Investigation of the Therapeutic Effect of Silymarin, the *Silybum marianum* Medicinal Plant Extract, in an Empirically Induced *In Vitro* Model of Non-Alcoholic Fatty Liver Disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common digestive disorder that can cause some additional liver problems. Nowadays developing efficient therapies for the prevention and control of hepatic diseases is crucial. Recently, replacing synthetic drugs with natural herbal remedies has been more favored due to their ever-growing demand and public acceptance. Our goals in this study were the experimental induction of non-alcoholic fatty liver disease in the hepatocyte cell line and the evaluation of the therapeutic efficiency of silymarin, the Silybum marianum medicinal plant extract. The HepG2 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 2 mM GlutaMAX. The effect of various concentrations of fructose in the induction of the fatty liver condition was assessed. Subsequently, the diseased hepatocytes were treated with different concentrations of silymarin. The therapeutic effect of silymarin was evaluated by fluorescent microscopy of the cells stained with a lipid-specific fluorescent dye and by quantifying the hepatic enzymes. Cultivation of HepG2 cells for seven days in the medium containing 30 mM fructose was found appropriate in the induction of the NAFLD condition. The lipid-specific staining, as well as the measurement of the hepatic enzymes, revealed the therapeutic efficiency of the 200 and 250 µM concentrations of silymarin. The experiments showed that Nile Red stain, silymarin, and DMSO were not notably cytotoxic. A high-energy diet provided by the added fructose in the culture medium was effective in resembling the NAFLD condition in HepG2 cells *in vitro*. Furthermore, silymarin was found to be effective in curing the NAFLD condition in a time and dose-dependent manner.

Keywords: NAFLD, Milk Thistle plant, HepG2, Silibinin, Nile red stain, Hepatic enzymes

Introduction

The mortality rate caused by gastrointestinal diseases is alarmingly high, both in Iran and around the world, making them a significant public health concern. The liver, as a digestive system organ, along with its role in the regulation of carbohydrates and phospholipids metabolism, lipid absorption,

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defense against microbes, and many other functions is in charge of detoxification and excretion of waste metabolic products which make it vulnerable to various acute and chronic diseases. Fatty liver, hepatitis B, and C are widespread liver disorders. Nowadays, appropriate vaccinations for hepatitis B and effective treatments for hepatitis C are available. But, the prevalence of fatty liver diseases is rapidly increasing among individuals because of a sedentary lifestyle and consuming unhealthy foods. Non-alcoholic fatty liver disease was introduced first by Mr. Ludwig and his colleagues in 1980. Accumulation of fat (usually triglycerides) in the cytoplasm of hepatic cells to an extent of more than 5 to 10% of the liver mass and with no relation to alcohol consumption is known as non-alcoholic fatty liver disease (NAFLD) (Yu and Keeffe, 2002). NAFLD is a high-prevalence liver disease, and on average, 4 to 35 percent of people around the world are suffering from it, and the reported number for Iran is about 30%. Insulin resistance, abdominal obesity, diabetes, high blood pressure, and unhealthy diets may result in NAFLD. The combination of cell damage, delayed detection, and inadequate treatment can result in severe and permanent conditions such as liver fibrosis and cirrhosis, often necessitating a liver transplant for patients (Yu & Keeffe, 2002; Reddy & Rao, 2006). Fatty liver is the third most common reason for liver transplantations worldwide. Although it can be successfully treated in its initial phases, diagnosis is often challenging due to vague symptoms. Despite some ambiguity in its pathogenesis, two hypotheses are attempting to explain the cause of the illness: the first one suggests that fat accumulation in the liver that may ultimately result in liver steatosis arises after insulin resistance as the primary cause of the disease; the second hypothesis believes in oxidative stresses and inflammatory mediators as the initiating factors in liver injuries (Zolfaghari et al., 2014).

The lack of suitable biomarkers in fatty liver disease diagnosis is a meaningful problem. Slight increases in Aspartate transaminase (AST) and Alanine aminotransferase (ALT) levels, the liver enzymes, usually occur in hepatic disorders, but sometimes the enzyme levels stay normal (Willebrords et al., 2015). Also, some studies claim that the level of gamma-glutamyl transferase (GGT) may increase in the serum of sick individuals (Pratt & Kaplan, 2000). Biomarkers related to inflammation, fibrosis, oxidative stress, and Liver cell death have occasionally been used in NAFLD diagnosis. Nowadays, no exact medicine is available for NAFLD treatment, and the therapeutic regimen mainly comprises some general advice, including physical activities, weight control programs, and the use of hepatoprotectives and lipid-lowering drugs such as the receptor blockers of angiotensin and antioxidants (Pratt & Kaplan, 2000.

A significant challenge for the global medical community is introducing novel effective drugs to treat and prevent NAFLD. Digestive disorders, kidney damage, and other discomforts are potential side effects of chemical drugs. Therefore, using natural compounds with lower side effects, higher safety, and affordability takes precedence.

In recent years, medicinal plants, or in other words, the use of non-toxic natural ingredients of plant origin are attracting attention for their potential to prevent and cure different diseases. Many scientists have been intrigued by the potential of dietary natural compounds, particularly flavonoids, in treating a range of illnesses. Indigenous medicine has a rich history of utilizing plants for treating liver diseases. The medicinal properties of Silybum marianum (Milk Thistle plant) have made it a popular choice for alleviating digestive ailments. This medicinal plant has plenty of flavonoid compounds with remarkable antioxidant activity, collectively called silymarin (Toklu et al., 2007; Gallo et al., 2003). There are pieces of evidence indicating the effectiveness of silymarin in liver problems. The protective role of silymarin in preventing and impeding the progression of fatty liver disease can be through reducing the destructive effects of oxidative stress and preventing the accumulation of fat in the liver and its dysfunction. According to the literatures, silymarin can play an important role in the treatment of liver diseases by inhibiting glycogenesis, gluconeogenesis, modulation of inflammation, apoptosis, and fibrogenesis. Silymarin exhibited effective antioxidant and antimicrobial activity in animal model studies and holds promise for extended utilization in humans down the line. Although there are numerous reports about the effects of silymarin on liver diseases, the published assays to investigate the effects of this drug directly on hepatocyte cell lines are limited and do not show a good agreement. In the present study, we intended to find a suitable model to investigate non-alcoholic fatty liver disease, so we attempted *in Vitro* stimulation of lipid droplet accumulation in hepatic cells using a high-carbohydrate regimen. Then, we further studied the therapeutic potency of silymarin in the developed NAFLD model, investigating the effect of various silymarin concentrations and different exposure times.

Material and methods

NAFLD induction in hepatocytes

HepG2 hepatocyte cell line was obtained from the cell bank of Pasteur Institute. Dulbecco's Modified Eagle Medium/ nutrient mixture F-12 (DMEM/F12) medium enriched with 10% fetal bovine serum (FBS), 2mM Glutamax, 1% penicillin/streptomycin, and 15 mM sodium bicarbonate was used as a basal medium for cultivation of the hepatocytes in an atmosphere with 5% CO_2 concentration at 37 °C. About 3 x 10^4 cells from the second passage of cells were seeded in each well of a 12-well plate and were used to conduct the experiments. Different concentrations of 25 to 50 mM fructose in the basal medium were used as the culture medium to stimulate the formation of lipid droplets in hepatocytes.

Confirmation of fatty hepatocytes

The progress of fatty liver occurrence in hepatocytes was investigated through two different approaches: measuring the ALT and AST enzymes' levels in the culture medium and fluorescence microscopy of fatty cells stained with lipid-specific Nile red dye. In order to see if any lipid droplet has been formed, the cells need to be fixed first with 4% paraformaldehyde and then stained

by the Nile red as a lipophilic fluorescent dye. A 10% stock solution of Nile red in dimethyl sulfoxide (DMSO) was prepared, and the working solution was made by 1000 times dilution of stock solution in 150mM sodium chloride solution. First, the hepatocytes were washed twice with phosphate-buffered saline (PBS) and fixed at room temperature with 20 minutes of exposure to 4% paraformaldehyde added to each well. Then, the cells were rinsed twice for 5 minutes with PBS, and one ml of the working solution was added to each well. After 15 minutes, the cells were washed with phosphate-buffered saline for 5 minutes three times. Fluorescence microscopy was used to see the stained lipid droplets.

Treating fatty cells with silymarin

After lipid droplet formation was confirmed, silymarin (Sigma, CAS No.: 65666-07-1) in 25 to 500µM concentrations was applied for 1 to 7 days to evaluate its potential for healing the fatty cells. The previously described double approaches, measuring the hepatic enzymes and lipid-specific staining, were used with the same procedure to evaluate the effectiveness of silymarin. Fluorescence microscopy was used to visualize lipid droplets in cells, and the enzyme levels were measured using a Roche/Hitachi autoanalyzer in the pathobiology laboratory of Dey Hospital, Tehran.

Toxicity test

The possible toxicity of DMSO, Nile red dye, and silymarin in the test conditions was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay method. The viability percentage of the treated cells was calculated based on the light absorption intensity of the developed color at 570 nm using the formula as follows.

Viability %: =(Optical absorption at 570 nm for treated cells)/(Optical absorption at 570 nm for non-traeted cells)×100

All tests were performed in triplicate, and the analysis of variances test was used for statistical analysis of the obtained data using GraphPad software (Version 9.0).

Results

HepG2 cells grew as attached clumps in the DMEM/F12 culture medium with a doubling time of 48 hours.

Toxicity tests

A toxicity test on DMSO as the silymarin solvent revealed it was non-toxic in the concentrations and exposure times used in the experiments. Also, the Nile red dye used to stain lipid droplets showed non-toxicity even in a two-day-long exposure time (Figure 1).

The silymarin toxicity tests revealed that the exposure times equal or longer than four days and three days to silymarin concentrations equal or higher than 300 and 350µM, respectively, are considered hepatotoxic, the 200 and 250 µM concentrations for five days were found to the appropriate conditions of silymarin treatment (Figure 2).

Microscopic examination of NAFLD-induced and silymarin-treated cells

Fluorescence microscopy of hepatocytes nourished with different concentrations of added fructose showed that seven-day cultivation of cells within the culture medium containing 30mM fructose was sufficient to form visible intracellular lipid droplets (Figure 3). The hepatocytes were

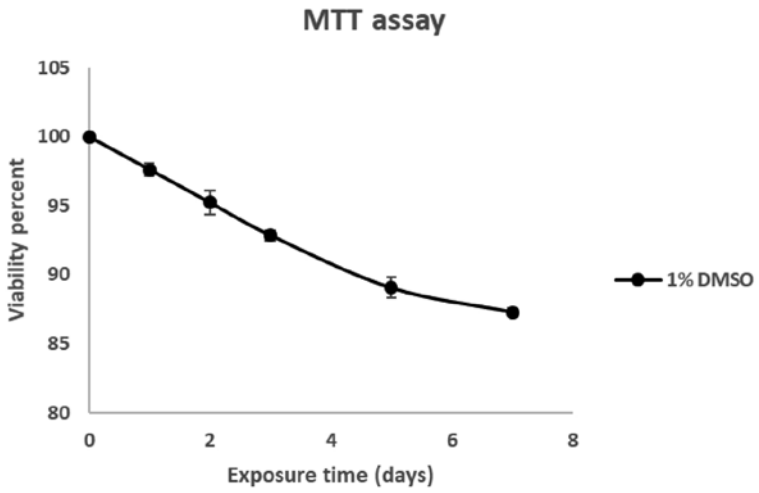

Fig. 1. Toxicity of DMSO on HepG2 cells evaluated by MTT assay

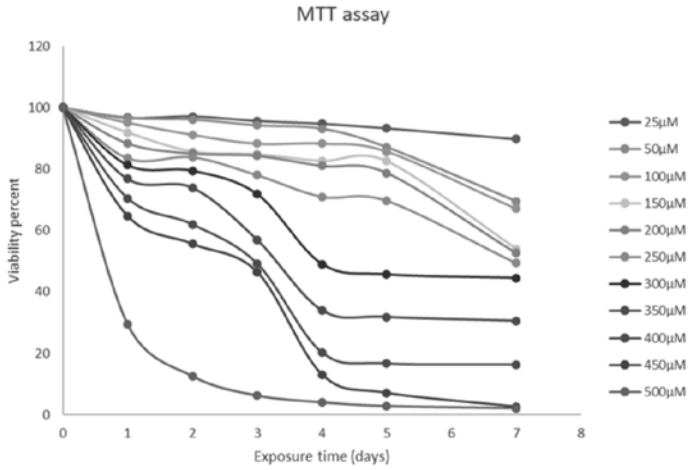

Fig. 2. Toxicity of different concentrations of silymarin on HepG2 cells determined by MTT assay

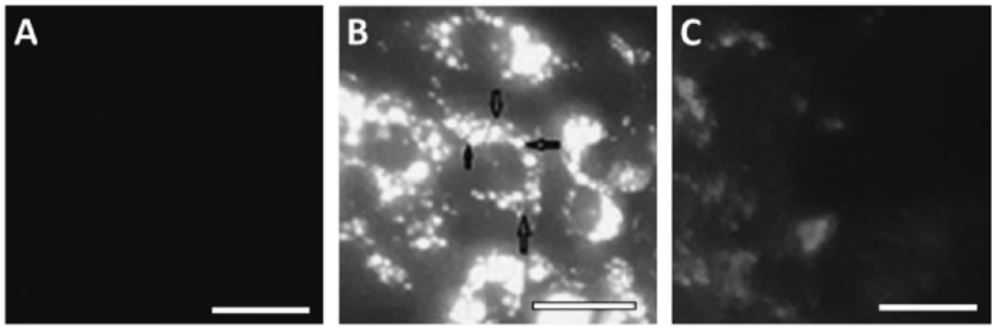

Fig. 3. Fluorescent microscopy images of HepG2 cells; A: normal cells, B: NAFLD-induced cells, C: fatty cells treated with silymarin; arrows indicate the accumulated lipid droplets. NAFLD was induced by cultivation in a 30 mM fructose-containing culture medium for seven days. The fatty cells were treated with 200 µM silymarin for five days. The scale bars are equal to 25 micrometers

selected to be cultivated in the 30 mM fructose-containing culture medium for seven days to induce NAFLD to be used later in the evaluation of silymarin efficiency in NAFLD treatment.

Considering the silymarin toxicity test results, NAFLD-induced cells were treated with 200µM silymarin for five days, and the subsequent fluorescent microscopy revealed a remarkable reduction in intracellular lipid droplets in the treated cells (Figure 3).

Changes in the hepatic enzymes' level

The effects of NAFLD induction and silymarin treatment at 200 and 250 µM concentrations for five days were investigated on the level of hepatic enzymes. The results of AST and ALT enzyme measurement and their ratio confirmed the efficiency of the high-energy diet in inducing fatty liver in hepatocytes. Also, silymarin in both the used conditions was effective in improving the functionality of hepatic cells based on the hepatic enzymes' level. The 250 µM silymarin concentration was capable of reducing the AST and ALT enzymes' levels and elevation of the AST to ALT ratio to almost the same level of normal cells with no significant differences (Figure 4).

Discussion

Extensive studies have demonstrated that many plants and spices possess valuable medicinal and biochemical properties, including the ability to act as antioxidants and anti-inflammatory agents. They are also involved in anti-malignant and anti-mutagenic activities. Meanwhile, the incidence of chronic illnesses is globally on the rise, and discovering medicinal compounds derived from natural sources to treat and prevent diseases attracts scientists' attention. Several reports suggest that incorporating antioxidant compounds and anti-inflammatory substances into treatment plans can effectively combat these diseases (Shukla and Singh, 2007).

In-vitro sudies show the cytotoxic effect of secondary metabolites of *Silybum marianum* on cancer cell lines in vitro. This influence may be due to their impact

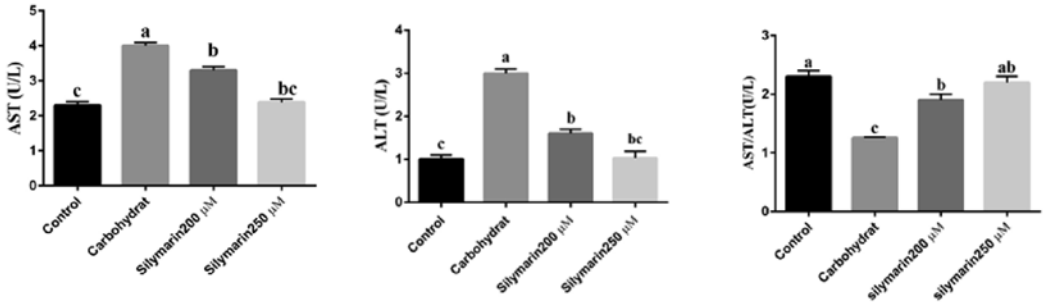

Fig. 4. Comparison of AST, ALT, and AST/ALT levels in normal HepG2 cells, NAFLD-induced cells (The carbohydrate group in which the cells were nourished with 30 mM fructose-containing culture medium for seven days to induce NAFLD), and the fatty cells treated for five days with either 200 µM or 250 µM concentrations of silymarin ($P \leq 0.05$)

on various metabolic reactions, such as energy metabolism and protein synthesis, or even by causing genetic alterations. Active components of silymarin, the *Silybum marianum* plant extract, mainly consist of silibinin, which accumulates mostly in seeds and sometimes to a lesser extent in the root. The impact of these compounds on chronic diseases, including chronic digestive disorders, has been frequently reported. Sobolová et al. (2006) investigated the effect of silibinin on liver fibrosis and cirrhosis caused by liver exposure to iron andfound that adding silibinin to the diet of mice successfully prevented the damage induced by injected iron and attributed this effect to its antioxidant properties. During an experiment, Ghosh et al. (2011) induced lipid peroxidation and alteration in the membrane lipids composition in the rats' liver by carbon tetrachloride administration in high doses. The subsequent administration of silymarin revealed a hepatoprotective effect that was attributed to the antioxidant properties of the silymarin flavonoid components. In a 6-month study on 17 patients with hepatitis, Ferenci et al. (2016) showed that silymarin at the dose of 140 mg twice a day led to the restoration of normal liver function. Ghasemi et al. (2013) demonstrated in a study the beneficial effects of silibinin administration to enhance liver, heart, and gall bladder functions. According to Tiwari and colleagues (2011), silibinin can trigger apoptosis in breast cancer cells through its impact on the mitochondrial pathway. Pesakhov et al. (2010) observed that silibinin along with some other flavonoids can activate caspase 8 and 9 proteins in myeloid cancer cells.

Abascal and Yarnell (2003) showed that the elevated level of transaminase caused by hepatic injuries and the malondialdehyde level as an indicator of oxidative stresses can be reduced by silibinin treatment in patients suffering from hepatic cirrhosis. In a study by Federico et al. (2017), it was discovered that daily use of silymarin for four weeks in patients with an apparent elevation in the serum level of hepatic enzymes significantly decreased the elevated level of AST and ALT enzymes. They further proved the hepatoprotective properties of silymarin through histological examinations.

Despite numerous studies carried out on the therapeutic effects of *Silybum marianum* extract on liver diseases, the findings have yet to be presented in a comprehensive and organized manner. Fatty liver disease is currently recognized as a prominent health concern for society. Although the number of individuals affected by this condition steadily becomes more significant, there hasn't been an assured therapy or medication that the public has deemed trustworthy and approved until this point. So, it is crucial to identify an appropriate solution for treating and preventing this disease. Recent studies have looked into the efficacy of herbal compounds in treating fatty liver, but their findings are limited to specific cases and cannot be generally applied. During a 12-week research project on fatty liver, Fukumitsu et al. (2010) incorporated 30 grams of crushed flax seeds into the daily diets of 50 patients. The results indicated a significant reduction of ALT, AST, GGT, Tumor Necrosis Factor-α (TNF-α), High-

sensitivity C-reactive protein (hs-CRP), body mass index values, glucose and insulin levels elevated due to fatty liver disease. Askari et al. (2014) reported that a daily intake of 750 mg of cinnamon for 12 weeks could significantly recover the fatty liver-related indications. Research conducted by Pezeshki et al. (2016) revealed that the inclusion of green tea, rich in catechin, in a daily diet for 12 weeks effectively combats fatty liver disease. The importance of preventive and therapeutic efforts in controlling the disease cannot be omitted, as it can result in severe challenges for society that demand immediate attention. This study directly assessed the impact of silymarin on human cells at various concentrations and time intervals.

The lack of advancement in identifying effective compounds for fatty liver disease could be attributed to the absence of a suitable model for the disease simulation. Multiple investigations seeking to explore the nature of non-alcoholic fatty liver disease have used fatty acids to induce fatty liver in hepatocyte cell lines, specifically HepG2 cells (Ricchi et al., 2009; Cao et al., 2016). Ricchi et al. (2009) examined the accumulation of lipids and their effect on steatosis and apoptosis in liver cells by treating WRL68, HuH7, and HepG2 cell lines with two fatty acids, palmitic and oleic acid and stained them with Oil Red to confirm lipid droplets formed in the cells. To simulate fatty liver disease, Im et al. (2016) subjected HepG2 cells to different concentrations of fatty acids for 24 hours as part of a trial investigating the therapeutic effect of *Dolichos lablab* extract. Nile Red and Oil Red staining was utilized to visualize the buildup of lipid droplets in cells. The current research focused on the process of lipid accumulation in hepatocyte cells and investigated the role of silymarin in ameliorating fatty liver conditions through two consecutive phases.

In the first phase of the present study, fructose was used to nourish cells with a high-energy diet. The results validated the effective induction of non-alcoholic fatty liver disease in HepG2 cells by the high-energy diet. The diseased cells were subjected to Nile red staining, which selectively stains triglycerides and makes it possible to see the lipid droplets formed within the cells using a fluorescent microscope. It was observed that administering the high-energy diet led to the accumulation of triglycerides and the formation of lipid droplets. In the second phase of the study, it was seen that the lipid droplet content was effectively diminished by silymarin treatment (Fig. 3). Reduction and elimination of lipid droplets in the cells treated with silymarin implies an improvement in the health condition of the hepatocytes. Additionally, measuring the levels of AST, ALT, and their ratio as the hepatic enzymes through the two study phases to assess the hepatic cell functionality provided us with further insights to analyze the impact of fructose-induced fatty liver and its treatment with silymarin.

Quantifying appropriate biomarkers in serum is a non-invasive strategy to qualify liver complications in NAFLD patients. This approach offers advantages such as feasibility, and reproducibility of test results in various laboratory settings (Castera et al., 2019). An increase in the blood level

of hepatic enzymes does not exclusively represent a liver-associated complication. They may increase in other disorders, such as muscle damage, but deviation in their levels in the hepatocytes culture medium compared to healthy hepatocytes culture is undoubtedly an immediate indication of the hepatocytes' performance. That may also represent the severity of the malfunction. As a typical indicator of hepatic disorders, the AST level increased in the present study due to the induced fatty liver condition. Similarly, the ALT level, as a more specific indicator of NAFLD, showed a remarkable elevation (Birjandi et al., 2016). Different factors can cause impairment in the function of hepatic cells, subsequently leading to abnormally high levels of both the mentioned enzymes. By analyzing their pattern of changes and considering histological indicators and serum biomarkers, liver complications are diagnosed, and their severity can be determined. Furthermore, the AST/ALT ratio is applied in defining several indices such as NAFLD liver fat score، BARD score، NAFLD fibrosis score، Palekar model, and Hepatic steatosis index, which are employed by a variety of designed models aimed at diagnosing and assessing the liver conditions (Kotronen et al., 2009; Harrison et al., 2008; Angulo et al., 2007; Palekar et al., 2006; Lee et al., 2010).

As a rule, a lower AST/ALT ratio is linked to a higher liver fat content (Kotronen et al., 2009). The AST/ALT ratio is decreased from the normal in patients suffering from NAFLD (Birjandi et al., 2016; Lee et al., 2010). This parameter is specifically valuable in distinguishing non-alcoholic steatohepatitis (NASH) from less severe types of NAFLD. Although both the AST and ALT enzymes increase in hepatic disorders, there is a higher increase in ALT levels in mild hepatic steatosis, first-degree, and second-degree steatohepatitis, resulting in lower AST/ALT ratios, differentiating them from the third and fourth-degree steatohepatitis and the more advanced conditions like the hepatic fibrosis (Harrison et al., 2008; Angulo et al., 2007; Palekar et al., 2006; Angulo, 2002). The initial phase of this study also revealed a noteworthy decrease in the AST/ALT ratio in HepG2 cells fed with a high-energy diet, confirming the successful induction of NAFLD conditions. On the other hand, administering silymarin during the second phase led to a favorable reduction in fatty cell enzyme levels, indicating its effectiveness in enhancing the hepatic cell function. As a result of silymarin treatment, a decrease in AST and ALT enzymes and an increase in the AST/ALT ratio were apparent, and there was no significant difference in the values between the group treated with a 250 μM silymarin and the control group. In the present study, a carefully produced highly standard commercial silymarin was used instead of conventionally provided *Silybum marianum* extract so that the observed therapeutic effect can be more reliably attributed to the active compounds of *Silybum marianum* plant and a more accurate evaluation of the time-dependent and dose-dependent therapeutic effects can be obtained by eliminating the impurities in the plant extracts.

The utilization of an *in vitro* model for inducing non-alcoholic fatty liver disease

to assess the efficacy of silymarin treatment doesn't appropriately simulate the natural state of the body and the complexity of interactions among the related various factors. However, this approach provides a suitable condition for investigating the pure and immediate effect of the drug on the target hepatocytes by eliminating many confounding factors, such as the incorporation of aminotransferase enzymes of non-hepatic origin and the interferences caused by other tissues.

The results of this study revealed a notable impact of silymarin in decreasing lipid droplets in hepatocytes in a non-alcoholic fatty liver model. The discoveries made through this investigation affirm the effectiveness of silymarin, as a pool of herbal components, in treating fatty liver diseases. Additionally, it highlights the possibility of using silymarin alone or in conjunction with other medications. Further investigations may provide adequate indications to propose silymarin as a beneficial therapeutic intervention for fatty liver disease treatment. The effectiveness of *Silybum marianum* extract revealed by the current study and numerous other studies necessitates undertaking comprehensive studies to understand molecular mechanisms and metabolic pathways involved in the silymarin effectiveness in fatty liver conditions in order to achieve a deeper understanding of its mode of action enabling develop new drugs for the successful treatment of non-alcoholic fatty liver disease.

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