



Antifibrotic Activity of *Phaleria macrocarpa* Extract in Rat Liver-fibrosis Model: Focus on Oxidative Stress Markers, TGF- β 1 and MMP-13

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Abstract

Edited by: Sinisa Stojanoski
Citation: Wardhani BWK, Sundari N, Tjandrawinata RR, Jusuf AA, Soetikno V, Louisa M. Antifibrotic Activity of *Phaleria macrocarpa* Extract in Rat Liver-fibrosis Model: Focus on Oxidative Stress Markers, TGF- β 1 and MMP-13. Open Access Maced J Med Sci. 2020 Sep 10; 8(A):555-562. https://doi.org/10.3889/oamjms.2020.4929
Keywords: Liver fibrosis; *Phaleria macrocarpa*; TGF- β 1; MMP-13; Oxidative stress
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Received: 12-May-2020
Revised: 25-Jul-2020
Accepted: 18-Aug-2020
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Funding: PT Dexa Medica, Jakarta, Indonesia and Universitas Indonesia
Competing Interests: The authors have declared that no competing interests exist.
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AIM: This study was aimed to determine the antifibrotic activity of *Phaleria macrocarpa* (PM) extract in liver fibrosis (LF) and its possible mechanism in the rat model.

METHODS: Sprague Dawley male rats were injected with 2 mL/kg BW of carbon tetrachloride intraperitoneally twice a week for 2 weeks, followed by 1 mL/kg BW for 6 weeks. Afterward, the treatments began from the 3rd week: Silymarin 100 mg/kg BW/day, standardized PM extract (Proliverenol) 75 or 150 mg/kg BW/day orally. Rats were sacrificed in the 8th week. Blood and liver were collected to analyze liver function, liver damage and fibrosis marker, oxidative stress markers, pro-fibrogenic cytokine, and antifibrotic marker.

RESULTS: Our study showed that the treatment of silymarin and PM resulted in the normalized activity of liver function, followed by the amelioration of oxidative stress, demonstrated by the decreased malondialdehyde levels and an increased ratio of glutathione and glutathione disulfide. All markers examined showed that PM extract has antioxidant activity due to decreased hepatic stellate cell activation. We also found a decrease in tumor growth factors- β 1 and protein expressions of matrix metalloproteinases-13 in all treatment groups compared to the carbon tetrachloride group. There were tendencies of the decreased fibrotic area following improvements of biochemical parameters.

CONCLUSION: PM extracts ameliorate carbon tetrachloride-induced LF. The proposed mechanism is by overcoming oxidative stress and regulating pro-fibrogenic cytokine and antifibrotic markers.

Introduction

Liver fibrosis (LF) is a long-lasting injury and inflammation in the liver. Scarring of the LF may lead to life-threatening complications such as cirrhosis and hepatocellular carcinoma (HCC) [1], [2]. LF is associated with a 45% mortality rate in Europe and the most common cause of non-neoplastic death in the United States [2], [3], [4]. Up to date, there are no antifibrotic agents available in the market. The approach used to treat LF is by managing the underlying disease [1], [3], [5].

Phaleria macrocarpa (PM) (Scheff.) Boerl., namely, Mahkota Dewa in Indonesia, is a member of the family Thymelaeaceae. Pits, stems, leafage, and fruits of PM were widely used as natural sources of medical plants in Indonesia [6]. PM, mainly grains, has been used empirically to treat cancer, allergy, and diabetes mellitus [6], [7]. Many studies have

elucidated the chemical compounds of PM, which consists of mahkoside A, mangiferin, kaempferol-3-O- β -glucoside, dodecanoic acid, palmitic acid, ethyl stearate, fevicordin-A, and sucrose [6], [7], [8]. PM also has been studied for its antioxidant, anti-inflammatory, cytotoxicity, antihistamine, and hepatoprotective effects [7], [8], [9].

The hepatoprotective activity of this PM extract was reported by Berlian *et al.* [10] using *in vitro* ethanol-induced LF model. PM extracts suppressed the expressions of NF- κ B, TNF- α , and caspase-8 [10]. Hendra *et al.* [6] also reported that PM fruit extract has antioxidant and anti-inflammation using an *in vitro* model. Antioxidant was proven by ferric thiocyanate (FTC), thiobarbituric acid (TBA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The anti-inflammatory effect was determined by nitric oxide (NO) inhibitory effect [6]. In accordance with their results, Sundari *et al.* [9] confirmed the protective effect using the *in vivo* model. PM extracts prevent CCl₄-induced fibrosis

through its antioxidant and anti-inflammatory activities [9]. Other studies also reported that the upregulation of the NF- κ B transduction pathway and lipid peroxidation is critical in LF [1], [2], [3], [11]. Considering all the above facts, we presume that PM extracts might have the antifibrotic activity to attenuate LF. Hence, this study was aimed to determine the antifibrotic activity of PM extracts and investigate its mechanism in carbon tetrachloride-induced LF in male rats.

Materials and Methods

PM aqueous extract (Proliverenol) and silymarin

The dried powder PM extract (Proliverenol) was prepared by Dexa Laboratories of Biomolecular Sciences (Batch No. RP130116), as described by Kim *et al.* [8] and Berlian *et al.* [10]. Proliverenol was a bioactive fraction extracted from *P. macrocarpa* fruit using subcritical water extraction, as described by Kim *et al.* [8]; Berlian *et al.* [10]; and Sundari *et al.* [9]. Slivers of PM fruits were macerated using water-based solvent and carried out extraction under temperatures of 373K, pressures 4.0 MPa for 5 h. The yield extract was then filtered, evaporated, dried, and collected in a tube. High purity silymarin (81.32%) was purchased from Plamed Science Technology Company (Xian, China).

Experimental design

Sprague Dawley male rats (200–350 g) were maintained on a 12-h light/dark cycle and allowed free access to food and water. The protocol was approved by the Animal Care Ethics Committee from the Faculty of Medicine, Universitas Indonesia. The flow of the experiments done in the study is depicted in Figure 1. The total sample in this experiment is 25 rats divided into five groups. The number of rats per group was decided based on sample calculation by Charan *et al.* [12]. Five rats were allocated to a healthy control group. The other 20 rats were treated with an intraperitoneal injection of 2 mL/kg CCl₄ (carbon tetrachloride, Merck, Germany) in olive oil (1:1) twice weekly for first 2 weeks and followed by 1 mL/kg weight for 6 weeks [13], [14], [15]. The 20 rats of the liver-fibrosis induced were randomly divided into four groups of five rats: CCl₄ only (untreated liver-fibrosis model); CCl₄ + silymarin 100 mg/kg/day (Sil), CCl₄ plus PM extract 75 (T75), and CCl₄ plus 150 mg/kg/day (T150) orally on the 3rd week. After 8 weeks, all animals were sacrificed by decapitation (3 days after the last injection of CCl₄) [14].

Biochemistry analysis

The whole blood was collected in a heparin tube and centrifuged 3000 rpm for 10 min [16]. Obtained

plasma was analyzed for alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP). Those were quantified using kit DiaSys (Int. Holzheim, Germany). Liver tissue was homogenized and used for oxidative stress and concentrations of TGF- β 1. Oxidative stress was determined by the MDA level and GSH/GSSG ratio. MDA level was analyzed by thiobarbituric acid (TBA) assay, while GSH/GSSG ratio was measured according to the manufacturer's protocol (Cell Biolabs Inc, San Diego, USA). The concentration of TGF- β 1 in liver tissue was quantified using ELISA kit TGF- β 1 according to the manufacturer's protocol (Novateinbio, MA, USA).

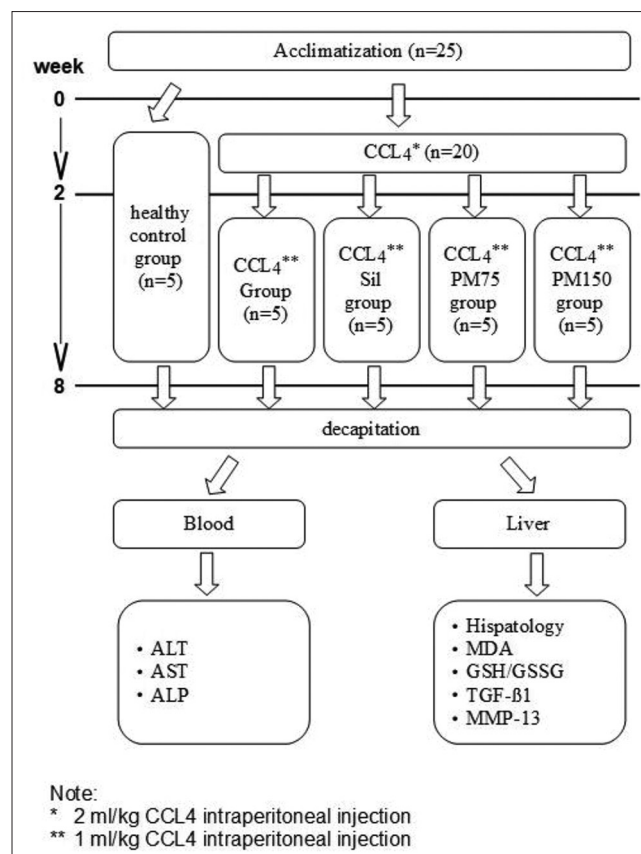


Figure 1: The flow of experiments throughout the study. CCl₄: Carbon tetrachloride, Sil: Silymarin 100 mg/kg/day, T75: PM extract 75 mg/kg/day; T150: PM extract 150 mg/kg/day. n: number of rats

Histopathology and Immunohistochemistry analysis

Section of the area around central vein from all rat livers was sliced, fixed in 10% buffered formalin, and made in a paraffin-embedded block. Histopathology analysis was done to determine the accumulation of connective tissue by Masson's trichrome staining [17]. While MMP-13 was detected by immunohistochemistry, as described in the manufacturer's procedure using primary antibody from Santa-Cruz (sc-101564, USA) and Novolink™ Polymer Detection System from Leica Biosystems (RE7290-K, USA). We analyzed the degree of fibrosis in histopathology and assessed quantitatively using the J-Image software image processor.

Statistical analysis

All the graphs and statistical tests were done using GraphPad Prism 8 (USA) software. The results were expressed as mean \pm standard deviation (SD). The differences among groups were analyzed by one-way analysis of variance (ANOVA) and followed by *post hoc* Tukey analysis when data were normally distributed and had homogeneous variance. Kruskal–Wallis followed by Mann–Whitney tests were used when the data did not meet the requirement for parametric analysis. A $p < 0.05$ is considered statistically significant.

Results

After 8 weeks of CCl_4 administration, the CCl_4 group showed a significant increase in ALT, AST, and ALP activity in plasma as hepatic injury markers. Sil, T75, and T150 groups showed ameliorated all of the markers significantly (Figure 2).

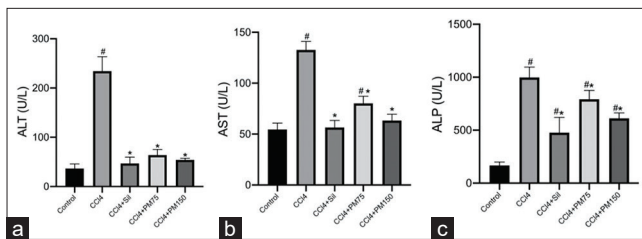


Figure 2: Silymarin and *Phaleria macrocarpa* extracts enhance the activity of (a) ALT, (b) AST, and (c) ALP after 8 weeks of CCl_4 -induced LF. Data are expressed as mean \pm SD. CCl_4 : Carbon tetrachloride, Sil: Silymarin 100 mg/kg/day, T75: PM extract 75 mg/kg/day; T150: PM extract 150 mg/kg/day. [#] $p < 0.05$ versus control group; ^{*} $p < 0.05$ versus CCl_4 group. There was a significant decrease in the activity of ALT, AST, and ALP in silymarin and PM extracts as compared to the CCl_4 group. Silymarin decreased the activity of ALT and AST, approaching the values of those in the control group

Our findings in the liver function markers are supported with histopathology results, as shown in Figure 3.

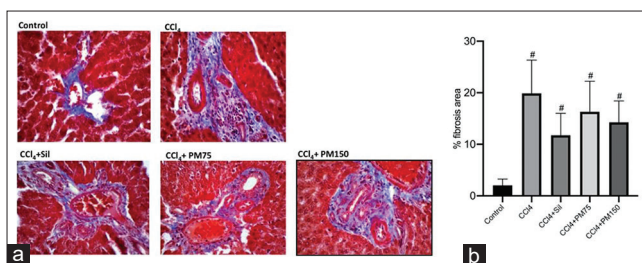


Figure 3: Silymarin and *Phaleria macrocarpa* extract attenuated the effect of 8-week of CCl_4 -induced liver fibrosis by decreasing fibrosis area. (a) Histopathology overview, which represents each treatment group using Masson's Trichrome Staining (magnification: $\times 400$). The blue color represents an accumulation of connective tissue surrounding area branches of the hepatic artery, portal vein, and bile duct. (b) Quantification results of fibrosis area using image processor. Data are expressed as mean \pm SD. CCl_4 : Carbon tetrachloride, Sil: Silymarin 100 mg/kg/day, T75: PM extract 75 mg/kg/day; T150: PM extract 150 mg/kg/day. [#] $p < 0.05$ versus control group; ^{*} $p < 0.05$ versus CCl_4 group

The percentage of fibrosis area in the CCl_4 treated group resulted in fibrosis was significantly

increased as compared to normal. Silymarin and PM extract reduced the percentage of fibrotic areas, although the decline had not approached the control group.

LF induced by CCl_4 was clearly shown in the CCl_4 group, with a significant increase in the area of fibrosis and hepatic oxidative stress marker (MDA) as compared to the control group (Figure 4a). The GSH/GSSG ratio was shown to decline to compensate for oxidative stress that occurs on LF pathogenesis. Both silymarin and PM extracts in two different doses ameliorate fibrosis area, reduced MDA levels, and increased the GSH/GSSG ratio significantly compared to the CCl_4 group (Figure 4b).

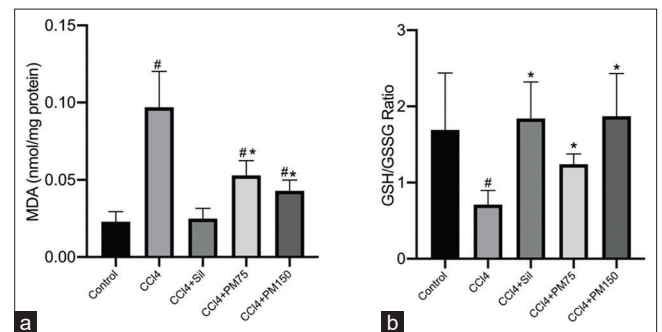


Figure 4: Silymarin and *Phaleria macrocarpa* extracts improve oxidative stress markers in CCl_4 -induced liver fibrosis as shown by (a) malondialdehyde levels, (b) ratio of GSH/GSSG. Data are expressed as mean \pm SD. CCl_4 : Carbon tetrachloride, Sil: Silymarin 100 mg/kg/day, T75: PM extract 75 mg/kg/day; T150: PM extract 150 mg/kg/day. [#] $p < 0.05$ versus control group; ^{*} $p < 0.05$ versus CCl_4 group

Data are expressed as mean \pm SD. CCl_4 : Carbon tetrachloride, Sil: Silymarin 100 mg/kg/day, T75: PM extract 75 mg/kg/day; T150: PM extract 150 mg/kg/day. [#] $p < 0.05$ versus control group; ^{*} $p < 0.05$ versus CCl_4 group.

Chronic CCl_4 administration for 8 weeks increased the TGF- $\beta 1$ levels and in line to a percentage of the positive expressions of MMP-13. Sil and PM extracts ameliorate levels of TGF- $\beta 1$ and reduced the percentage of the positive expressions of MMP-13 (Figure 5).

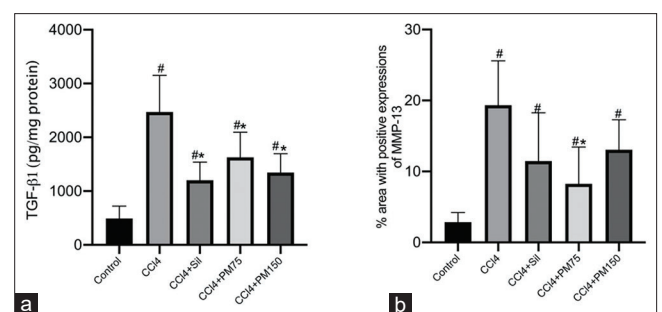


Figure 5: *Phaleria macrocarpa* extracts elevated (a) levels of TGF- $\beta 1$ and (b) the percentage of area with positive expressions of MMP-13 (immunohistochemistry). Data are expressed as mean \pm SD. CCl_4 : Carbon tetrachloride, Sil: Silymarin 100 mg/kg/day, T75: PM extracts 75 mg/kg/day; T150: PM extracts 150 mg/kg/day. [#] $p < 0.05$ versus control group; ^{*} $p < 0.05$ versus CCl_4 group

Discussion

The present study aimed to investigate PM extracts' efficacy and mechanism for the treatment of LF. CCl_4 was used to induce LF that involved oxidative stress and followed persistent inflammation as same as happened in chronic liver disease [12], [13], [14]. Chronic administration of CCl_4 for 8 weeks caused liver damage characterized by an increase of ALT, AST, and ALP activity in plasma. As described by Hayashmi *et al.* [12], Truong *et al.* [16], and Li L *et al.*, [18], the mechanism of CCl_4 is trichloromethyl and trichloromethyl peroxide radicals is attacked the endoplasmic reticulum membrane and leading to lipid peroxidation [12], [16], [18]. CCl_4 chronic treatments lead to impaired membrane permeability so that the enzymes in the cytosol and mitochondria of hepatocytes will come out into plasma. Consequently, there are increased activities of those in plasma [16].

ALT and AST are enzymes located in the cytosol of hepatocytes that are involved in the gluconeogenesis process. Hepatocyte injury can lead to increased activity of them up to more than 2 times normal [18], [19], [20]. We showed a significant increase in ALT and AST enzyme activity in the CCl_4 group, more than 2 times from normal value. These results are in line with other studies by Constandinou *et al.* [13], Tzeng *et al.* [21], Bona *et al.* [22], and Deng *et al.* [23]. In contrast to our results, Tsai *et al.* [24] reported that the treatment of CCl_4 only caused an increase of ALT and AST less than twice compared to the control group, though they found clear signs of LF in histopathology samples.

ALP is an enzyme that catalyzes the hydrolysis process of organic phosphate to inorganic phosphate. Increased activity of ALP for 3–10 times from normal value is the result of cholestasis and obstruction of intra and extra biliary [16]. CCl_4 in this study has caused an increase of ALP activity more than 3 times the standard value. It indicated that chronic administration of CCl_4 causes bile duct disorders. These results are consistent with the findings by other researchers similar to Bona *et al.* [22], Tuncer *et al.* [25], and Amin *et al.* [26] that used CCl_4 to induce fibrosis.

The increase in the three markers showed signs of chronic injury of the liver caused by CCl_4 , which is a good fibrosis model. This fibrosis model was used to study PM extract's antifibrotic activity (75 and 150 mg/kg/day). Doses used in this study were determined from the previous research [9], [10]. Silymarin (Sil) has been reported to stop the progression of LF effectively *in vitro* and *in vivo* by improving histopathology and decreased biochemical parameters [24], [27]. It has been used for patients who have NAFLD and alcoholic liver disease (ALD). It improved biochemical parameters (AST, ALT, and GGT), ameliorate inflammation through the reduction in TNF- α serum levels, and decrease hepatic steatosis degree, which followed by improvement in

histopathology [28], [29]. Silymarin is leading to inhibit HSCs activation and protect hepatocytes from apoptosis when oxidative stress occurs in LF [3], [27]. Silymarin was known to scavenge free radicals, thereby inhibiting lipid peroxidation. It also has an anti-inflammatory effect by inhibiting intrahepatic activation of the transcription factor NF- κ B. Thus, decreasing the release of TNF- α , IFN- γ , IL-2, and inducible NO synthase (iNOS) that plays a role in the process of chronic inflammation as well as LF [29], [30]. In the previous study, PM extract inhibits the NF- κ B pathway and decreases lipid peroxidation [9], [10]. Therefore, silymarin was chosen as a comparator. In line with our findings, a study by Mostafa *et al.* reported that the administration of silymarin 100 mg/kg/day decreased ALP activity significantly compared with the fibrosis group [31]. Studies by Tsai *et al.* and Amin *et al.* had also shown a decrease in ALP enzyme activity after administration of silymarin 200 mg/kg [24], [26].

Improvement in liver function tests by all treatment groups was in line with the findings in the percentage of fibrosis area. In agreement with our study, Tsai *et al.* [24], Amin *et al.* [22], and Lee *et al.* [32] reported that the administration of silymarin caused liver improvement in histopathology examination.

CCl_4 leads to oxidative stress, causes damage to hepatocytes, and further induces fibrogenesis in chronic exposure [13], [16], [18]. Imbalance redox status occurred due to the increased free radicals from CCl_4 metabolites and the lack of endogenous antioxidant capacity. Those were described by elevation MDA levels (lipid peroxidation) and declining GSH/GSSG ratio (endogenous antioxidant capacity) [33], [34], [35]. MDA levels were higher in CCl_4 than the control group, which showed elevation of lipid peroxidation. All therapy groups improved the level of MDA. CCl_4 produced those due to their ability to scavenge free radicals, thus inhibiting lipid peroxidation in hepatocytes [10], [27], [30]. GSH/GSSG ratio declined in the CCl_4 group compared to the control group. Silymarin and PM extract improved GSH/GSSG ratio, which is higher than the CCl_4 group and approached the control group. Studies done by Tzeng *et al.* [21] and Kiruthiga *et al.* [36] showed that silymarin increased endogenous antioxidant activity by improving the glutathione peroxidase activity (GPx), manganese superoxide dismutase (Mn-SOD), and cooper/zinc-superoxide dismutase (Cu/Zn-SOD) [21], [36]. The increase of endogenous antioxidant activity illustrated elevation GSH availability to form conjugates with $\text{CCl}_3\text{OO}^\cdot$ and COCl_2 (free radical, which is CCl_4 metabolites). The antioxidant activity of the PM extract might mediate the anti-fibrosis effect.

The possible mechanism of antifibrotic activity is by regulating TGF- β 1 and MMP-13. Decreased TGF- β 1 levels reduce the activation of hepatic stellate cells, which further inhibits fibrogenesis. While as a marker for antifibrotic activity, we measure MMP-13, which acts by degrading ECM in LF [13], [37], [38]. We found that silymarin and PM extracts reduced the TGF- β 1 levels. However, the improvement has not reached the normal

level, which indicates that silymarin and PM treatment have not been able to normalize TGF- β 1 levels. In line with our findings, Li *et al.* [39] showed the decline of TGF- β 1 in a higher dose (200 mg/kg/day), and longer duration of silymarin still could not normalize profibrotic cytokines. Reversal of LF normalization has also not been successful with other drugs such as pioglitazone and phloridzin [14], [23].

MMP-13 is a specific protease capable of degrading collagen type I in rodents [10], [14], [34]. MMP-13 in rodents has functions similar to MMP-1 in humans and is required to maintain the homeostasis of ECM on rodents [40], [41].

In LF, Kupffer cells, hepatic stellate cells, and activated myofibroblast will produce MMP-13 for degrading collagen I, resulting in decreased ECM composition [13], [40]. We showed an elevation percentage of the positive expressions of MMP-13 in the CCl₄ group, even not approaching the healthy group. This finding was in agreements with studies done by Iridale *et al.* [40] and Chen *et al.* [42] Iridale *et al.* reported that the expression of MMP-13 increases in experimental fibrosis model, which is induced by CCl₄ intraperitoneally in the same regimen in this study for 4 weeks [41]. The decline was not as apparent as the improvement of histopathology examination [41]. While Chen *et al.* reported that there were increased expressions of MMP-2, MMP-8, MMP-9, MMP-13, TIMP-1, and TIMP-2 in the fibrosis model group due to administration of TAA [42].

Iridale *et al.* [40] and Leclercq *et al.* [14] found that CCl₄ induction similar dose in this study can increase MMP-13, which differs significantly from the control group. Ginandrea *et al.* [43] reported that in the early phase of fibrogenesis, and myofibroblast activation occurs, followed by the release of pro-inflammatory cytokines (such as TNF- α and IL-1) and pro-fibrogenic cytokines (TGF- β 1). TNF- α induces activation and expression of MMP-13. Both cytokines are released by extra-hepatic macrophages, Kupffer cells, and HSCs when activated [40], [43]. Uchinami reported that the elevation TNF- α during peak LF is accompanied by increased expression of MMP-13 [44]. In our experiments, the MMP-13 in the CCl₄ group was increased. As described by Iridale *et al.* [40] and Uchinami *et al.* [44], it possibly occurred due to the continuous activation of hepatic stellate cells, which resulted in myofibroblast activation.

There was a tendency to decline in the positive expressions of MMP-13 in all treatment groups, silymarin, and PM extracts. The phenomenon might occur due to inhibition of hepatic stellate cells and myofibroblast activation by silymarin and PM extract. Therefore, activation of MMP-13-producing cells has decreased, although those are higher than the control group. It is in accordance with a study, which is conducted by Chen *et al.* [42]. They reported that silymarin could reduce the expression of MMP-13.

In contrast, Leclercq *et al.* [14] reported that pioglitazone for 3 weeks and 7 weeks led to MMP-13 elevation, which was significantly different compared to the CCl₄ group and baseline [14]. Another study done by Kawaguchi *et al.* found that pioglitazone could prevent the activation of HSCs by reducing the mRNA expression of collagen type I, MMP-2, TIMP-1, and TIMP-2 and increased the expression of MMP-13 mRNA [41]. However, an *in vivo* study by Kawaguchi *et al.* showed declining mRNA expression of TIMP-1 and TIMP-2 with no increase in MMP-13 [45].

MMP-13 alone could not adequately describe the process of ECM degradation. However, the process of ECM degradation can be seen from the percentage of fibrosis area. It tends to decrease, although not significant, compared to the CCl₄ group. It happened because there are two working mechanisms of MMP-13. First, MMP-13 will directly degrade collagen types I and III then resulting in decreased ECM accumulation. Its process is leading to LF resolution [40], [41], [42], [43], [46]. Second, MMP-13 can activate pro-HGF (hepatocytes pro-growth factors) to HGF. HGFs bind to the c-Met receptor and subsequently induce the expression of MMP-2 and MMP-9 [47]. In accordance, Iridale *et al.* showed that increasing MMP-13, followed by decreasing of TIMP-1 and TIMP-2 in fibrosis [40]. Then, the expressions of TIMP-1 and TIMP-2 reduce when LF occurs without MMP-13 reduction [40].

Furthermore, the presence of pro-inflammatory (TNF- α and IL-1) and pro-fibrogenic (TGF- β 1) cytokines can affect the expression of MMPs and TIMP [40], [44]. Elevation of pro-inflammatory cytokines, lead to the induced expression of MMP-13. Nevertheless, pro-fibrogenic cytokine PM extract at T75 and T150 groups decreases the activity of ALT and AST significantly compared to the CCl₄ group. Our findings are consistent with results from the previous study, in which proven PM extract is shown to ALT and AST activity in rats induced ethanol [9], [11]. ALP levels decreased in all treatment groups, however, not enough to reach the value of the control group. Mostafa *et al.* [31] reported the administration of Sil, 100 mg/kg/day decreased ALP activity significantly compared with the fibrosis group (CCl₄) to near normal values. TGF- β 1 only induced expression of TIMPs, not MMPs [40], [47], [48]. Thereby, silymarin and PM extracts have improved liver function test markers through decreased ALT, AST, and ALP activity, followed by oxidative stress markers improvement through declined MDA levels and as the elevation of GSH/GSSG ratio.

PM extracts are shown to have antioxidative activity by the prevention of hepatocyte damage due to CCl₄. The antioxidant activity may decrease HSCs activation and myofibroblast and further decrease the pro-fibrogenic cytokine (TGF- β 1) has decreased. Along with that, expressions of MMP-13 declined in all treatment groups compared to the CCl₄ group. This

shows PM extracts' antifibrotic activity on LF reversal through the improvement of all the biochemical parameters, followed by the tendency to decrease the fibrosis percentage area. The activation of HSCs by PM was not determined, which was the limitation of our study. This activation has a pivotal role in LF. Therefore, it remains unclear the mechanism of PM extract on HSCs activation, as well as TIMPs and MMPs regulation.

Conclusion

PM extract has anti-fibrosis by improving liver functions (AST and ALP), declining MDA levels, elevating GSH/GSSG ratio, decreasing TGF- β 1 concentrations, and MMP-13 expressions. However, these effects were not enough to normalize the fibrosis effect.

Data availability statement

All the data used to support this study's findings are available from the corresponding author on request.

Acknowledgment

The authors would like to thank PT Dexa Medica (Jakarta, Indonesia) for funding this study, Universitas Indonesia for International Publication grant and drh. Vetrizah Juaniantito, Ph.D. from Bogor Agricultural University, Faculty of Veterinary Medicine, Bogor, Indonesia, for helping us in immunohistochemistry work.

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