INSULIN RESISTANCE OR NONALCOHOLIC STEATOHEPATITIS: WHICH IS THE TARGET OF SILYBUM MARANUM?

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Summary

We tried to answer whether the crude extract of *Silybum marianum* is able to prevent the incidence of insulin resistance in rats with nonalcoholic steatohepatitis (NASH). NASH was induced in Male N-Mary rats using a methionine/ choline deficient (MCD) diet. MCD diet led to grade 1 liver steatosis, inflammation and ballooning degeneration establishing the presence of NASH. In crude extract treated-rats the histopathological findings revealed normal hepatocyte status in 80% of rats, lipoprotein profiles and the sera ALP, AST, ALT and GGT activities had significantly improved relative to those not receiving the extracts. The antioxidant defense capability was also enhanced; however the insulin resistance status remained unchanged. Although *S. marianum* extract could reverse the adverse effects of MCD diet on some of the biochemical markers, but it was not capable of attenuating the insulin resistance status.

Key words: *Silybum marianum*, methionine/ choline deficient diet, nonalcoholic steatohepatitis, oxidative stress, insulin resistance.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of liver disease from benign simple fatty liver disease to non-alcoholic steatohepatitis (NASH), which is associated with fibrosis and progression to cirrhosis. Fat, diabetic, hyperuricemic and hypertriglyceridemic individuals are considered to be the vulnerable population of NAFLD (1). The prevalence of NAFLD is as high as 20%, with about 2% of the population having the more serious case of NAFLD called NASH (2). Diet abnormalities like protein calorie malnutrition and methionin/choline deficient (MCD) diet have been considered as two of the causative factors of NASH (3). MCD diet, mainly due to inhibition of phosphatidylcholine biosynthesis, tends to build up lipids in hepatocytes by preventing VLDL synthesis and export. Thereby fat will not be eligible to leave the liver and consequently accumulates in the liver (4). Increased intrahepatic levels of fatty acids provide the appropriate environment for lipid peroxidation particularly if the system is under the condition of oxidative stress (5). This might account for disease progression from steatosis to steatohepatitis and cirrhosis due to enhanced levels of lipid peroxidation cytokine and Fas ligand induction.

Silybum marianum (Asteraceae) is used for more than 2000 years to treat a range of liver and gallbladder disorders, including hepatitis, cirrhosis, jaundice, and to protect the liver against toxins. As a medicinal plant, *S. marianum* is well tolerated and exhibits low adverse effects (6). Efficacy of *S. marianum* in preventing membrane absorption of toxins via avoiding cell surface binding and suppressing the membrane transporting system for some toxins has been documented (7). *S. marianum* capability in treatment of NASH is checked here with a glimpse on its outcomes on insulin resistance status.

Materials and methods

Animals and experimental protocols

Male N-Mary rats, obtained from Pasteur institute (Tehran, Iran), weighting 176-217 g were housed in groups of five at 60 ± 5% relative humidity and 22 ± 2 °C with a 12 h light/dark cycle. All rats had free access to water and food *ad libitum*. Nonalcoholic steatohepatitis was induced by administration of a MCD diet as described by Ustundag *et al* (8). There were 6 rats in group A receiving normal diet for 13 weeks. Fifteen rats were on MCD diet for an 8 week period to induce NASH. At the end, five rats were sacrificed to assay the NASH status and the remaining ten rats were divided into two groups randomly. In group B, rats continued to receive the MCD diet and in group C rats were given MCD diet along with *S. marianum* extract (equivalent to 1g seeds powder /kg body weight/day) by intragastric administration. After 4 weeks, rats of all three groups were fasted overnight and then sacrificed under diethyl ether anesthesia. All experiments were carried out according to the guiding principles of care and use of experimental animals approved by state veterinary administration of University of Tehran. Blood and liver tissue samples from the rats of all groups were taken, each liver was immediately washed with normal saline, blotted on a filter paper, weighted and cut into small pieces and homogenized in Tris–HCl buffer (0.025 M, pH 7.5) using a homogenizer to give a 10% (w/v) liver homogenate. The homogenate was then centrifuged at 12,000 rpm for 15 min and the supernatant was aliquoted and frozen until use. The serum was separated from each blood sample and then stored at −70 °C pending biochemical analyses.

Measurement of body weight and diet intake

Diet intakes were measured daily and the body weights were recorded before starting the extract administration up to the end of the experiment.

Plant material

Seeds of *S. marianum* were collected from Hamadan (Iran) during summer. Seeds were air-dried, protected from direct sunlight, and finely grounded. The powder was kept in a closed container at 4°C.

Preparation of *S. marianum* extract

Fifty grams of the seed powder was initially defatted with 200 ml petroleum ether twice at room temperature (RT) for overnight, and then it was extracted four times with ethanol %96 at room temperature. The accumulated extract was concentrated under reduced pressure on a rotary evaporator to a volume of 50 ml. The aliquoted samples were stored at -20°C.

Biochemical analyses

The sera levels of Albumin (Alb), Glucose, Triglycerides (TG), High density lipoprotein cholesterol (HDL-C) and Low density lipoprotein cholesterol (LDL-C) were determined using enzymatic kits (Pars Azmoon, Iran). Alkaline phosphatase (ALP), Aspartate aminotransferase (AST) and Alanin aminoteransferase (ALT) activities were assessed as a measure of hepatic cell damages using the corresponding commercial kits (Pars Azmoon, Iran). Total protein content of each liver homogenate was estimated by the Lowry's method (9) and the liver antioxidant status was measured in terms of the activities of glutathione reductase, according to Jollow et al (10), and glutathione peroxidase according to Paglia et al (11). The amount of reduced glutathione and malondialdehyde (MDA) were determined according to Jollow and Drapel et al (10), respectively.

Superoxide dismutase (SOD) activity assay

SOD activity was measured based on the extent of inhibition of amino blue tetrazolium formazan formation in the reaction mixture of nicotinamide adenine dinucleotide, phenazine methosulphate and nitroblue tetrazolium (NADH–PMS–NBT), according to the method of Kakkar et al (12). The assay mixture contained 0.1mL of the supernatant, 1.2 mL of sodium pyrophosphate buffer (pH 8.3; 52 mM), 0.1mL of phenazine methosulphate (186 μM), 0.3 mL of nitroblue tetrazolium (300 μM). The reaction was started by addition of 0.2 mL of NADH solution (750 μM). After incubation at 30°C for 90 s, the reaction was stopped by addition of 0.1mL of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 mL of *n*-butanol. Colour intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as that amount of enzyme which caused 50% inhibition of NBT reduction/mg protein.

Glutathione peroxidase (GPx) activity assay

Liver GPx activity was assayed in a cuvette containing 0.89 mL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1mM NaN_3, 0.2 mM NADPH, 1 U/mL GSH reductase and 1mM GSH. Each liver homogenate (10 μL) was added to make a total volume of 0.90 mL. The reaction was initiated by the addition of 100 μL of 2.5 mM H_2O_2, and the conversion of NADPH to $NADP^+$ was monitored with a spectrophotometer at 340 nm for 3 min. The GPx activity was expressed as nmoles of NADPH oxidized to NADP+/ (min. mg protein), using a molar extinction coefficient of 6.22×10^6 ($cm^{-1}M^{-1}$) for NADPH (11).

Glutathione reductase (GR) activity assay

The liver GR was assayed in a reaction mixture containing 0.99 mL of 100 mM potassium phosphate buffer (pH 7.0), 1.1 mM $MgCl_2$, 5 mM oxidized glutathione (GSSG) and 0.1 mM NADPH. The liver homogenate (10 μL) was added to trigger the NADPH conversion reaction. Changes in absorbance were monitored at 340 nm for 5 min at 25°C. The specific enzyme activity was expressed as nmol NADPH oxidized to $NADP^+$/ (min. mg protein) with 6.22×10^6 ($cm^{-1}M^{-1}$) as the molar extinction coefficient of NADPH (11).

Histopathological examinations

The liver tissue samples were fixed in 10% formalin and then the paraffin blocks were prepared. The sections from blocks were stained with hematoxylin-eosin (HE) and masson trichrom. The histopathological evaluations were performed blindly by an expert pathologist by means of a scoring system proposed by Kleiner et al (13): steatosis (0-3), lobular inflammatory changes (0-3) and hepatocyte ballooning (0-2). Fibrosis was evaluated as absent/present.

Insulin resistance status

Serum insulin was assayed by an ELISA kit specific for rat insulin (Kits Crystal Chem. Inc., USA). The physiological index of insulin resistance used was homeostasis model assessment of insulin resistance (HOMA-IR) (14, 15), assessed from fasting glucose and fasting insulin concentrations using the following formula: HOMA-IR = [fasting insulin (ng/mL) × fasting glucose (mg/dL)] / 22.5.

Statistical analyses

All values are expressed as mean ± SD. The significance of differences between the means of the tests and the controls were calculated by unpaired Student's t-test and p values less than 0.05 were considered significant.

Results

Histopathological findings

Normal histology was observed in group A, however, in group B pathological damages were observed. MCD diet has led to grade 1 liver steatosis, inflammation and ballooning degeneration. In group C, these factors consciously declined to grade 0 in 80% of the rats (Table 1). Based on data shown in Table1, fibrosis was found neither in group B nor among the rats of group C (Fig 1).

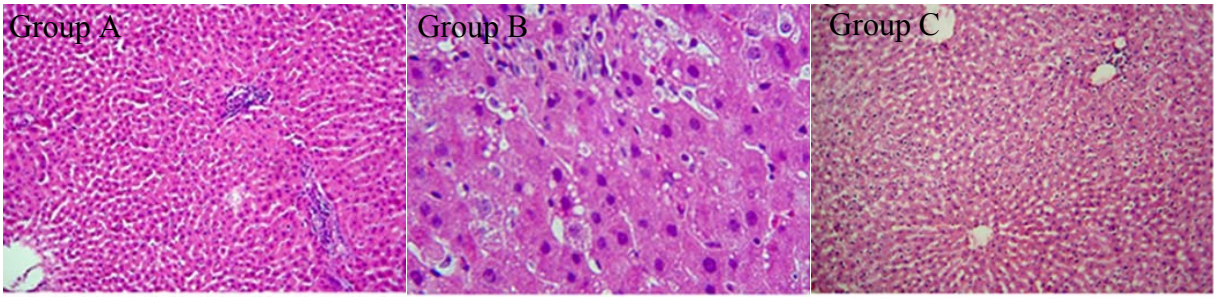
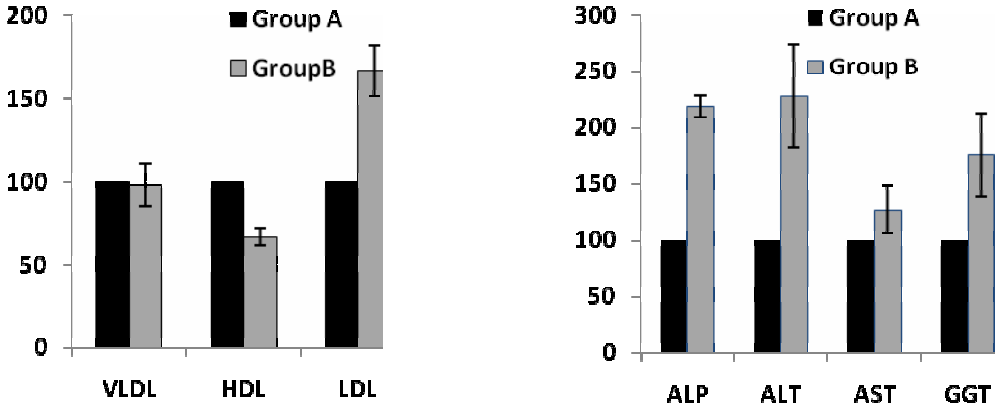


Fig1. Histological evaluation of rats' livers. Group A (normal diet), Group B (MCD diet), Group C (MCD diet+ *S. marinum*). (Group A): normal liver histology. (Group B): macro and microsteatosis, ballooning degeneration and lobular inflammation. (Group C): marked reduction in steatosis, ballooning degeneration and lobular inflammation.

Effect of S. marianum extract on serum lipoprotein profile and hepatic enzymes

The MCD diet feeding caused 67% elevation in LDL level among group B rats. However, the levels of VLDL and HDL were reduced by 2 and 33%, respectively. In group C, receiving the S. *marianum* extract, LDL concentration dramatically reduced (29% with $P<0.05$) relative to group B. The sera levels of HDL and VLDL increased in group C compared to group B by about 33% and 83%, respectively. The ALP, AST, ALT and GGT sera levels were determined to assess the liver function. In group B the levels of ALT, ALP, AST and GGT were all increased by 128, 119, 27 and 76 %, respectively relative to group A rats. Treatment with the plant extract (group C) significantly reduced the sera levels of aforementioned enzymes by 45, 51, 24 and 49% ($P<0.05$), respectively relative to group B, implying that the plant has protecting effect against the MCD diet-induced liver damages (Fig 2).

a)



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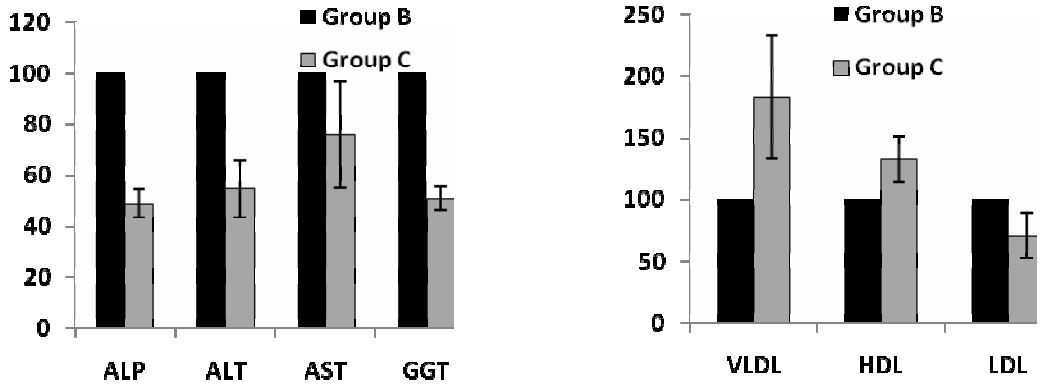
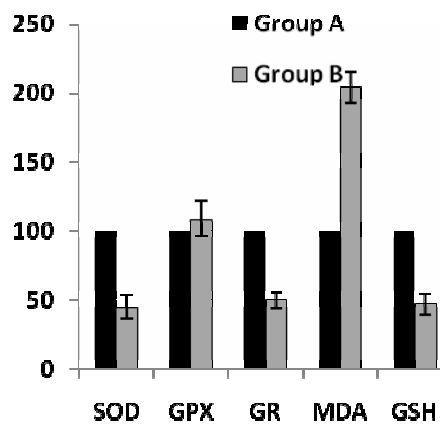


Fig2. Effect of *S. marinum* on serum lipoprotein profile and hepatic enzymes. Group A (normal diet), Group B (MCD diet), Group C (MCD diet + *S. marinum*). The results are the mean ± SD (n=5) of triplicate measurements of each sample. (a) LDL concentration dramatically increased in group B by 67% ($P<0.05$). However, the levels of HDL and VLDL decreased by 33 and 2 %, respectively ($P<0.05$) compared to Group A. Group B revealed 128, 119, 27 and 76% elevation respectively in ALT, ALP, AST and GGT comparing to group A. (b) LDL concentration dramatically declined in group C by 29% ($P<0.05$). However HDL and VLDL concentrations increased by 33 and 83%, respectively ($P<0.05$) compared to Group B. Significant reduction is made in hepatic enzymes in group C as 45, 51, 24 and 49% respectively in ALT, ALP, AST and GGT comparing to group B.

Effect of Silybum marianum extract on the liver oxidative status

In order to determine the effect of *S. marianum* extract on the liver antioxidant status the activities of SOD, GPx and GR enzymes, as the main antioxidant enzymes of the system were verified. The *S. marianum* extract increased the activity of SOD, GPx and GR by 61%, 41% and 64%, respectively in group C compared to group B. Meanwhile, the extent of MDA production, as an index of the extent of lipid peroxidation, was also evaluated in all groups. The results indicated 104% elevation in the MDA level in group B rats relative to group A rats. Treatment with the *S. marianum* extract made a 36% reduction in the level of MDA in group C rats compared to group B rats as shown in Fig 3.

a)



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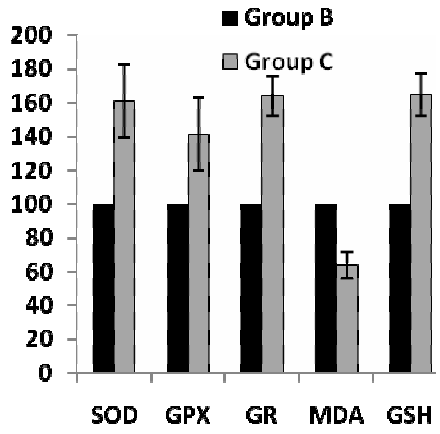

Fig3. Effect of *S.marinum* extract on the liver oxidative status.Group A (normal diet), Group B (MCD diet), Group C (MCD diet + *S. marinum*). The results are the mean ± SD (n=5) of triplicate measurements of each sample.(a) The activities of SOD and GR enzymes in group B evidently decreased by 55 and 50%, respectively compared to group B ($P<0.05$). GPX revealed 9% elevation in the same groups. (b) Administration of *S.marinum* extract increased the activities of SOD, GPx and GR enzymes by 61, 41 and 64%, respectively in group C relative to group B ($P<0.05$).

Effect of S. marianum extract on the insulin resistance status

The physiological index of insulin resistance was assessed from fasting glucose and insulin concentrations using HOMA- IR. Although the MCD diet led to 100% elevation in insulin resistance in group B compared to group A, treatment with the *S. marianum* extract made no improvement in the extent of insulin resistance in term of HOMA- IR, among the rats of group C relative to rats of group B (Fig4). These data clearly indicate that oxidative stress is not the sole cause of insulin resistance.

Discussion

Non-alcoholic fatty liver disease (NAFLD) is considered as the most common root of chronic liver diseases worldwide. Non-alcoholic steatohepatitis (NASH) is the progressive type of NAFLD which can lead to cirrhosis, hepatocellular carcinoma (HCC), liver dysfunction and ultimately to death (Powell, 1990; Clark, 2003). From the histological point of view, NAFLD is accompanied by the liver accumulation of macrovesicular lipids (16).

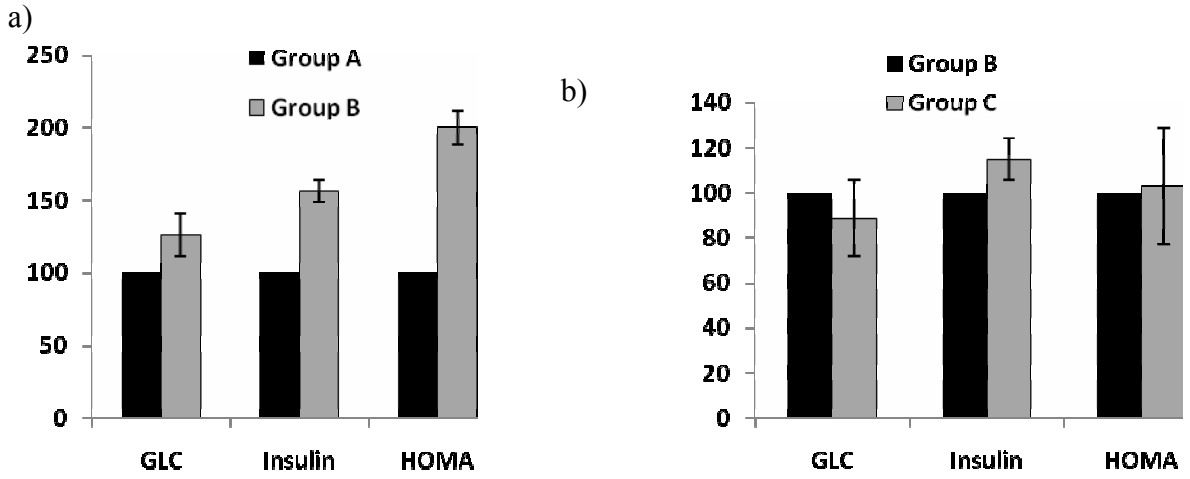

Fig4. Effect of *S. marinum* extract on the insulin resistance status. Insulin resistance is presented here as HOMA index. Group A (normal diet), Group B (MCD diet), Group C (MCD diet + *S. marinum*). The results are the mean ± SD (n=5) of triplicate measurements of each sample.(a) Insulin resistance is elevated by 100% in group B relative to group A.(b) Treatment by *S. marinum* extract made no considerable difference in group C compared to group B.

Although the hepatoprotective action of *S. marianum*, through avoiding cell surface binding and suppressing the membrane transporting system for some toxins, is documented in the literature (17), we were interested to evaluate whether the *S. marianum* extract is also capable of attenuating the extent of insulin resistance associated with NASH.

As it was anticipated, the MCD diet caused NASH associated elevation in insulin resistance. The administration of the *S. marianum* extract to rats with NASH caused noticeable improvements in the liver function which was evident through the sera levels of aminotransferases. In a 4 week treatment period, the extract caused a significant drop of 45% in serum ALT level ($P<0.001$). Serum AST level was also decreased by 24% ($P<0.001$). The extract feeding has also enhanced the hepatic levels of some of the antioxidant enzymes such as SOD, GPX and GR confirming the high antioxidant activity of the plant extract. Histological changes also revealed a dramatic improvement among the extract-treated rats.

While the reactive oxygen species (ROS) are considered as the major mediator in the etiology of NASH, it is postulated that the *S. marianum* extract probably acts by neutralizing free radicals leading to decreased lipid perioxidation; increased protein synthesis (thereby promoting regenerating processes) and stabilization of cell membranes. It has also been reported that the active components of *S. marianum* might bind to the estradiol binding site situated on a subunit of RNA polymerase I. This might stimulate the synthesis of ribosomal RNAs, possibly restoring structural proteins and damaged enzymes (18, 19, 20).

Despite the significant valuable protecting effects of the *S. marianum* extract on the liver histology, function in terms of liver aminotransferases (ALT and AST) and antioxidant defense enzymes, our data clearly indicated that no improvements in the levels of insulin resistance has occurred fallowing one month of plant extract administration. These data clearly indicate that although oxidative stress is involved in the onset and propagation of NAFLD to NASH and the involvement seems to be reversible, however, the insulin resistance associated with NASH is either an irreversible outcome of NASH or other causative mechanisms might be involved in its etiology. Further molecular investigations are certainly needed to disclose these predictions.

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