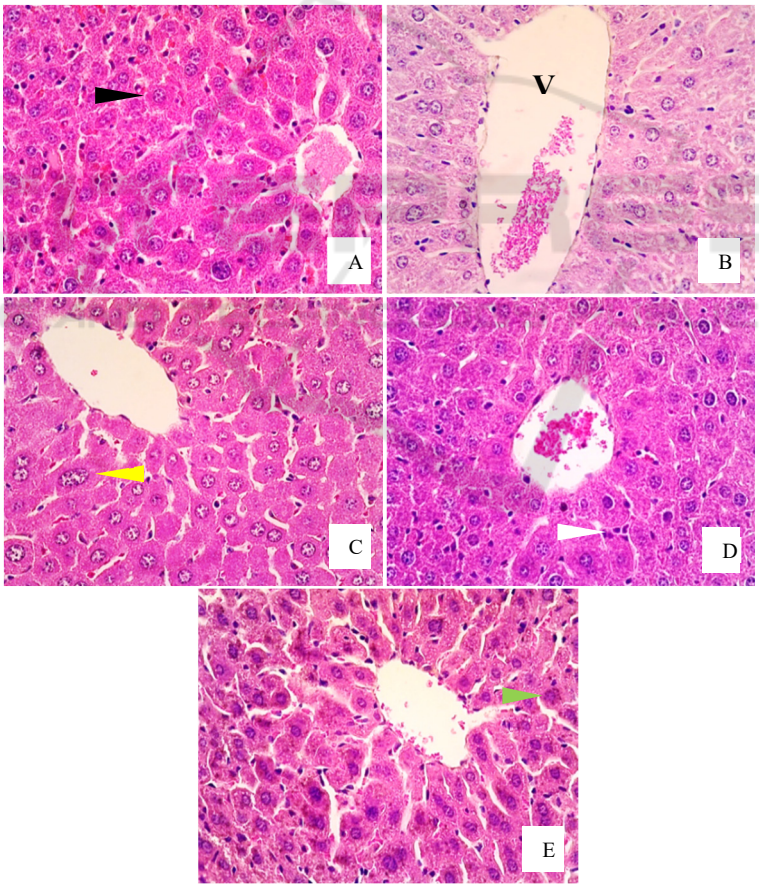

Figure 1. Hepatic histological features with HE staining and 40x objective magnification in all groups (in alphabetical order for K1, K2, K3, K4, K5). Black arrow: normal liver, white arrow: inflammation, yellow arrow: degeneration, green arrow: necrosis, VS: central vein.

Table 2: Mean Anova of hepatocyte damage in different groups.

Group	n	Mean+SD	p
K1	4	73.5±2.39	0.029
K2	4	92.00±5.24	
K3	4	69.00±2.79	
K4	4	76.25+4.42	
K5	4	77.25+6.48	

*: ($p<0.05$)

Table 3: Total score of hepatocyte damage from Post-Hoc test.

	K1	K2	K3	K4	K5
K1		0.011*	0.493	0.674	0.567
K2	0.011*		0.003*	0.027*	0.036*
K3	0.493	0.003*		0.276	0.218
K4	0.674	0.027*	0.276		0.878
K5	0.567	0.036*	0.218	0.878	

4 DISCUSSION

This research shows that D-Galactose induces cell death in the liver. The highest degree of liver damage was found in the D-Galactose-induced group with no treatment (neither AQMC leaf extract nor vitamin C). The administration of AQMC leaf extract provides improved features of hepatic architecture in that the dose of 35mg/kgBW mirrored the normal features while the dose of 70 mg/KgBW approximated the features in the group given vitamin C. The total score of liver damage was significantly different in all study groups. A significant difference was particularly evident between the group with AQMC leaf extract at a dose of 35 mg/kg BW and the positive control group. There was an insignificant difference between the hepatic histological features of the mice receiving AQMC leaf extract and those of the healthy group. Therefore, it indicates that the administration of AQMC leaf extract (especially at a dose of 35mg/kgBW) can improve the histological features of aging liver (D-galactose-induced). Such histological changes have the same features with those of the liver of healthy mice (not induced by D-galactose).

When induced to the body, D-Galactose increases the levels of galactose, and galactose will reduce to galactitol, a compound that cannot be further metabolized, thereby accumulating intracellularly and increasing the cell osmotic pressure. This process will eventually lead to swollen cells.

Histologically, this feature is recognized as ballooning degeneration/cell swelling with clear cytoplasm (Ye et al., 2014). D-Galactose will also reduce free amine groups in amino acids or proteins, thus leading to AGEs formation through glycation as well as ROS formation (Parameshwaran et al., 2010). The administration of D-galactose for 6 weeks correspondingly led to an elevated level of free radicals (plasma MDA levels) by approximately 3-fold of that of the healthy group (Sulistyoningrum et al., 2019).

Muntingia calabura has a hepatoprotective effect. A study shows that the administration of methanol extract of Muntingia calabura leaves (MMC leaf extract) followed by soft drink administration can significantly prevent increases in SGOT and SGPT levels (Siddiq et al., 2019). MMC leaf extract administered for 7 days followed by the administration of hepatotoxic substances (CCL4 or paracetamol) can prevent further liver damage (Zakaria et al., 2019) (Mahmood et al., 2014). MMC leaf extract as premedication for CCl4 administration is able to prevent elevated ALT levels, increased pro-inflammatory cytokines (NO, TNF-α, IL-β, IL-6), and higher ratio of the liver weight to the body weight. MMC leaf extract administered to rats at therapeutic doses of 250 mg/kgBW and 500 mg/KgBW results in a liver weight ratio closer to that of the healthy group. ALT levels in the 500 mg/KgBW treatment group are similar to those of the positive control group (receiving N-acetyl-cysteine therapy and induced by CCl4). MMC leaf extract (doses of 50.250 and 50 mg/kgBW) as premedication before induction by CCl4 is also able to significantly increase the antioxidant levels (SOD and CAT) in the body. Histopathological features of liver damage become minimal in the 500 mg/kgBW treatment group (Zakaria et al., 2019). MMC leaf extract as premedication is also able to prevent liver damage induced by paracetamol. In line with the report by Zakaria et al., (2019), MMC leaf extract as premedication is able to prevent an increase in the relative weight of the liver but unable to prevent increased levels of liver enzymes (ALT, AST, and ALP). Minimal necrosis and inflammation are found in the group receiving MMC leaf extract as premedication (Mahmood et al., 2014).

The doses of 35 mg/KgBW and 70 mg/KgBW for the mice in this study are equivalent to the doses of 250 mg/KgBW and 500 mg/KgBW in experimental rats. This indicates that the findings of this study are in accordance with previous studies in which the potency of MMC leaf extract is found at both doses (Mahmood et al., 2014; Siddiq et al., 2019; Zakaria et al., 2019). In contrast to the potential dose of MMC leaf extract at 500 mg/kgBW, that of AQMC leaf extract in this study is found at 250 mg/kgBW. This

difference is likely caused by the different treatment given to the experimental animals. The main objective of this study was to examine the therapeutic effects of AQMC leaf extract on damaged liver while the study using MMC leaf extract aimed to investigate the protective effects on the induction of liver damage. Another reason for the difference is the compound concentrations in MC due to the extraction process. Kolar, Kamble and Dixit, (2011) report that AQMC leaf extract has a total flavonoid content exceeding that of MMC leaf extract but with a lower phenolic content. The antioxidant activity of MMC leaf extract is apparently higher than that of AQMC leaf extract.

AQMC leaf extract has more benefits than other types of extract. Compared to petroleum ether and ethyl acetate extracts, the total phenolic content of AQMC leaf extract is twice higher with a better hepatoprotective effect (indicating only minimal inflammation). In addition, premedication using AQMC leaf extract is able to suppress increasing hepatic enzyme levels (ALT, AST, and ALP) in rats induced by paracetamol. By comparison with the doses of 50 and 250 mg/KgBW, the dose of 500 mg/KgBW in rats shows the best potency (in terms of the liver weight ratio, hepatic histological features, antioxidant levels, and liver enzyme levels) (Zakaria et al., 2018).

The protective effect of MC is associated with its antioxidant activities in the flavonoid compounds, such as catechin, gallocatechin, epigallocatechin narigenin, and quercetin in MC (Pereira et al., 2018). The hydroxyl complex in the phenol compounds in MC can inhibit proton donation in ROS formation (Balakrishnan et al., 2011). The polyphenol compounds in MC inhibit glycosidation reactions and have an anti-glycation activity by inhibiting RAGEs signaling (Sadowska-Bartosz & Bartosz, 2015). The saponins in MC also slow down aging through activation of the AKT FOXO3a pathway and the Nuclear factor-erythroid 2-related factor-2 pathway. This process will improve the expression and functions of antioxidant enzymes, such as superoxide dismutase-2 (SOD-2), catalase, glutathione reductase, glutamate-cysteine ligase, and heme oxygenase-1 (Khan Y et al., 2015). In addition to the antioxidant potential, research by Rofiee et al., (2015) shows that the protective effect of MC is manifested through the bile acid biosynthesis and arachidonic acid metabolism.

This study has a limitation in that the effects of AQMC leaf extract on impaired liver function (levels of ALT, AST and ALP enzymes) were not investigated. However, the findings of this study have been able to illustrate that MMC leaf extract has a therapeutic effect on liver damage, particularly on an aging liver. Further research with reference to the therapeutic effects of AQMC leaf extract is required by taking into account the various stimuli of both acute and chronic tissue damage.

5 CONCLUSION

The administration of AQMC leaf extract can reduce liver and hepatocyte damage of BALB/c *Mus musculus* induced by D-Galactose. The potential dose of AQMC leaf extract is 35 mg.

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