Effects of lactulose and silymarin on liver enzymes in cirrhotic rats.
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ABSTRACT

Silymarin (SM), a mixture of anti-hepatotoxic flavonolignans is being used for liver diseases and lactulose (LAC), a non-absorbable synthetic disaccharide were used in this study to analyze their probable synergic effects on hepatic cirrhotic rat model to see whether they cause improvement of this illness or vice versa. Upon thioacetamide (TAA) withdrawal and treatment administration. The curative effects of SM and LAC were confirmed by significant decrease of liver enzymes and malondialdehyde (MDA) levels. In SM+LAC group, the liver enzymes and malondialdehyde levels were decreased significantly compared with TAA group. All treatments were able to regenerate and trigger an enhanced regeneration. Silymarin and lactulose alone or in combination had potent curative effects and reduced thioacetamide effects induced liver damage.

Keywords: Liver Cirrhosis; Rat; Silymarin; Thioacetamide.
Introduction

Cirrhosis is a result of chronic liver injury defined by development of regenerative nodules bordered by fibrous bands that leads to portal hypertension and end-stage liver disease (Schuppan and Afdhal 2008) and in some patients to formation of portosystemic collateral veins which in high blood ammonia concentrations leads to portosystemic encephalopathy (Tarantino et al. 2009). Liver transplantation remains the only beneficial option for some patients, but pharmacological treatments that can stop progression or even reverse cirrhosis are currently being developed (Schuppan and Afdhal 2008).

Thioacetamide induces hepatotoxicity, originally used as fungicide. Earlier literature suggests that an obligate intermediate metabolite of thioacetamide which binds to proteins, forming acetylimiodolysine derivatives, is responsible for TAA-induced hepatotoxic effects (Kumar et al. 2014).

At present, one of the therapeutic strategies for liver fibrosis includes complementary and alternative medicine. Animal and clinical evidence have confirmed that any degree of fibrosis and even cirrhosis are potentially reversible by reasonable therapeutic strategies (Zhou et al. 2014).

We shouldn’t consider cirrhosis as the irreversible end stage of chronic liver disease, but rather as a stage in an active process whose advance course (progression or reversion of fibrosis) depends on the providing of proper treatment and the avoidance of further harmful effects. Appropriate treatment can stabilize cirrhosis or even reverse it (Wiegand and Berg 2013).

Milk thistle (Silybum marianum) has been the dietary supplement taken more frequently by patients with chronic liver diseases. As a result, this plant is now being more widely used in
the treatment of liver diseases such as toxic hepatitis, fatty liver, ischemic injury, cirrhosis, viral hepatitis and radiation toxicity (Luper 1998). Both milk thistle and its active ingredient silymarin are pharmacologically safe and well tolerated (Feher and Lengyel 2012). Silymarin is a lipophilic extract of the milk thistle seeds containing various flavonolignans (which silybin is the major one), has received a tremendous attention over the last decade as an herbal remedy for liver treatment over 30 years. In many cases, the antioxidant properties of silymarin are considered to be responsible for its protective actions (Surai 2015), through alleviating oxidative stress and inflammatory response (Feng et al. 2016). Silymarin is commonly prescribed in cases of viral hepatitis or cirrhosis (Del Prete et al. 2012) because it has hepatoprotective activity (Al-Sayed and Esmat 2016).

Lactulose modifies the bacterial profile in intestinal contents, increasing the lactobacilli and bifidobacteria counts, up-regulating short-chain fatty acids production (Algieri et al. 2014). Lactulose used for the treatment of hepatic encephalopathy (HE) and is effective for primary prevention of overt hepatic encephalopathy in patients with cirrhosis (Sharma et al. 2012). Lactulose decreases the blood endotoxin level and reduce liver damage. It is suggested that lactulose can prevent absorption of endotoxin from the gut and may have an effect on gut-derived endotoxemia (Hou 1991).

Liver regeneration evolved presumably to protect wild animals from catastrophic liver loss caused by toxins or tissue injury (Wu et al. 2015). Lactulose administration accelerates post hepatectomized liver regeneration in rats by inducing hydrogen, which may result from attenuation of the oxidative stress response and excessive inflammatory response (Yu et al. 2015). Long-term administration of silymarin significantly increased the survival time of cirrhotic alcoholic patients (Feher and Lengyel 2012). But in case of silymarin, Wu and Tsai et al. have stated that silymarin plays a crucial role in accelerated liver regeneration after Partial hepatectomy. It accelerates the cell cycle in the G1 to S phases (Wu et al. 2015). In the
same time Cetinkunar and Tokgoz et al. demonstrated that silymarin does not contribute to proliferative regeneration of the liver—which has very important metabolic functions—after partial hepatectomy; instead it will decrease serum levels of transaminases (Cetinkunar et al. 2015).

Therefore, for clarifying this confliction we designed present study aimed to investigate the potential curative effect of silymarin, lactulose or a combination of both against TAA-induced liver cirrhosis in rats.

**Materials and Methods**

**Materials, Drugs and chemicals**

Formaldehyde solution was purchased from (Dr. Mojallali Industrial chemical complex Co., Iran). Lactulose was purchased from (Alborz Daru, Iran), ketamine %10 and xylazine %2 were purchased from (alfasa, Holland), silymarin from the fruit of *Silybum marianum* extract was purchased from (SIGMA-ALDRICH, USA), and thioacetamide, hydrochloric acid fuming and 2- thiobarbituric acid were purchased from (Merck, Germany) and trichloroacetic acid was purchased from (Central drug house (p) Ltd., India).

**Animals**

All the animal experiments were conducted in accordance the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and approved by the Bu- Ali Sina research committee. Forty Wistar albino male rats weighting 210-275 g were used (Pasture Institute, Karaj, Iran). The animals were kept in an agreeable environment in transparent plastic cages under standard laboratory conditions (light period 7.00 a.m. to 7.00 p.m., 22±2 °C, and humidity, 55±5%) with free access to food and water, they were acclimatized for at least 2 weeks before the start of the study. The experiment lasted for 26 weeks.
**Induction of liver cirrhosis**

Liver cirrhosis was induced according to the method of Li et al. (Li et al. 2002) and Laleman et al. (Laleman et al. 2006). Thirty-two male Wistar rats were administered by TAA with drinking water for 18 consecutive weeks (124 days). Eight rats, drinking natural water, were randomly chosen as the healthy controls. In the 12th week, three rats were sacrificed to monitor the development of cirrhosis (Figure 5.). In cirrhotic rats only 2 rats were died. After 18 weeks TAA water was withdrawn and replaced by tap water or treatment according to rat groups.

**Preparation of silymarin**

Experimental diet was prepared by 50 mg/kg/day silymarin (Alshawsh et al. 2011), mixed into their chow before forming it into pellets weekly for 8 weeks (Toyoda-Hokaiwado et al. 2011).

**Experimental design**

Rats were divided randomly into five groups: (Table 1) Control group, TAA group: TAA-induced cirrhotic group, Silymarin (SM) group: hepatic cirrhosis treated with silymarin (50 mg/kg/day in diet), Lactulose group: hepatic cirrhosis treated with lactulose (2 g/kg/day in drinking water), SM + LAC group: hepatic cirrhosis treated with silymarin (50 mg/kg/day in diet) and lactulose (2 g/kg/day in drinking water) for 8 weeks (Figure 1.).

**Endpoints to evaluate hepatic injury and regeneration**

The following endpoints were evaluated: (1). Liver injury: Serum alanine amino transaminase (ALT) = Glutamic Pyruvic Transaminase (SGPT), aspartate amino transferase...
(AST) = Glutamic Oxal acetic Transaminase (SGOT) and alkaline phosphatase (ALP). (2). Liver regeneration: Calculating liver index (LI) according to the following formula: liver index = liver weight (g) / bodyweight (g) × 100%.; (3). Oxidative stress: Malondialdehyde (MDA) (Amin et al. 2012; Gu et al. 2011a; Gu et al. 2011b).

Collection of blood samples and liver tissues

At the end of the experiment, all rats were fasted for 12 h then 5-8 ml whole blood samples were taken directly from the heart under ketamine 10% - xylazine 2% anesthesia. The blood samples were centrifuged at 10000 rpm for 20 min for serum separation. All animals were sacrificed and their liver dissected out. Right lobe of the liver was cut and fixed in 10% formalin for histopathological examination.

Biochemical analysis

Biochemical analysis in serum were done for determination ALT, AST and ALP, serum total bilirubin, total protein (Pro), albumin (ALB) and gamma glutamyl transferase (GGT) levels (Roche Hitachi 912 Chemistry Analyzer, Japan) using commercial kits (Man, Iran) for total protein (Parsazmun, Iran) for others to evaluate the liver injury degree.

Preparation of liver homogenates

At the end of the experiment, all of the rats were decapitated and the liver was rapidly isolated and weighted. This tissue was subsequently homogenized in cold 0.9% normal saline. The homogenate was kept for lipid peroxidation assay.

Measurement of malondialdehyde
MDA measurement has been used frequently to measure lipid peroxidation. The lipid peroxidation assay was performed by determining the MDA, which is the end product of lipid peroxidation, and reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex that has a peak absorbance (A) at 535 nm. A mixture of trichloroacetic acid (TCA), TBA, and HCl were added to 1mL of serum, and the mixture was heated for 45 min in a boiling water bath. After cooling and centrifuging it at 1000 g for 10 min, the cleared supernatant absorbance was measured using SECOMAM S.750 Spectrophotometer at 535 nm. The level of TBARS was calculated by: 

\[ \text{C (M)} = \frac{A}{1.65 \times 10^5} \] (Rajaei et al. 2013).

**Histopathological examination**

Formalin 10% fixed liver tissues were embedded in paraffin, and then they were cut into 5 µm thick sections. The slides were stained with the conventional hematoxylin and eosin (H&E) and masson's trichrome protocol, mounted with neutral resin, and examined with optical microscopy.

**Assessment of necroinflammatory activity grades and staging:**

Liver samples stained with H&E and trichrome masson were assessed for total and individual activity parameters guided by ishak's histological activity index (Ishak et al. 1995). Classified according to the following parameters: confluent necrosis (0-6), periportal or perisepetal interface hepatitis (piecemeal necrosis) (0-4), portal inflammation (0-4), focal (spotty) lytic necrosis, apoptosis and focal inflammation (0-4), and architectural changes, fibrosis and cirrhosis (0-6). Additional features which were noted but not scored: bile-duct inflammation, damage and proliferation and steatosis, mild, moderate or marked.
Statistical analysis

All data were presented as mean ± SD. The analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 22. Statistical analyses were conducted by One-way Analysis of Variance (ANOVA) and followed by Least Significant Difference (LSD) analyses to compare various groups with each other. Statistical analyses for scoring of histological parameters were done by Mann-Whitney U from two independent sample tests. Significant differences were analyzed at $P < 0.05$.

Results

Mortalities and liver function

During the whole experimental period, there were minor rat deaths. A total of three rats died: one rat died from the normal group; two rats died in making cirrhotic model. The mortality rate was not more than 7.5% which makes the survival rate of the model 92.5% (Table 1). Levels of serum ALT and AST were assayed as markers of liver injury. thioacetamide produced liver cirrhosis characterized by a significant increase in serum ALT and AST levels compared with control group. Although thioacetamide have been withdrawn for 8 weeks but in TAA group serum ALT and AST levels was high and significant in AST comparing with control group ($P < 0.05$), while treatment with treatments, decreased AST and ALT levels. Silymarin administration decreased AST and significantly decreased ALT level compared with a TAA group ($P < 0.05$). Lactulose and both silymarin and lactulose significantly decreased liver enzymes ($P < 0.01$ and $P < 0.05$) as shown in (Figure 2, A and B). The plasma ALP level of the thioacetamide group increased significantly ($P < 0.001$) when compared with the control group (Figure 2C). ALP levels was significantly decreased in SM, LAC and SM+LAC groups by comparing with TAA group.
Liver dysfunction improved upon thioacetamide withdrawal and treatment administration, with decrease of ALT, AST and ALP it almost resume to the levels as that of the healthy rats (Figure 2A, B and C). In the silymarin group, liver dysfunction improved upon thioacetamide withdrawal with decrease of ALT, AST and ALP and resume to the levels lower as that of the healthy rats in ALT. In lactulose group, recovery of liver dysfunction noted to improve after thioacetamide withdrawal as compared with the controls and with the decrease of ALT and AST resume to the levels lower as that of control rats but ALP did not resume to the levels as that of control rats. In SM + LAC group, recovery of liver dysfunction resume to the levels of control rats in AST and lower of control rats in ALT but still ALP is higher than control group. There was a significant increase ($P < 0.05$ and $P < 0.01$) in serum total bilirubin of lactulose group (2 g/kg/day) when compared with control, TAA and SM groups. Therefore there are no significant differences between TAA, SM and SM + LAC groups and the control group, as shown in (Table 2).

It seems that after 8 weeks liver dysfunction improved upon thioacetamide withdrawal and liver ability to synthesize the albumin and other proteins resume to the levels as that of the healthy rats so there are no significant differences between all groups and the control group, except for SM+LAC which are significantly lower than TAA group as shown in (Table 2) which it could be explained by albumin’s long half-life.

Table 3 indicates the effect of silymarin and lactulose and their combination on the GGT levels in the serum of control and experimental groups of rats. This marker enzyme is significantly ($P < 0.05$) increased in TAA and SM + LAC animals when compared with control animals. (Table 2)

**Effect of silymarin and lactulose on lipid peroxidation**
MDA was measured to evaluate oxidative stress. MDA, as an oxidation product of lipid peroxidation injury, increased non-significantly in serum after exposure to thioacetamide (Figure 3). Treating with lactulose or silymarin or their combination non-significantly lowered MDA changes, respectively (Figure 3).

**Effects of silymarin and lactulose and their combination on the liver index**

After 18-weeks of thioacetamide administration and 8-weeks of treatment, the weight of rats in the TAA group was non-significantly less than rats in control group (Table 3). The weight of rats in LAC and SM+LAC groups non-significantly increased compared with control and TAA groups. Weight of liver in LAC and SM + LAC groups were significantly higher than liver weight in control group ($P < 0.05$).

The liver index used to assess liver damage. LIs of LAC and SM+LAC rats increased and remained at significant higher levels to that of the control ($P < 0.05$), except for SM group which wasn’t remarkably higher than control group (Figure 4). Liver index in the thioacetamide group increased compared with those of normal group, while silymarin group was decreased compared with thioacetamide group but wasn’t significant. The results showed that hepatomegaly alleviated after the treatments (Figure 4.).

**Making cirrhotic model**

Thioacetamide-induced cirrhosis was established at week 18 after administration thioacetamide, confirmed both macro and microscopically (Figure 5.). Thioacetamide increased amount of collagen deposition in liver tissue stained with masson's trichrome significantly.

**Effect of silymarin and lactulose on hepatic cirrhosis**
The liver tissue has been examined by light microscope for histopathological studies. For determination of liver injury, histological examinations have been done on sections stained with H&E and masson stain. For clarifying the extent of hepatic cirrhosis induced by thioacetamide, masson’s trichrome had been used for liver sections staining. The amount of collagen deposition has increased significantly by thioacetamide in liver tissue, which stained with masson trichrome. Cirrhotic pattern of affected liver was micro nodular, macro nodular, abnormal polyhedron architecture collagen fiber hyperplasia and liver cell necrosis in portal area that extended into the surrounding liver lobules, this leads to criteria of hepatic cirrhosis. In control group of rats we observed normal architecture of liver cells, containing uniform granulated cytoplasm and small nuclei. Collagen fibers are thin and delicate (Figure 5).

**Effect of silymarin and lactulose and their combination on hepatic necroinflammatory activity**

Examination of TAA group showed moderate to severe necroinflammatory activity with average score of (8.86) with marked confluent necrosis and moderate apoptosis and focal inflammation with mild portal inflammation. Treatment with silymarin and lactulose alone or in combination healed the liver from hepatotoxic effects of thioacetamide reflected by increase of necroinflammation to (9.29), (9.71) and (9.71), respectively but there wasn’t any significant difference between SM, LAC and SM+LAC groups and TAA group as shown in (Figure 5 and 6).

**DISCUSSION**

Key to the first step was to making a cirrhotic animal model. Prolonged exposure to thioacetamide has been reported to result in liver cirrhosis. The toxin affects both perivenular and periportal areas. This model develops macro nodular cirrhosis with portal hypertension,
the instauration of overt hyper dynamic circulation, associated with the typical histological,
biochemical and hemodynamic changes observed in man (Laleman et al. 2006), fibrosis
remains stable for weeks after thioacetamide withdrawal (Abraldes et al. 2006). In this study,
cirrhotic rat model was successfully developed via drinking thioacetamide water for 18 weeks
and was confirmed by histological evidence.
Analysis of histopathology (H&E staining and masson staining) showed that structure of rat
liver in the control group was integrity, the central vein was visible, liver cells were arranged
radially and neatly, and lobular contour was clear. However, in liver cirrhosis group the
structure of rat liver was abnormal, hepatic lobular architecture was destroyed, pseudo lobule
is formed, collagen deposition was around the portal region and connects to each other to
form nodules and distort the architecture, and extensive fibrosis with broad septa was
observed (Figure 5). These results definitely indicated that the rat model of liver cirrhosis was
successfully established.
Thioacetamide-induced hepatotoxic effects characterized by confluent necrosis, piecemeal
necrosis, apoptosis and focal inflammation and focal (spotty) lytic necrosis on tissue levels
(Figure 5) which reflected in our study by significant elevation of serum liver enzymes
(Figure 2) and GGT (Table 2) as well as increased collagen deposition and fibrotic areas
significantly. The statistically significant increase of the GGT concentrations is due to the
metabolic consequence of TAA induced dysmetabolism leading to severe hepatic steatosis, of
which GGT is a marker. These results are in accordance with many investigations on
thioacetamide - induced liver fibrosis and cirrhosis in rats (Gu et al. 2011a; Gu et al. 2011b;
Kawai et al. 2012; Laleman et al. 2006).
In order of interfere avoidance of thioacetamide with our observation endpoints,
thioacetamide has withdrawn before the treatment, which was based on the following
considerations. In the present study, the intention was to investigate regeneration of the
cirrhotic liver after treatment. The existence of thioacetamide, a hepatic toxin, would interfere, which would obscure treatment induced regeneration. In general, when used in combination with treatment, it would change treatments effect. Because the treatment period was as long as 56 days, thioacetamide continuously feeding up to 56 days would make most of the rats develop cholangiocarcinoma (Abraldes et al. 2006; De Minicis et al. 2013; Moreira et al. 1995) or die of thioacetamide toxicity. Upon thioacetamide withdrawal, hepatic cirrhosis continuously resolve, but persisted up to 120 days, and liver regeneration significantly decelerates (Gu et al. 2011b). Hence, we have enough time to make treatment on cirrhotic rats. In the literature, withdrawal of thioacetamide prior to the study was also acceptable as in Prasad study on cirrhotic rats (Prasad et al. 2010).

Thioacetamide had been confirmed to induce hepatic injury and trigger proliferation of liver, noticeably increased the ratio of liver to overall body weight as presented in LI, which used to scrutinize potential changes in the liver size, in comparison with control rats (Figure 4). Thioacetamide, a potent centrilobular hepatotoxicant, undergoes a two-step bio activation mediated by microsomal CYP2E1 to thioacetamide sulfoxide (TASO), and further to thioacetamide -S, S-dioxide (TASO2), a reactive metabolite that initiates cellular necrosis (Chilakapati et al. 2005). Thioacetamide provokes a remarkable increase in the activity and amount of total mRNA, as well as in the size of the nucleoli of liver cells. During the biotransformation of thioacetamide both cytochrome P450 and flavin-containing monooxygenase (FMO) reduce dioxygen to superoxide anion, which is catalyzed to form hydrogen peroxide. Therefore, biotransformation of thioacetamide precedes oxidative damage associated liver injury. This had been already implicated in the disappearance of tetraploid hepatocytes and the presence of an increase in MDA in liver cells following thioacetamide administration (Constantinou et al. 2007). By considering the role of oxidative stress in disease, malondialdehyde assay is the most generally used test. Malondialdehyde is
one of the several products formed throughout polyunsaturated fatty acids breakdown induced by radicals (Lefevre et al. 1997). Silymarin and lactulose alone or in combination protected the liver from oxidative stress caused by thioacetamide as they decreased significantly the elevated serum MDA (Figure 3). In this study, oxidative stress after cirrhosis manifested as increased lipid peroxidation. After silymarin treatment silymarin acts as a scavenger of free radicals, so it decreases oxidative stress. The antioxidative property of hydrogen might play a main role in the molecular mechanism of lactulose-enhanced liver regeneration.

GGT is one of the important markers of liver injury, and their elevation discloses cholestasis and bile duct necrosis. When liver cells are damaged, the serum AST and ALT are released into the blood from the cells. Therefore, ALT and AST are two of the most reliable markers of necrosis or hepatocellular injury. Data from the present study have demonstrated that SM and LAC and SM+LAC groups protected liver from tissue inflammation, which exhibited an obvious decrease in serum markers of liver function brought back these enzymes to near routine level by possibly preserving the functional integrity of the hepatocytes, showing their defense action against thioacetamide -induced hepatotoxicity as compared to the model control (TAA group) (Figure 2). Silymarin decreased serum GGT level but it didn’t happen in LAC and SM+LAC groups (Table 3). Treatments decreased fibrosis scores in LAC and SM+LAC groups compared to TAA group although it wasn’t significant due to the short time of observations. (Data not shown) (Figure 5) indicate effectiveness of silymarin, lactulose or both in the recovery of liver function after cellular damage. Amin et al. and Fadillioglu et al., respectively (Amin et al. 2012; Fadillioglu et al. 2010) demonstrated that silymarin and lactulose significantly reduced the increased ALT and AST caused by thioacetamide. These are evidence for the important role of silymarin and lactulose alone or in combination in protecting cells from lipid peroxidation and restoring cell membrane function so they have
hepatocurative effects. In accordance to these results, observations indicated that an increase in serum liver enzymes levels in TAA group may be result of massive necrosis and late stage of fibrosis (Gu et al. 2011a; Gu et al. 2011b). The elevation of the serum activities of ALP and GGT indicated the induction of cholestasis (Kawai et al. 2009) while decreased serum liver enzymes activities in SM and LAC groups may be due to recovery, as decreasing serum liver enzymes activities in hepatic injury occur with recovery, given that lactulose has a function of reducing blood ammonia levels, and hydrogen produced by anaerobic organisms was the key for its therapeutic effects.

We found that silymarin, lactulose and their combination have dramatic effects on MDA, as serum MDA significantly decreased. The probable antioxidant mechanisms of silymarin are direct scavenging free radicals and chelating free Fe and Cu, preventing free radical formation, improving the integrity of mitochondria’s electron transport chain in stress conditions, maintaining an optimal redox balance in the cell, activating vitagenes, and providing additional protection in stress conditions, affecting the microenvironment of the gut, have hepatoprotective effects, decreasing inflammatory responses, is an emerging mechanism of silymarin protective effects in liver toxicity and various liver diseases (Surai 2015). The exposure of hepatocytes to free fatty acids in the context of dyslipidemia, resulting in increased ROS production and mitochondrial damage (Tarantino et al. 2010), rapid fermentation of lactulose into organic acids at rates exceeding colonic buffering capacity to reduce formation of toxic fatty acids and ammonia from amino acids, polypeptides, and blood in the colon maybe is the probable mechanism of lactulose (Bonnkn 1990).

CONCLUSIONS
In conclusion, our findings of this study are: (1) As proved by serum markers for liver function and histological findings, silymarin, lactulose and silymarin & lactulose combination could protect against thioacetamide-induced liver cirrhosis in rats. (2) Silymarin & lactulose combination should be useful for the treatment of liver cirrhosis at physiological concentrations. (3) Silymarin, lactulose, and silymarin & lactulose combination exert significant curative effects against thioacetamide-induced oxidative stress and liver damage by augmenting host antioxidant defense mechanisms. (4) Silymarin & lactulose combination curative effects are due to increase of antioxidative effects as well as lowering the extent of lipid peroxidation.

**Declarations**

**Ethics approval and consent to participate**

All the animal experiments were conducted in accordance with the guideline and approved by the Bu-Ali Sina research committee.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.
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Authors' contributions

Mozhgan Ghobadi Pour: Done the entire project.

Naser Mirazi: Project supervisor and corresponding author.

Hojjatollah Alaei: Supervisor assistant, project done in Dr. Alaei’s lab and he provide all what was needed.

Shirin Moradkhani: Proofreading the article.

Ziba Rajaei: Supervised the MDA test.

Alireza Monsef Esfahani: Professor in Pathology histopathological expert.

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Collaborating author names:


References:


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Table 1- Experiment design and mortalities.

<table>
<thead>
<tr>
<th>Experiment groups</th>
<th>Mortalities</th>
<th>Subgroups(7 rats)</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy rats(8 rats)</td>
<td>1/8</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Cirrhotic rats</td>
<td>2/32</td>
<td>Negative Control</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Silymarin</td>
<td>50mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Lactulose</td>
<td>2000mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Silymarin &amp; Lactulose</td>
<td>50mg/kg/day +2000mg/kg/day</td>
</tr>
</tbody>
</table>

Table 2- Effect of thioacetamide, silymarin, lactulose alone or in combination on some serum parameters.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Total bilirubin (mg/dl)</th>
<th>ALB (g/dl)</th>
<th>Total protein (g/dl)</th>
<th>GGT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.227 ± 0.048</td>
<td>3.141 ± 0.434</td>
<td>6.200 ± 1.184</td>
<td>3.86 ± 3.185</td>
</tr>
<tr>
<td>TAA</td>
<td>7</td>
<td>0.197 ± 0.011</td>
<td>3.498 ± 0.202</td>
<td>7.004 ± 0.629</td>
<td>8.14 ± 5.640 *</td>
</tr>
<tr>
<td>SM</td>
<td>7</td>
<td>0.198 ± 0.052</td>
<td>3.088 ± 0.433</td>
<td>6.085 ± 0.915</td>
<td>6.14 ± 5.336</td>
</tr>
<tr>
<td>LAC</td>
<td>7</td>
<td>0.285 ± 0.050 * ## $</td>
<td>3.078 ± 0.531</td>
<td>5.912 ± 1.469</td>
<td>8.14 ± 3.671</td>
</tr>
<tr>
<td>SM + LAC</td>
<td>7</td>
<td>0.225 ± 0.023 ¥</td>
<td>2.915 ± 0.760 #</td>
<td>5.590 ± 1.708 #</td>
<td>10.86 ± 7.603 *</td>
</tr>
</tbody>
</table>

Note: Values represent mean ± SD. Values are significantly different (*P < 0.05 vs. Control), (#P < 0.05 vs. TAA), (## P < 0.01 vs. TAA), ($ P < 0.05 vs. SM), (¥ P < 0.05 vs. LAC).
Table 3- Effects of treatments on body and liver weights of rats for 8 weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg/day)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>291.29 ± 13.671</td>
<td>8.045 ± 0.535</td>
</tr>
<tr>
<td>TAA</td>
<td>-</td>
<td>287.57 ± 16.582</td>
<td>9.320 ± 1.538</td>
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<tr>
<td>SM</td>
<td>50</td>
<td>284.57 ± 13.661</td>
<td>9.140 ± 1.503</td>
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<td>LAC</td>
<td>2000</td>
<td>297.71 ± 15.108</td>
<td>10.195 ± 2.039 *</td>
</tr>
<tr>
<td>SM + LAC</td>
<td>50 + 2000</td>
<td>299.00 ± 15.384</td>
<td>10.422 ± 1.808 *</td>
</tr>
</tbody>
</table>

Note: Results were presented as the mean ± SD. (*P < 0.05 vs. Control).
Figure captions

Figure. 1. Schematic representation of the experimental design used for the study.

Figure. 2. Changes of liver function after thioacetamide withdrawal and treatment administration. Effect of (thioacetamide for 18 weeks), silymarin (50 mg/kg daily) and lactulose (2 g/kg daily) alone or in combination on liver functions by serum AST(A), ALT(B) and ALP (C) activities. Values: (Mean ± SD). * P < 0.05 vs. Control group. ** P < 0.01 vs. Control group. *** P < 0.001 vs. Control group. # P < 0.05 vs. TAA group. ## P < 0.01 vs. TAA group. ### P < 0.001 vs. TAA group.

Figure. 3. Effect of thioacetamide (for 18 weeks), silymarin (50 mg/kg daily) and lactulose (2 g/kg daily) alone or in combination on serum MDA levels. Values: (Mean ± SD). # P < 0.05 vs. TAA group. ## P < 0.01 vs. TAA group.

Figure. 4. Changes of the liver index (LI). In all groups, LIs were higher in all than that of healthy rats. Values: (Mean ± SD). * P < 0.05 vs. Control group.

Figure. 5. The changes in liver photomicrographs of rats. The livers were stained with H&E and Masson’s trichrome. Central vein (CV), Hepatic cords (H), Portal Vein (PV), periductal Fibrosis (F) and Fatty Change in a diffuse manner (FC). (→) hepatocyte necrosis, destruction of lobular architecture, and extensive fibrosis. H&E and Masson’s trichrome, magnification 100×.

Figure. 6. Effect of thioacetamide (daily for 18 weeks), silymarin (50 mg/kg daily) and lactulose (2 g/kg daily) alone or in combination on (A) liver necroinflammatory score and values: (Mean ± SD). ** P < 0.01 vs. Control group.
Figure 1

- **Group 1:** Drinking water
- **Group 2:** Drinking thioacetamide
- **Group 3:** Drinking thioacetamide, followed by Silymarin (50 mg/kg/day)
- **Group 4:** Drinking thioacetamide, followed by Lactulose (2 g/kg/day)
- **Group 5:** Drinking thioacetamide, followed by Silymarin (50 mg/kg/day) + Lactulose (2 g/kg/day)

Time of sacrifice:
- Drinking water
- Drinking thioacetamide
- Drinking or feeding treatment

Thioacetamide withdrawal
Figure 2A

Figure 2B

Figure 2C
Figure 3

![Graph showing Serum MDA (µMol/L) levels for CONTROL, TAA, SM, LAC, and SM+LAC groups.](https://mc06.manuscriptcentral.com/cjpp-pubs)
Figure 4

Liver Index (%)

CONTROL  TAA  SM  LAC  SM+LAC

* *
Figure 5

1. Gross
2. Healthy
3. Cirrhotic (12 weeks)
4. Cirrhotic (18 weeks)
5. Silymarin
6. Lactulose
7. Masson
8. Healthy
9. Cirrhotic (12 weeks)
10. Cirrhotic (18 weeks)
11. Silymarin
12. Lactulose
13. H&E
14. CV1
15. FC
16. CV1
17. FC
18. CV1
Figure 6

![Bar graph showing necroinflammatory scores for different groups: Control, TAA, SM, LAC, SM+LAC.](image)

- **Control** group has the lowest necroinflammatory score, significantly different from other groups.
- **TAA**, **SM**, and **LAC** groups have higher scores, with **SM+LAC** showing the highest.

Significance levels: **p < 0.01**, ***p < 0.001*.