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Antifibrotic Potential of *Nigella sativa* Extract: Study on Male Wistar White Rats Induced by Hepatotoxic Agents

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Abstract. Liver fibrosis is the initial stage of the liver injury response, which is still reversible. Continued injury causes damage to hepatocytes, activating Hepatic Stellate Cells to become myofibroblasts, producing type 1 collagen. Untreated collagen type 1 and fibril tissue piles can lead to liver cirrhosis. The administration of herbal antifibrotics has become the focus of current research. One herbal treatment that has antifibrotic functions is black cumin seeds. This study aimed to prove the effect of black seed extract (*Nigella sativa*) on decreasing expression of collagen type 1 in the liver parenchyma of male Wistar white rats (*Rattus norvegicus*) liver fibrosis model. The method in this study was True Experimental, Post Test Only Control Group Design with one positive control group and one negative control group, as well as three papaya leaf extract treatment groups, namely doses of 1.2 g/kg, 2.4 g/kg, and 4. 8 g/kgBW. The One Way Anova test results showed a significant difference (sig=0.000). The Post-Hoc Tukey test results showed significant differences between each treatment group. The correlation test results showed a strong relationship, namely a correlation coefficient value of -0.978. The regression test yielded 95.3% results, which means that black cumin extract (*Nigella sativa*) had a 95.3% effect on the expression of type 1 collagen in the liver parenchyma of male Wistar white rats so that the administration of black cumin extract (*Nigella sativa*) affected decreasing the expression of type 1 collagen liver parenchyma of male white Wistar rat (*Rattus norvegicus*) liver fibrosis model.

Keywords: Liver fibrosis, collagen type-1, black cumin extract

1. INTRODUCTION

Liver fibrosis is a response to healing injury from various stimuli resulting from myofibroblasts' activation, which are generally produced by hepatic stellate cells (HSC) (1). With continued damage, fibrosis can form excessive connective tissue and organ failure such as liver cirrhosis (2). According to the World Health Organization (WHO), liver cirrhosis ranks eighteenth for diseases with the highest number of deaths worldwide, namely 800,000 cases (3).

Injury to hepatocytes caused by various factors results in the activation and transformation of HSC into myofibroblast cells, which will regulate type 1 collagen, matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), and other proteins (4). Type 1 collagen is specifically produced by the Golgi apparatus of myofibroblasts (5). The extracellular matrix (ECM) becomes rich in glycoproteins, glycans, and especially type 1 collagen in large quantities (6). Activated HSC and chronic exposure to repeated injuries will lead to ECM deposition and fibrosis. HSCs produce ECM proteins and matrix metalloproteinase (MMP), which functions in the degradation of type 1 collagen and other proteins in the ECM, allowing fibrosis regression to occur (7)(8).

If treatment is not given to improve the regression mechanism, there is a possibility that fibrosis will become cirrhosis. One of the treatments that is the focus of current research is the administration

of antifibrotics (9). Appropriate administration of antifibrotics has not been found in daily practice due to the many side effects of existing drugs. Nowadays, the potential of antifibrotic sources is often studied in herbal ingredients to minimize side effects. One of the herbal ingredients that has antifibrotic potential is black cumin (*Nigella sativa*) (10).

Based on the content of black cumin (*Nigella sativa*), which has an anti-inflammatory and antioxidant effect, researchers want to conduct a study that aims to determine the effect of black cumin extract on the expression of type 1 collagen in the liver parenchyma in white Wistar rats (*Rattus novergicus*) hepatic fibrosis model.

2. MATERIALS AND METHODS

This research uses True Experimental Research. The design of this study is the Posttest Only Group Design. Each group was randomized to be considered the same before treatment. The research location was in the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University for 14 weeks. The population in this study was male white rats of the Wistar strain (*Rattus norvegicus*). The sample used was a male white rat of the Wistar strain (*Rattus norvegicus*) weighing 150-280 grams, aged 2-3 months, with a healthy condition characterized by active movements and clear eyes. The sample consisted of 5 groups determined randomly. The sample in one treatment group is seven rats divided into five treatment groups.

The research doses used were 1.2 g, 2.4 g, and 4.8 g, which were given to each rat in treatment groups 1, 2, and 3 for four weeks, which had previously been given intraperitoneal injections of CCl4 for eight weeks.

The extraction process is done by grinding the black cumin seeds and then soaking them in 90% ethanol, after which the solution is left for 24 hours to evaporate. Black cumin seed extract is dissolved in corn oil and given orally with a sonde. The extraction process was carried out at the Pharmacology Laboratory, Brawijaya University.

At the end of the 4th week of administering black cumin extract, the mice were dissected to remove the liver organs, then made histology preparations and stained using the immunohistochemical method to observe the expression of type 1 collagen in each preparation. The data will be analyzed using the One Way Anova test, Tukey post-hoc test, Pearson correlation test, and linear regression test.

3. RESULTS

3.1 Data Analysis Result

The results of this study showed that the average number of type 1 collagen expression in KN = 18.4 cells, KP = 161.6 cells, P1 = 69.8 cells, P2 = 51.6 cells, and P3 = 18.8 cells. One-way ANOVA test result obtained a significant effect ($p < 0.05$). Post-Hoc test results showed significant differences ($p < 0.05$) in each treatment group. The results of the Pearson correlation test obtained a value of -0.978. The results of the linear regression test accepted adjusted $R2 = 0.953$.

Positive control group (K+) treated with CCl4 injection had more expression of myofibroblasts stained with type 1 collagen than the expression of type 1 collagen in the normal control group (KN). This difference shows that intraperitoneal injection of CCl4 at a dose of 1 ml/kgBW can increase the expression of type 1 collagen in the liver parenchyma of male Wistar rats. In the three treatment groups, P1, P2, and P3, the expression of type 1 collagen was significantly reduced compared to the positive control (K+). Research data shows a difference in the decrease in type 1 collagen expression that occurred in the P1, P2, and P3 treatment groups. In the P1 and P2 treatment groups, the type 1 collagen expression reduction had not yet reached normal. However, the P3 treatment group reduced type 1 collagen expression until it reached the KN group's average amount of type 1 collagen expression.

Based on the Pearson correlation test shows that there is a robust correlation with a negative and significant correlation direction between the increase in the dose of black cumin extract (*Nigella sativa*) and the decrease in the expression of type 1 collagen in the liver parenchyma of Wistar male rats (*Rattus norvegicus*) thus indicating that the greater the dose given the extract Black cumin (*Nigella sativa*) causes decreased expression of type 1 collagen in the liver parenchyma of male Wistar rats (*Rattus norvegicus*). Meanwhile, the results of the linear regression test showed that the adjusted R^2 value was 0.953, which means that the antifibrotic effect of administering black cumin

extract (*Nigella sativa*) reduced the expression of type 1 collagen in the liver parenchyma of male Wistar rats (*Rattus norvegicus*) by 95.3%. The remaining 4.7% is influenced by factors not examined.

4. DISCUSSION

4.1 Black Cumin Affects Type I Collagen Expression

This research shows that the administration of black cumin extract (*Nigella sativa*) affects the expression of type 1 collagen in the liver of male Wistar rats (*Rattus norvegicus*). In this study, the expression of type 1 collagen was used as a marker of liver fibrosis produced by Hepatocyte Stellate Cells (HSC), which are activated to become myofibroblasts.

Liver fibrosis results from an imbalance between the overproduction of extracellular matrix, especially type 1 collagen, and its resolution mechanisms (1). Type 1 collagen is a protein of the triple-helix fibril network that forms strong bonds in the extracellular matrix to maintain the anatomical function of the liver in the event of injury. However, this pile of fibrils will become excessive if the balance between production and degradation does not work well (14).

4.2 CCl_4 Effect on Liver Metabolism and Liver Fibrosis

The most sensitive organ is the liver when CCl4 is exposed to the human or animal body. In the liver, it will undergo metabolism by the enzymes CYP2E1, CYP2B1, CYP2B2, or CYP3A to produce CCl3, which is a free radical. One of the most damaging is CCl3 reacting with oxygen to form trichloromethyl peroxide (OOCCl3), which is a cause of oxidative stress that can damage cytochrome P-450. Trichloromethyl peroxide will then affect the phospholipid and cholesterol membranes, thereby initiating the formation of malaldehyde, which will cause loss of calcium and cell homeostasis and damage hepatocyte cells as a whole (15). Damage to hepatocyte cells will cause the release of proinflammatory cytokines Transforming Growth Factor β1 (TGF-β1), Tumor Necrotizing Factor α (TNF-α), Reactive Oxygen Species (ROS), Platelet-Derived Growth Factor (PDGF), interferon γ (IFN- γ) as well as activation of Kupffer cells via Toll-Like Receptor 4 (TLR4). These cytokines will cause HSC activation, which then differentiates into myofibroblasts and produces type 1 collagen, accumulating extracellularly, leading to liver fibrosis (11).

Positive control group (K+), which was treated with CCl4 injection, had more expression of myofibroblasts stained with type 1 collagen than the expression of type 1 collagen in the normal control group (KN). This result in line with research by Domitrovic (2009) and Amin (2010), which shows that peritoneal induction of CCl4 at a dose of 1 ml/kgBW can cause damage to hepatocyte cells, which will then activate HSCs so that type 1 collagen expression increases. HSC in normal liver functions as a deposit of vitamin A and is located in the space of Disse (1). And in the treatment group showed type 1 collagen expression was significantly decreased compared to the positive control. This is in accordance with the theory that the thymoquinone compound contained in black cumin has an anti-fibrotic effect by reducing TLR4 expression in Kupffer cells, inhibiting lipid peroxidase, and increasing NK cell activation through TRAIL. Thus, it can cause apoptosis of HSC, which will decrease TIMP-1 and increase MMP13, which specifically works on type 1 collagen so that fibrotic tissue in the extracellular matrix can be degraded (8).

Treatment groups P1 and P2 decreased the expression of type 1 collagen and did not reach normal levels. This could be because the thymoquinone compound contained in black cumin extract in groups P1 and P2 was still inadequate or required longer to reduce hepatic parenchyma type 1 collagen expression to reach normal conditions. While P3 group showes that decreased type 1 collagen expression until it reached the average amount of type 1 collagen expression in the KN group. This is in accordance with Saricicek's study (2014), where a dose of black cumin extract of 2.4 g/kg has improved liver histopathology but has not returned to normal liver. From the study results, it was found that the highest dose (4.8 g/kg BW) showed the best results by approaching the average expression of type 1 collagen in normal liver parenchyma.

4.3 Factors Affeting Antifibrotic of Black Cumin

There are lots of factors that could affect antifibrotic in the administration of black cumin extract (*Nigella sativa*) in reducing the expression of type 1 collagen in the hepatic parenchyma of male Wistar rats. Factors that can influence include other ingredients contained in black cumin extract, which not only function as an anti-fibrotic. Besides thymoquinone in black cumin extract, the active compounds are linoleic acid and oleic acid. As in the research of Karacor and Cam (2015) and Silva-Santi, Antunes, & Caparroz-Assef et al. (2016), linoleic acid and oleic acid function as anti-inflammatories by reducing IL-6, IL-10, and TNF-α. It was explained by Biswas and Sharma (2016) that anti-inflammatories have a significant influence on the fibrosis regression process by reducing inflammatory conditions in the extracellular matrix so that MMP13 activity can be maximized to degrade type 1 collagen piles. In addition, reducing inflammatory diseases in the extracellular matrix can increase TRAIL (TNF-) activity. Related Apoptotic Induced Ligand), which is secreted by NK cells (Natural Killer) to support the apoptosis of HSC. When administering black cumin extract orally, there can also be confounding factors, namely the possibility of interaction with food before administering the extract, which can reduce the bioavailability of the extract. Likewise, another factor that can influence is the condition of the cage environment, which can influence stress in mice, which also affects the metabolic function of rats.

4.4 Study Limitation

This study only discusses the effect of black cumin extract (*Nigella sativa*) on the expression of type 1 collagen in the liver of male Wistar rats (*Rattus norvegicus*) in a liver fibrosis model, where it is known that the fibrosis stage is reversible in the liver injury mechanism. However, in practice, liver fibrosis is rarely detected and is often found when it reaches the cirrhosis stage. Thus, further research is needed on whether the administration of black cumin extract (*Nigella sativa*) can provide an improvement mechanism at the stage of liver cirrhosis.

This study's limitation was the immunohistochemical staining procedure for type 1 collagen. In the staining procedure performed in this study, positive controls were made after staining the liver preparations. Making a positive control is done on bone marrow tissue, which aims to determine whether the procedure applied can bring out the expression of type 1 collagen. Making a positive control is done repeatedly until the correct procedure is obtained. This procedure should be carried out before staining the liver preparations, assuming that if type 1 collagen expression is seen in the positive control preparations, the staining procedure is correct and ready to be applied to the liver preparations. However, in this study, the positive control was made only once and was carried out after staining the liver preparations. Because of that, several liver preparations showed many false positives with stained hepatocytes.

After discussing the theoretical studies and reviewing some of the facts found in this study, the hypothesis about the effect of black cumin extract (*Nigella sativa*) is proven to decrease the expression of type 1 collagen in the liver parenchyma of male Wistar rats (*Rattus norvegicus*).

5. CONCLUSION

Based on the results and discussion of this study, the following conclusions can be drawn: Black cumin extract (*Nigella sativa*) has an effect on the expression of type 1 collagen in the liver parenchyma of male Wistar rats (*Rattus norvegicus*) liver fibrosis model with an adjusted R2 value = 0.953. It was found that the optimal dose for black cumin extract (*Nigella sativa*) was 4.8 g/kgBW/day, as indicated by the mean expression of collagen type 1 in the liver parenchyma of male Wistar rats (*Rattus norvegicus*) did not differ significantly from the mean expression of collagen in the normal control group. There was a significant difference in the expression of type 1 collagen in the liver parenchyma of male Wistar rats (*Rattus norvegicus*) between treatment groups, as indicated by $p<0.05$ from the One Way ANOVA test results.(12)

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