Hepatoprotective and hepatoregenerative therapeutic effects of polyherbal medicine on *Rattus norvegicus* Wistar with liver fibrosis

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ABSTRACT

Background: Chronic injury severely impairs liver regeneration through excess inflammation, scarring, and epithelial abnormalities since injured cells may shift response from proregeneration into profibrosis which leads to cirrhosis and total liver failure. An Indonesian registered polyherbal medicine, Heparmin, has been indicated as "hepatoprotector and helps healing liver disease." It combines extracts of *Curcuma xanthorrhiza, Nigella sativa, Kleinhovia hospita, Arcangelisia flava,* and *Ophiocephalus striatus*. **Objective and Methods:** In this report, using rats induced with iterative carbon tetrachloride, we examined whether administration of silymarin, L-ornithine-L-aspartate or the polyherbal medicine (at 37.8, 810, and 315 mg/kg BW/d, respectively) modulate the development of liver fibrosis. **Results and Conclusion:** Compared to other drugs, we showed that the polyherbal medicine was better as it (i) increases antioxidant enzyme activity, (ii) decreases oxidative stress (malondialdehyde) and inflammatory markers (aspartate aminotransferase, alanine aminotransferase), (iii) improves lipid profiles, (iv) normalizes liver metabolic function (bilirubin direct-indirect-total, alkaline phosphatase, albumin), (v) prevents fibrosis progression (liver histology, Ishak score), and (vi) activates hepatocyte regeneration based on BrdU staining.

INTRODUCTION

Chronic injury severely impairs liver regeneration through excess inflammation, scarring, and epithelial abnormalities [1]. Injured liver cells may shift response from repair into fibrosis which leads to cirrhosis and total liver failure [2,3]. Management of chronic injury could be directed toward avoiding the cause(s), stopping the processes, and alleviating the complications of injury, while promoting the regeneration and repair. In clinical setting, drugs have been used mostly to control symptoms of liver fibrosis complications, for example, L-ornithine-L-aspartate (LOLA), lactulose, silymarin, propranolol, octreotide, and many others [4]. Several agents with potential hepatoregenerative properties have been explored, at preclinical and clinical

trial stages, for example, captopril [5-7], losartan [5,7], oroxylin A [8], granulocyte-colony stimulating factor [9,10], and thyroid hormone [11], with mixed results and varied mechanisms. In the market, some herbal remedies are claimed to have hepatoprotective and/or hepatoregenerative effects, or beneficial to manage liver injury complications, for example, extract of *Elephantopus scaber* [12,13], *Artemisia capillaries – Lonicera japonica – Silybum marianum* [14,15], Diwu Yanggan [16], and daikenchuto [17].

In Indonesia, among many herbal medicine, a commercial registered polyherbal Heparmin combines extracts of *Curcuma xanthorrhiza*, *Nigella sativa*, *Kleinhovia hospita*, *Arcangelisia flava,* and *Ophiocephalus striatus* has an indication as hepatoprotector [18]. *C. xanthorrhiza, N.*

sativa, K. hospita, and *A. flava* have been known and used to protect liver against disease progression as alternative and complementary medicine. Their hepatoprotective properties are mainly associated with antioxidant and anti-inflammatory of the phytochemical components of the herbs [19-24]. The hepatoprotective and hepatoregenerative activities of the four herbs plus *O. striatus* as a single combination medicine, however, have not been much studied. Extract of *O. striatus* has been used traditionally to substitute albumin injection to rescue patients with hypoalbuminemia [25], including in chronic liver disease. Thus, in this study, we aimed to determine both the liver protective and regenerative properties of the polyherbal Heparmin, and compared their activities with silymarin [14] as a standard herbal medicine and LOLA [26] a mixed of two amino acids commonly used in chronic (fibrotic or cirrhotic) liver disease. In this report, the term polyherbal medicine and Heparmin were used interchangeably.

We evaluated whether the polyherbal medicine could stop the progression of liver fibrosis following iterative carbon tetrachloride (CCl_4) administration by alleviating oxidative stress, while improving the complications of injury by normalization of lipid profiles, serum bilirubin, alkaline phosphatase and albumin levels, and promoting the hepatocyte regeneration and/or liver repair.

MATERIALS AND METHODS

Materials

CCl_4 was from Fluka Chemicals (UK), silymarin (#S0292), LOLA (#O1725), goat anti-mouse IgG-AP antibody (#3562), 5-bromo-2'-deoxyuridine (#B5002), tetramisole HCl ((#L9756), BCIP/nitro blue tetrazolium ((#B1911), and Nuclear Fast Red (#N8002) were purchased from Sigma–Aldrich (St. Louis, MO), mouse monoclonal antibody anti-BrdU (#SC-32323) from Santa Cruz Biotechnology (Santa Cruz, CA), Vectamount (#H5000, Vector Labs.), and Superoxide Dismutase Assay Kit (#7500-100-K) from R&D systems (Minneapolis, MN). The polyherbal medicine Heparmin was generously supplied by Royal Medicalink (Makasar, Indonesia).

Phytochemical Screening

Qualitative assays for alkaloid, triterpenoid, saponin, flavonoid, tannin, polyphenol, volatile oil, and thymoquinone contents of Heparmin were performed as described previously [27].

Animals and Treatments

Male Wistar rats were obtained from D'Wistar Laboratory - Animal Supplier, Bandung, Indonesia. The animals were allowed access to food and tap water *ad libitum* throughout the acclimatization and experimental periods. Five groups of five Wistar rats were treated with CCl_4 followed by drug administration. Liver fibrosis was induced by administration of 40% CCl_4 in olive oil intraperitoneally at 2.5 mL/kg BW/d twice weekly during week 1-2, followed by 1 mL/kg BW/d twice weekly from week 3 to 7. Drugs were started orally from week 5 to 7: (i) 37.8 mg/kg BW/d silymarin in 0.5% carboxymethyl cellulose in distilled water; (ii) 810 mg/kg BW/d LOLA in distilled water; or (iii) 315 mg/kg BW/d polyherbal

medicine in distilled water. Normal group was given olive oil 2.5 mL/kg BW/day from week 1 to 7, whereas fibrotic control group was given CCl_4 alone, as described above. Doses used in these experiments were calculated based on the registered usual dose used for human [18]. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals. All treatments were in accordance to standard protocol agreed by Animal Ethics Committee for Health Research, Faculty of Medicine, Brawijaya University (#312/EC/KEPK/05/2015).

Sample Collection

All animals were sacrificed at the end of week 7, euthanized by CO_2 inhalation. 1 day prior, animals were given 50 mg/kg BrdU intraperitoneally. Blood was obtained by cardiac puncture and collected without anticoagulant, then centrifuged at 3000 RPM for 10 min to get serum. Livers were removed, weighed, and cut for evaluation, including for oxidative stress (superoxide dismutase [SOD] and malondialdehyde [MDA] determination) and histological analysis.

For urine collection during week 6, animals were placed individually in metabolism cages (Techniplast, Kettering, Northants, UK), with water *ad-libitum,* but not diet. Urine was collected for 12 h for urine analysis including volume measurement.

Histopathological Evaluation

Samples of the right, middle, and left lobes of the liver were fixed in 10% neutral buffered formalin solutions for 24 h. Standard histopathological techniques were followed for processing the tissue and preparation of paraffin blocks. A qualitative analysis of liver was performed on sections (4 µm thick) stained with hematoxylin and eosin for histopathological examination and Masson's trichrome using the Ishak modified HAI system [28]. The following scores were used to quantify fibrosis: 0 - no fibrosis; 1 - fibrous expansion of some portal areas with or without short fibrous septa; 2 - fibrous expansion of most portal areas with or without short fibrous septa; 3 - fibrous expansion of most portal areas with occasional portal-to-portal (P–P) bridging; 4 - fibrous expansion of portal areas with marked portal bridging (P–P as well as portal-to-central [P–C]); 5 - marked bridging (P–P and/or P–C) with occasional nodules (incomplete cirrhosis); and 6 - cirrhosis, probable, or definite. Sections were observed using an Olympus CX 21 series microscope and OlyViA image viewer analytical system. Each sample was observed at a magnification of 40× and 400×. The degree of hepatic fibrosis was expressed as the mean of five fields per section, and determined by two trained investigators who were unaware of the experimental groups.

Immunohistochemistry Evaluation

Fixed liver sections on slides were processed for immunohistochemistry evaluation. A primary mouse anti-BrdU monoclonal antibody was employed in a standard alkaline phosphatase immunostaining protocol [29]. Color was developed using tetramisole HCl plus BCIP/nitro blue tetrazolium to produce a blue precipitate. Slides were counterstained with Nuclear Fast Red, and then mounted

using Vectamount. Stained hepatocytes (polygonal cells with purple cytoplasm) were examined microscopically and images recorded. Microscopic images (at least five fields/slide) were analyzed using Image J v. 1.45 and expressed as percentage of positive cells.

Measurement of Hepatic SOD Activity

SOD activity was measured using Superoxide Dismutase Assay Kit as manufacturer described. Absorbance at 550 nm was determined for samples of liver homogenates. The percentage of inhibition for the samples was calculated by the aid of running a control with no sample under the same conditions. SOD enzyme activity was expressed as unit/100 mg protein, where one unit was defined as the amount of the enzyme inhibited absorbance increase from NBT formazan formation by 50%.

Measurement of Lipid Peroxidation

Liver oxidative stress was assessed as MDA equivalents determined by thiobarbituric acid assay [30].

Evaluation of Liver Functions

Serum samples were analyzed spectrophotometrically on an automated clinical chemistry analyzer according to manufacturer's instructions (Cobas Enzymatic Colorimetric Test, Roche Diagnostics, USA) according to the manufacturer's instructions. The following parameters were measured: Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, bilirubin direct, albumin levels and total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C).

Statistics

The results were expressed as median and range or means and standard deviation, as appropriate. Statistical analyses (GraphPad Prism v.7, GraphPad Inc., CA, USA) were performed using one-way ANOVA followed by Tukey *post-hoc* test, Kruskal-Wallis followed by Mann–Whitney, or Pearson, where appropriate, and considered significant at $P < 0.05$.

RESULTS

Phytochemical Screening

The results of phytochemical screening indicated that polyherbal medicine Heparmin contains alkaloid, triterpenoid, saponin, flavonoid, tannin and phenolic compounds, volatile oils, and thymoquinone (data not shown).

Histopathological Evaluation

Compared to normal group, chronic iterative administration of CCl_4 resulted in severe liver fibrosis (Figure 1). Histological observation revealed that Ishak scores among experimental groups differed significantly ($P = 0.0011$). Rats in CCl_4 only group reached incomplete liver cirrhosis. Administration of silymarin or polyherbal Heparmin resulted in better Ishak scores than CCl_4 only group, and statistically not different compared to the normal group ($P > 0.999$ and $P = 0.836$, respectively), thus silymarin and Heparmin both are liver anti-fibrotic agents. LOLA did not improve histological profile of liver fibrosis ($P > 0.999$).

Immunohistochemistry Evaluation

In the present study, we determined whether polyherbal medicine could induce hepatocyte regeneration. Using immunohistochemistry, we analyzed BrdU stained hepatocytes and showed (Figure 1) that compared to the normal and fibrotic groups, administration of the polyherbal medicine augmented hepatocytes proliferation ($P = 0.0025$ and $P = 0.0189$, respectively).

Measurement of Hepatic SOD Activity and Lipid Peroxidation

In this study, rats with incomplete cirrhosis showed decreased liver SOD activity up to ~50% ($P = 0.0033$), and two-fold increase of MDA ($P = 0.0003$) (Figure 2). Administration of silymarin improved both parameters, whereas LOLA and the polyherbal medicine normalized SOD activity and oxidative damage marker MDA value, thus all tested agents protected liver from oxidative stress.

Evaluation of Liver Functions

Serum AST, ALT, ALP, and bilirubin (direct, indirect, and total bilirubin) were slightly higher than normal group but statistically not all significant (AST, $P = 0.628$; ALT, $P = 0.049$; ALP, $P = 0.481$; and bilirubin, $P = 0.0897$) (Figure 2). Lipid profiles of all groups with and without CCl_4 treatment did not differ significantly (TC, $P = 0.021$; LDL, $P = 0.185$; and HDL, $P = 0.221$) (Figure 2). Serum albumin level decreased significantly in CCl_4 only, silymarin, and LOLA groups ($P = 0.0166$). On the contrary, polyherbal Heparmin normalized serum albumin in all rats treated with chronic iterative CCl_4 (Figure 2).

From urine analysis data, CCl_4 alone may reduce rats' urine volume ($P = 0.339$), and administration of silymarin or LOLA exacerbated such situation ($P = 0.0027$ and $P = 0.0254$, respectively). The polyherbal medicine neither worsened ($P = 0.467$) nor improved reduction of urine volume in CCl_4-induced fibrotic rats.

DISCUSSION

Liver fibrosis is usually preceded by inflammation and oxidative stress induced by chemicals including CCl_4. A sustained hepatic insult resulted in persistent inflammation activates fibrogenic cascade of hepatic stellate cells, progresses into cirrhosis, and eventually liver function failure. Consequently, attenuating the upstream inflammatory responses or oxidative stress has been considered as therapeutic approach to stop fibrogenesis [31]. In this study, we used CCl_4 to induce chronic liver insult and administered complementary medicinal agents to see whether administration of complementary medicinal agents prevent the progression of liver fibrosis into cirrhosis. As expected, CCl_4 administration damaged the liver, histologically and functionally, whereas treatment with medicinal agents could stop the progression of such damages.
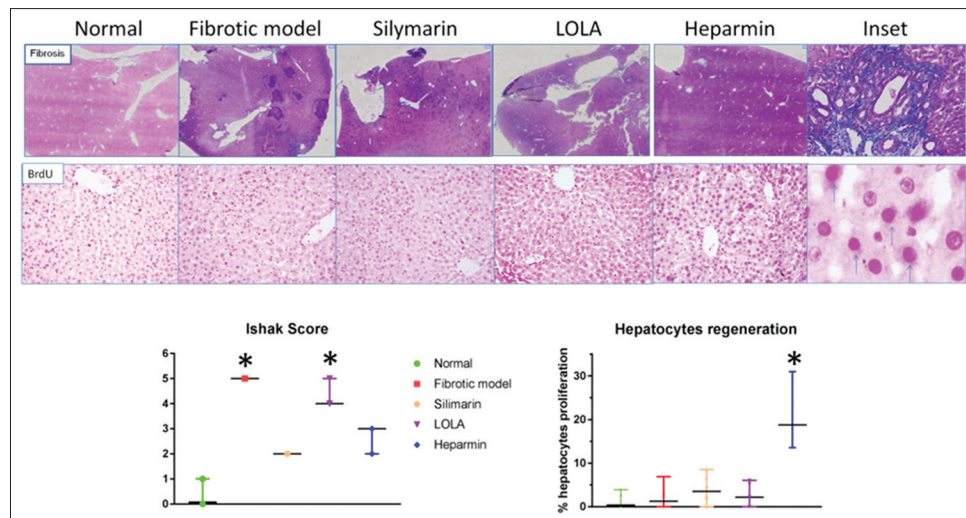
Figure 1: Polyherbal Heparmin improved liver histology. Masson's trichrome staining showed silymarin or polyherbal Heparmin administration resulted in lower score of liver fibrosis. Polyherbal Heparmin showed highest percentage of hepatocyte proliferation (BrdU signals). Original magnification was 40×. Inset images show a representative of nodule in fibrotic model group and BrdU stained cells in Heparmin group. Arrows point to representative individual BrdU-stained hepatocyte cells. Data were shown as median and 95% confidence interval. Asterisks indicate that values statistically different compared to that of normal group

Lipid profiles of all groups with and without CCl_4 treatment did not differ significantly, however, the lipid values of group treated with CCl_4 alone and group treated with silymarin were widely spread from very high to very low, outside the lipid range of normal group. The reported normal values of lipids in *Rattus norvegicus* are usually 10-54 mg/dL of TC, 7-27.2 mg/dL of LDL, and >35 mg/dL of HDL. In our study, such wide value range in silymarin group may due to hepatocellular damage, biliary dysfunction, or both, which in agreement with high serum ALP and bilirubin of the silymarin group. In clinical setting, the use of silymarin may cause elevated liver enzymes and high serum bilirubin [32]. At early stage of fibrogenesis, lipid peroxidation triggered lipogenesis following activation of sterol response element binding protein 1c regulator of genes associated with fatty acid synthesis. At later stage, when fibrosis progresses, hence, further dysfunction of hepatocytes, very LDL and HDL secretions would be compromised. Lipolysis from adipose tissues would compensate to maintain homeostasis [33]. When adipose lipid storage depleted, hypolipidemia could not be corrected, usually associated with liver cirrhosis and fatal/poor prognosis [34], or hepatobiliary dysfunctions. Results of lipid profiles suggested that Heparmin prevent hypolipidemia and biliary complications.

Serum AST, ALT, ALP, and bilirubin (direct, indirect, and total bilirubin) are usually helpful to differentiate whether the disease associated with inflammatory condition originated from hepatocellular and/or biliary damage [35]. Of note, one of rats in CCl_4 only group showed three-fold increase of ALP, and high serum bilirubin, whereas from silymarin group three of four animals showed very high serum ALP and bilirubin (higher than CCl_4 only group). Those rats with values outside range of the normal group may have biliary damage. It is not clear why silymarin associated with high serum bilirubin level. Probably silymarin interferes directly with metabolism or secretion of bilirubin, or silymarin did not protect hepatocytes or bile duct/biliary cells. Values in rats from polyherbal group were within range of the normal group. Overall, polyherbal Heparmin was a better hepatoprotector than the other complementary medicinal drugs.

From urine analysis data, administration of silymarin or LOLA may exacerbate reduction of rats' urine volume caused by CCl_4. The further reduction of 24-h urine volume may be associated with worsening of kidney function or hepatorenal syndrome. Polyherbal Heparmin neither worsened nor improved reduction of urine volume in CCl_4-induced fibrotic rats, thus safer than the comparator drugs.

Serum albumin level decreased significantly in CCl_4 only, silymarin, and LOLA groups indicated that comparator drugs failed to correct conditions associated with low albumin level. Instead, silymarin has recently been shown to bind to albumin [36]. Polyherbal Heparmin normalized serum albumin in all rats treated with chronic iterative CCl_4. The normalized albumin level suggests that Heparmin improve liver anabolic function in rats with liver fibrosis. The mechanism of such improvement may due to inhibition of oxidative stress on hepatocytes by *C. xanthorrhiza, N. sativa, K. hospita,* and *A. flava* of our polyherbal mixture [19-24], thus stopping the liver insults while regenerating liver cells. The newborn cells, particularly hepatocytes, could synthesize albumin. Our unpublished data showed that combination of *C. xanthorrhiza, N. sativa, K. hospita,* and *A. flava* without *O. striatus* extract improve serum albumin level, albeit not reach the normal range values. Normalization of albumin level may due to oral albumin intake as the *O. striatus* extract itself contains albumin 30.2%. *O. striatus* extract has been analyzed by Biotechnology Laboratory of Indonesia Institute of Science (unpublished data) and reported contains omega 3 (2.03%); omega 6 (2.11%); omega 9 (0.92%); Vitamin A (1,500 IU/100 g); B1 (0.9 mg/100 g); B6 (0.7 mg/100 g); B12 (0.76 mg/100 g); E (9.11 mg/100 g) and D3 (51.5 IU/100 g); calcium(186mg/100g);phosphorous(126mg/100g);magnesium(39
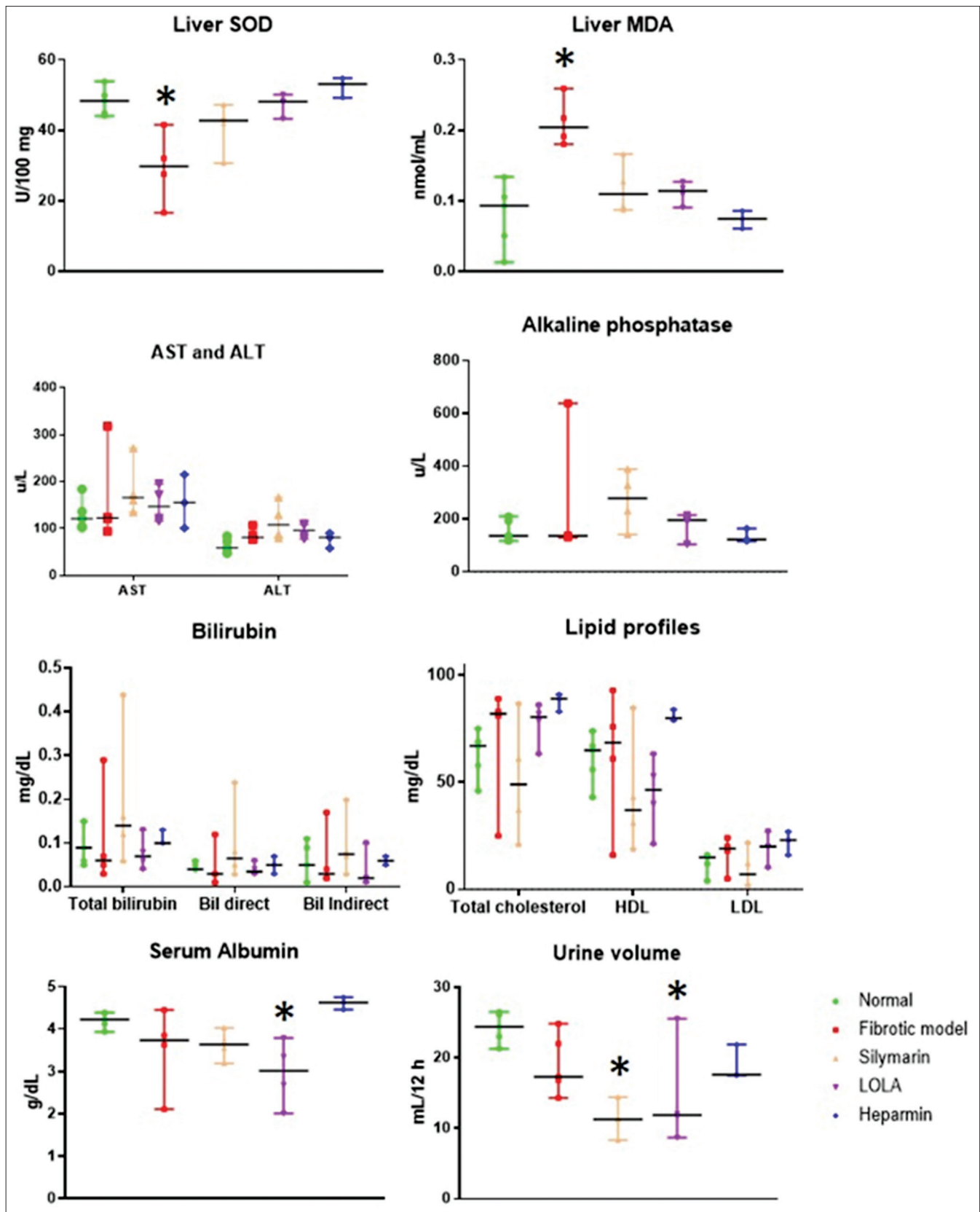
Figure 2: Tissue levels of malondialdehyde (MDA) and superoxide dismutase (SOD) activity and clinical parameters. Polyherbal Heparmin normalized SOD activity, decreased oxidative damage marker MDA, prevented biliary complications and hypolipidemia, and normalized serum albumin. Heparmin did not decrease further urine volume. All data were shown as median and 95% confidence interval. Asterisks indicate values statistically different compared to that of normal group

mg/100 g); Zn (3 mg/100 g); antibacterial immunoglobulin (2.11 IU/100); arachidonic acid (20.11 mg/100 g); and amino acids aspartate (1.04 g/100 g); glutamate (15 g/100 g); serine (1 g/100 g); glycine (1.11 g/100 g); alanine (2.11 g/100 g); leucine (1.6 g/100 g); valine (2.11 g/100 g); tryptophan (3 g/100 g); hydroxyproline (8.1 g/100 g); proline (1 g/100 g); phenylalanine (0.81 g/100 g); histidine (1 g/100 g); cysteine (1.07 g/100 g); lysine (1.46 g/100 g); and tyrosine (0.92 g/100 g). Such complex contents of *O. striatus* extract may help not only through direct provision of albumin but also through *de novo* albumin synthesis, liver regeneration, and wound healing. The *O. striatus* extract has been shown to increase proliferation and differentiation of other cell type in different clinical conditions/diseases [25].

In the present study, using BrdU immunohistochemistry, we showed that administration of polyherbal Heparmin augmented hepatocytes proliferation. It has been known that healthy liver has a remarkable capacity to regenerate after a major loss through a compensatory hyperplasia. In fibrotic liver, however, fibrogenesis is sustained by autocrine and paracrine mediators including profibrogenic growth factors, cytokines, and chemokines [37], whereas cell regeneration inhibited. Stopping the CCl_4-induced fibrogenesis through antioxidant activities of the Heparmin would inhibit the progression of fibrosis, along with supplying the necessary chemicals/nutrients for tissue healing, shift the balance from profibrosis toward proregeneration. Cell proliferation occurs to replace the lost hepatocytes. From the histological observation, reformation of normal liver architecture seems to occur following Heparmin administration. Further studies are required to know whether polyherbal Heparmin could facilitate reestablishment of liver function, including biosynthesis and detoxification activities.

CONCLUSION

Taken together, this study showed that the polyherbal medicine confers both hepatoprotection and hepatoregeneration in CCl4-induced chronic liver injury, better than silymarin and LOLA. Thus, polyherbal Heparmin could prevent disease progression and reverse fibrosis into regeneration and repair. Studies are required to see whether similar effects will be seen in different etiology such as liver injury due to alcohol or viruses.

ACKNOWLEDGMENTS

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