Evidence Based of Effects and Protection Mechanism of Lycium Barbarum Polysaccharide on Ethanol-Induced Liver Injury

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Abstract

Excessive ethanol intakes have been reported to be linked to liver diseases such as hepatitis and cancer-related diseases of ethanol. Excessive ethanol can lead to the accumulation of reactive oxygen species (ROS) and to the depletion of glutathione (GSH) in the liver. Ethanol-induced oxidative stress substantially increases peroxidation of the lipid and decreases GSH levels in human hepatocyte and animal models. Ethanol contributes to sterol regulating element-binding protein activation (SREBP)-1 by regulating main genes such as Acetyl-CoA carboxylase (ACC) and Stearoyl-CoA desaturase 1(SCD1) to facilitate lipid accumulation in the liver. The liquid fraction derived from wolfberry is lycium barbarum polysaccharide (LBP). In many animal disease models, it is an effective antioxidant and tissue protective agent. Researchers have shown hepato-protective functions in acute hepatic injury, NAFLDs, drugs-induced liver injury and hepatocellularcarcinoma models. The protection effect of L. barbarum polysaccharides (LBP) in this study on ethanol-induced injury in human hepatocytes was explored. In addition, the influence of LBP on oxidative stress protein activity was examined.

Keywords: *L. barbarum polysaccharides (LBP) - ethanol-induced injury - drugs-induced liver injury - reactive oxygen species (ROS).*

Introduction

A spectrum of hepatic injuries, from steatosis to cirrhosis, is encompassed by alcoholic hepatic disease (ALD). Alcohol misuse continues to be a significant global health and social burden. Alcohol-related diseases account for 9.2 percent of all lifespan adjusted for disabilities in the developing world[1] .Chronic alcohol intake contributes to steatosis in more than 90% of heavy drinkers[2].

Advanced hepatitis, fibrosis, and even hepatocellular carcinoma (HCC) may be advanced from prolonged alcoholic fatty liver intake. Whilst several key events have been identified during the development of ALD, the disease's comprehensive pathological mechanisms and associations with other risk factors (for instance, age, obesity and smoking) are still not clear[3]. Increased inflammation of the liver and oxidative stress are thought to play crucial roles in the development of ALD among these defined mechanisms[4,5].

From 2006–2010, excessive alcohol consumption caused about 88 000 deaths, shortening lives in the United States by 30 years[6,7] While many new, target drugs are currently being tested, strict abstinence and nutritional support appear to be the best choice for alcohol diseases of the liver[8].

Ethanol is first metabolized by alcohol dehydrogenase to acetaldehyde and cytochrome enzymes P450 in hepatocytes after consumption. Excessive acetaldehyde induces oxidative hepatic stress

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which reduces β-oxidation and increases lipogenesis in fatty acids[9].

Excessive ethanol intakes have been reported to be linked to liver diseases such as hepatitis and cancer-related diseases of ethanol[10]. Excessive ethanol can lead to the accumulation of reactive oxygen species (ROS) and to the depletion of glutathione (GSH) in the liver[11]. Ethanol-induced oxidative stress substantially increases peroxidation of the lipid and decreases GSH levels in human hepatocyte and animal models [12,13] .

Ethanol contributes to sterol regulating element-binding protein activation (SREBP)-1 by regulating main genes such as Acetyl-CoA carboxylase (ACC) and Stearoyl-CoA desaturase 1(SCD1) to facilitate lipid accumulation in the liver [14].

It is widely agreed that women are affected by alcohol-mediated liver disease[15]. Whilst the underlying mechanism is not completely determined, clinical results clearly indicate an association of sex hormones with ALD[16]. Estrogen receptors (ERs), primarily expressed in the cells of Kupffe and in sinusoidal endothelial cells[17] and have a very low expression level in hepatocytes[18] are believed to mediate the production of pro-inflammatory cytokines and reactive oxygen species during hepatic ethanol metabolism[19].

The evidence has shown the closely associated pathogenesis of alcoholic liver disease (ALDs) with ROS and malondialdehyde (MDA) as well as ethanol metabolism by-products and toxic metabolization. Because of their few side effects, herbal medicine was commonly applied in research into drug discoveries. Lycium Barbarum is part of a Solanaceae family plant and its fruits have been used in China for thousands of years as traditional medicine and edible food. L. barbarum fruit usage was first recorded about 2,300 years ago. L. barbarum fruits have a number of biologic and pharmacological functions and they prevent and treat chronic conditions like diabetes, hyperlipidemia, hepatitis, the function of hypo-immunity, thrombosis and infertility among males[20].

The treatment of ALDs includes free radical scavengers or antioxidants[21]. L. Barbarum is effective for the eyes, liver, and kidney as an anti-oxidative and nutritive agent. Polysaccharides have been isolated from fruits and tested in various models as the major component of antioxidant activity [22]. Extracts of L. barbarum, for instance, by the increased superoxide dismutase (SOD) activity is used to exert an anti-oxidative effect in brain and heart tissue of the mice [23] .

The liquid fraction derived from wolfberry is lycium barbarum polysaccharide (LBP). In many animal disease models[24], it is an effective antioxidant and tissue protective agent. Researchers have shown hepato-protective functions in acute hepatic injury[25,26], NAFLDs[27,28], drugs-induced liver injury[29] and hepatocellular carcinoma models[30].

This study aimed to explore the protection effect of L.barbarum polysaccharides (LBP) on ethanol-induced injury in human hepatocytes. In addition, the influence of LBP on oxidative stress protein activity was examined.

Materials and Method

Material Preparation: For 2 hours in a water bath at 60°C, LBP powder (100 g) was extracted with 1.000 ml water. The extracts have been purified and concentrated at a reduced pressure of 348 K to 200 ml in the revolving evaporator RE-52C. In addition to the concentrated extract, ethanol (75%) allowed for 4 hours, was filtered, concentrated and dried to obtain a polysaccharide extract. At −20°C, the dry extract was screened and placed in a refrigerator. In order to obtain sufficient concentrations, the extract was diluted with deionized water.

Experimental Cell Culture and Treatment: Human normal liver cell l-02 in humidified 5 percent CO2 atmosphere has been developed at 37°C in the medium of RPMI-1640 with an addition of 10 percent fetal serum bovine as well as 1 percent antibiotic penicillin and streptomycin. The cells (5 = 103 cells/ well) have been seeded and grown overnight on the 96 - well plate and treated at various LBP concentrations for 24 hours, followed by 5% ethanol (0, 12, 24, 48, 96, 192 μg/ml) treatment for another 4 hours. Or 5% ethanol treated for 4 hours and followed by a LBP treatment (0, 12, 24, 48, 96, 192 μg/ml) with different concentrations for an additional 24 hours.

The MTT assay assessed the cell viability. A 96-well plate has been used in cultivating and growing cells overnight (5 × 103 cells/well). 20 μl MTT (5 mg/ml) were applied to each well after LBP and ethanol were treated. The culture medium was extracted and formazan precipitate dissolved in 150 μl DMSO after incubation for a period of 4 hours at 37°C. A spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) was

used to test absorbance at 492 nm. Cell viability ratio was determined using the formula:

Cell viability ratio (%) = 1 −[($OD_{control}$/$OD_{treated}$)/($OD_{control}$)] × 100%

The cell apoptosis rate was analyzed by flow cytometry. L-02 was harvested and re-suspended at a density of 5×10^5 cells/ml in PBS buffer. Cells were stained with FITC-Annexin V and propidium iodide (PI) and the findings were analyzed using an Amnis ISXmkII flow cytometer fitted with IDEAS v6.0 image analysis software according to manufacturer's instructions. At least three times all the experiments have been replicated.

ROS generation measurement in vitro: In the complete growth medium L-02 cells (1,5×105 cells/well) were seeded and grown overnight in 12-well plates. Cells were trypsinised and centrifuged at 1500 rpm at room temperature for 10 min following treatment with lbp and ethanol. Then removed the supernatant and re-suspend the cellular pellet in a full medium of 0,5 ml, then added 1 ml DCFH-DA at a final concentration of 10 µmol/L, incubated in a water bath for 20 minutes at 37 ° C, and mixed and inverted once every 3–5 min to permit the spectrum to fully contact the cells. Then, the cells were washed three times with serum-free medium, and laser confocal microscopy (Olympus, Japan) was used to observe for the fluorescent state of the cells, with a fluorescence spectrophotometer measuring the amount of the reactive oxygen species in the cells (Perkin Elmer, USA). Finally, the findings are shown in relation to control as the percentage of cells with a rise in ROS levels.

Biochemical Assay: The biochemical experiments were carried out in compliance with the instructions of the manufacturer using commercial detection kits.

Total protein detection and automated capillary western blot: Plated cells were treated with LBP, Silybin, and ethanol, in six-well plates and 5-10 × 10^6 L-02 cells extracted and washed with PBS. Cytoplasmic proteins, nuclear proteins, and total proteins were collected from the cytoplasmic nuclear protein extraction kit and the RIPA cell lysate, respectively. The extracted protein has been tested with a BCA protein quantification kit for protein concentration and is ready for machine use.

Ethical consideration: Taking acceptance from ethical committee faculty of medicine, Assuit University in March 2019.

Statistical Analysis: The statistical software SPSS 21.0 was used for data analysis. In the case of variances not uniform, the ranking sum test was used for results analysis and data analysis results were mean±standard deviation. The Multi-group comparisons of the means were calculated in the analysis of a one-way variance analysis (ANOVA) test with post hoc comparisons. The α-test level was statistically important when compared to the model group * $p < .05$, * * $p < .01$. * $p < .05$, * * $p < .01$ was statistically relevant compared with the control group.

Results

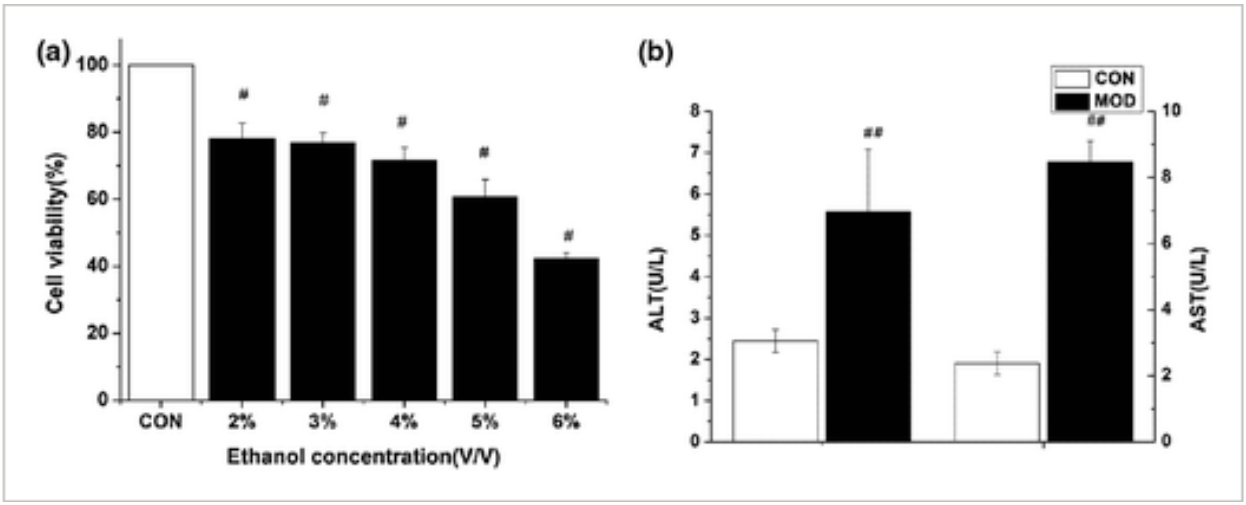

Figure (2): Effect of ethanol on the viability of cells as well as ALT and AST content.

Establishment of the liver cell model induced by alcohol: The final concentrations of L-02 cells were 2%, 3%, 4%, 5% and 6% (v/v) in the medium ethanol for 4 hours, and changes to the cell viability were studied. Figure 2(a) indicates the results. Their operation was significantly lower than normal control group (CON, p < 05) following treatment of ethanol cells at different concentrations during 4 hours. With increasing concentration of ethanol, cell viability steadily declined. The ethanol levels were set to 5 % (v/v) for hepatocyte damage and to ensure an adequate numbers of cells for the subsequent step of the test. Their vitality was 60.7%±4.19%.

After ethanol injury to hepatic cells, the content of ALT and AST was detected in the supernatant. In comparison with the model group, the ALT and AST contents of cells in the group CON increased respectively by 128.27% and 254.45% (MOD, p < .01). The AST content in the model group was greater than the ALT content (p<-01, Figure 2b). A model was successfully developed for ethanol-induced hepatocyte injury in vitro. Induced injury to the L-02 cells at 5 percent ethanol (v/v) for 4 hours.

(a) The effect on the viability of liver cells of various ethanol concentrations. In regular L-02, cell viability 2%, 3%, 4%, 5%, 6%: cell viability 2%, 4%, 5%, 6% in alcohol-intoxicated L-02, 5%, 6%. Cell viability in normal l-02

(b) The ethanol-induced hepatocyte injury ALT and AST content. Con: normal L-02 ALT and AST; MOD: Ethanol-induced L-02 ALT and AST content. *p < .05, **p < .01

LBP intervention concentration screening: Figure 3a demonstrates the effects on the viability of L-02 cells of different LBP concentrations. LBP interfered without proliferation or toxicity 24 hours with L-02 cells at concentrations of 12, 24, 48, 96, and 192 μg/ml. The LBP and L-02 cells were co-cultured at test concentration over 24 hours without the exception of LBP's false-positive and false-negative effects on hepatocytes and had no effect on the hepatocytes. The concentration gradient could therefore be used to screen the intervention concentration.

Figure 3b, c show cell viability at different LBP concentrations in the 24-hour prevention group (24 hours after incubation and 4-hour ethanol injury) and 48-hour prevention group (48 hours after incubation and 4-hour ethanol injury) respectively. The protective effect of LBP was compared with a hepatic protective agent in the same test. The positive control was Silybin (SM) at 24 μg/ml. LBP has achieved its maximum capacity of 24 μg/mL on increasing cell viability. The disparity between model group is statistically significant (p<0.05) but is not statistically significant (78.34 %±3.31 %, p>.05), as compared to models. When 24 μg/ml of LBP was added to cells for 24 hours, cell viability was up to 81.61 %±3.12 %.

Figure 3d demonstrates cell viability at variable concentrations of LBP in the 24-hour repair group (first with ethanol for 4 hours before LBP for 24 hours). Figure 3e shows cells in a 48-hour repair group with different concentrations of LBP (first ethanol for 4 hours and LBP for 48 hours).

Figure 3e indicates a higher cell viability at 24 μg/ml than at the other four stages. The viability of cells at 24 hour repaired was 87.51 percent±3.02 percent (p < 0.05), and the percentage was above that of SM group 83.89 %±3.69 %(p < 0.05). Cells in the LBP repair group were selected at 24 μg/ml and were better than the preventive model (p < -05, for the 24-hour treatment).

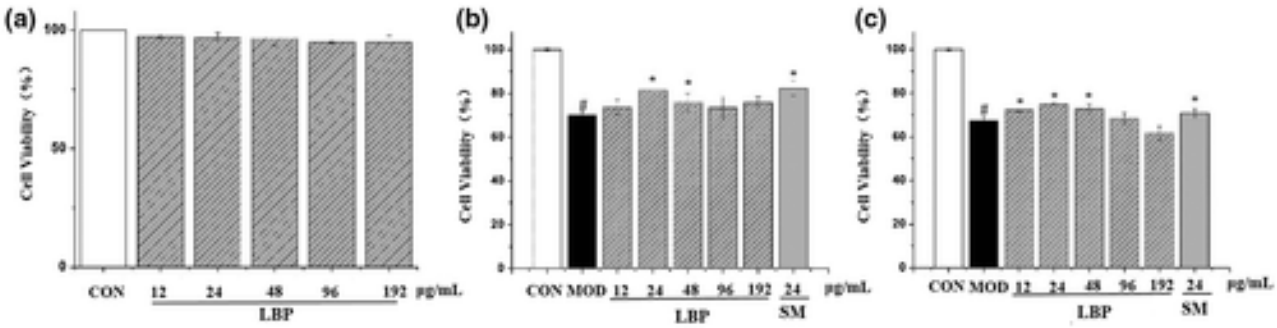

Figure (3): Cell viability effect of LBP.

(a) The effect on the viability of L-02 cells of various LBP concentrations.

(b-c) Effects of LBP prevention for viability of PA hepatocyte wound induced with alcohol at various concentrations for 24 hours (b) and 48 hours (c).

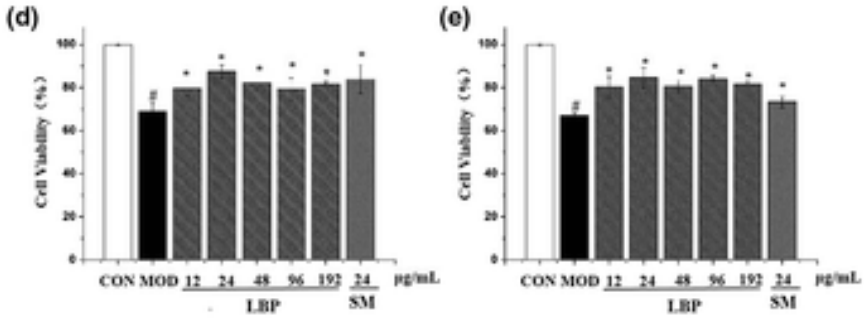

Figure (3): Cell viability effect of LBP. (d-e) LBP repair of alcoholic hepatocyte cell viability injured at different concentrations for 24 h (d) and 48 h (e).

Evaluation of the LBP Hepatic Function in Alcoholic Hepatocyte Injury Prevention and Repair: Figure 4 shows the levels of ALT and AST to avoid and restore hepatocyte injury from alcohol with LBP. The ALT content in the LBP repair group was higher than in the CON Group (2.489±0.34 U/L) and lower than in the MOD. The ALT content in the LBP repair group is less than in the CON group. The differences in cells that were treated with alcohol were statistically significant ($p < 0.05$). These findings have shown that after ethanol injury, LBP repair decreased ALT and AST levels. The activity in the LBP prevention group was greater than in the control group, with the ALT (4.40±0.64 U/L) and AST (3.818 + 0.54 U/L), and statistically relevant differences ($p < .05$). The prevention effect of LBP was stated by these findings. Following ethanol injury, LBP also decreased ALT and AST levels.

ALT content in normal L-02; LBP-Pre: LBP followed by ethanol followed by ethanol; Silybin-Pre: Silybin-Pre: LBP; LBP-Pre: Ethanol followed by ethanol; Silybin-Post: ethanol followed by silybin; LBP-Pre: ethanol followed by LBP: Con: ALT in normal L-02

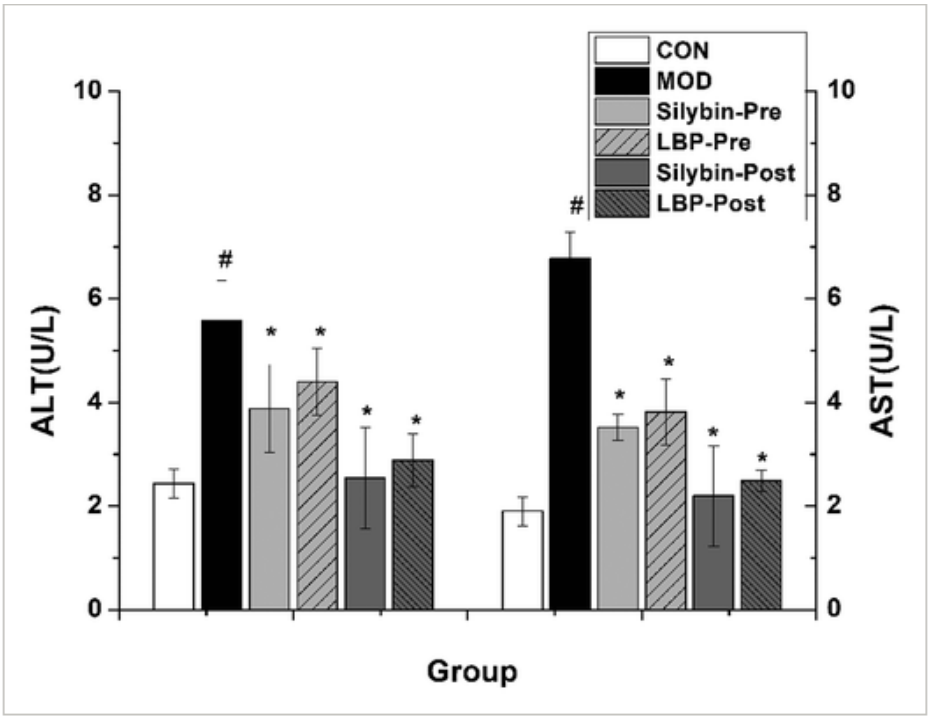

Figure (4): ALT and AST LBP content in alcoholic hepatocyte injury prevention and repair

LBP effect in L-02 cells on ethanol-induced ROS: The effect on ROS levels in ethanol-injured hepatocytes is shown in Figure 5. The impact of LBP action on the ROS levels in alcoholic hepatocytes has been identified qualitatively and quantitatively and oxidative stress in L-02 cells has already been observed. The control group had a large density, a transparent cytoplasm and the fluorescence intensity was weakest and the ROS was 147.83±10.55 the lowest. The cell density was considerably decreased, the nucleus condensed and after ethanol stimulation cell debris occurred. The intensity of the fluorescence within the cells increased dramatically and the amount of ROS rose by 2.99. The cell density increased and the intensity of fluorescence decreased following LBP interference. The rate of LBP repairs and prevention decreasing in comparison with the model group by 56,91% and 56,89% respectively ($p < 01$). LBP decreased the amount of ROS intracellularly, likely due to LBP ROS inhibition or clearance. The cell state of the fluorescence channel under the Laser Confocal Spiegel is observed in Figures A1, B1, C1, and D1. In non-fluorescent channel microscopes, cells are A2, B2, C2, and D2, respectively. The fluorescence spectrophotometer will detect A3, B3, C3, and D3 quantitatively.

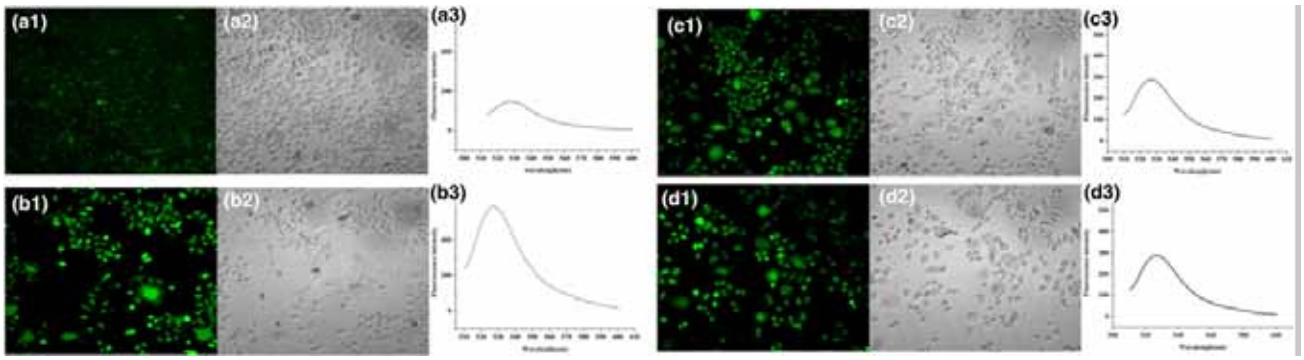

Figure (5): LBP reaction to ROS levels in ethanol-injured hepatocytes. (a) Control group (b) model group (c) LBP prevention group (d) LBP group repair group.

The LBP effect in the L-02 cell liver-induced liver-injury anti-oxidation pathway of a nuclear factor (Nrf2)/heme-oxygenase-1 (HO-1): Hepatocyte expression levels were observed in hepatocytes of Nrf2 expression levels, NRf2 expression levels, NQO1 extension, and GCLC proteins. The expression of Nrf2 and its HO-1, NQO1 and GCLC downstream proteins is shown as illustrated in Figure 6. The Nrf2 protein expression bands in cytosol and nucleus are shown in Figure 6a. The expression of Nrf2 in model group cytoplasm was substantially greater than in the nucleus, suggesting the nuclear expression of cytosolic Nrf2 for the internal cytosolic reference β-actin and the internal nuclear reference PCNA. The volume was minimal and the injury incurred by free radicals could not be resisted. Increased nuclear translocation of the NoRF2 cytoplasm in a varying degrees was demonstrated by the LBP prevention and repair groups.

The expression Nrf2 in the LBP repair group decreased by 30,44% ($p<.01$) in the quantitative expression histogram as shown in Nrf2 in Figure 7c, compared to models for the LBP prevention group, up from 232,2% ($p<.01$). In the cytoplasm of the LBP prevention group, Nrf2 exposition also declined by 20.05 percent, compared to a rise of 153.04% ($p<.01$) of the LBP repair group. Thus the nuclear transport of the cytoplasm of NRF2 to the nucleus was encouraged by LBP. Nuclear Nrf2 was higher than the preventive group ($p < 05$) in the repair group. The results indicate that LBP is controlling the Nrf2 protein and that its repair effect could increase the promotion of Nrf2 ‘s nuclear expression.

In this paper the alterations in the Nrf2 pathway’s downstream protein expression after LBP intervention were examined. Figure 6b will use the protein expression bands to qualitatively demonstrate that HO-1, NQO1 and GCLC are the weakest in the model group. Expression levels of these three proteins have increased to various degrees in the LBP repair and prevention groups. The expression levels of HO-1, NQO1, and GCLC in the LBP repair groups increased by 109.65%, 754.81% and 74.76% respectively in comparison with the model cells, as shown by the quantitative histogram in Fig. 6d. The LBP prevention group did not have an improvement in expression of HO-1, compared to NQO1 and GCLC, which respectively grew by 418.27% and 186.18%.

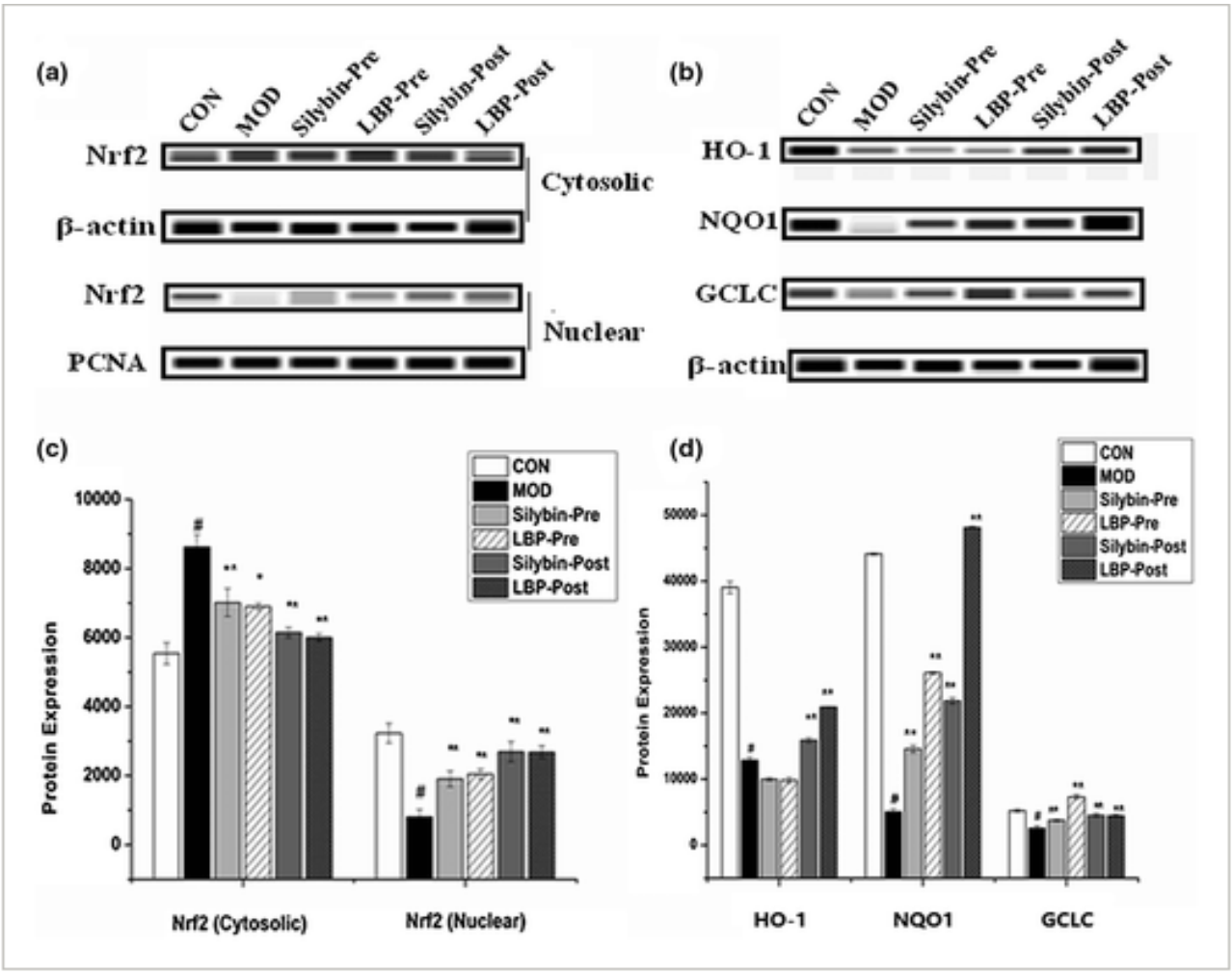

Figure (6): LBP effect on expression of the cytoplasmic and alcohol hepatocyte nucleus of Nrf2, HO-1 and GCLC proteins Effect of LBP (a) LBP and ethanol have been treated for cells. Nuclear and cytosolic extracts were extracted and Western blot analysis established Nrf2 levels. (b) HO-1, NQO1 protein expression, the Western blot analysis was carried out for GCLC and three separate studies have been carried out in terms of the findings.

Discussion

The development of liver disease including alcoholic steatosis, alcoholic hepatitis, cirrhosis and hepatocellular cancer can be caused during long-term alcohol consumption[31]. Ethanol flows into the liver and causes hepatic damage as a result of oxidative stress and hepatocytic lipid accumulation [32]. Therefore, inhibiting oxidative stress and ethanol-induced lipid accumulation will stop and treat ALD.

Edible foods or medicinal herbs are generally compatible with natural antioxidants, and it is thus an important method for the prevention and treatment of ALD. In this study, a regulation of oxidative stress associated with the enzymes in L-02 cells elucidated its protective potential against ethanol-induced oxidative stress.

The cell culture medium in the preliminary experiments included a dose-dependent dose of ethanol at 2 percent – 6 percent (v/v) gradient concentration and four hours of co-incubation. The rate of survival was decreased to 40% following 5% of ethanol injury for 4 hours. However, the cell survival rate improved significantly when LBP was pre-treated for 24 hours, treated for 4 hours with ethanol, and pre-treated for 4 hours with LBP and then treated with LBP for 24 hours, which indicated a hepatitis protection effect of LBP. LBP ‘s maximum capacity was reached at 24 μg/ml in terms of rising cell viability. The cell viability increased

sharply at lower concentrations, steadily rising with the LBP concentration. It was believed. The levels ALT and AST are commonly tested as biochemical liver injury markers[33].

In this research, LBP reduced the ALT/AST of ethanol-treated cells significantly and demonstrated its anticipatory and repair effects on hepatotoxicity of ethanol. LBP decreased significantly the ALT and AST levels, generation of MDA and ethanol-induced intake of GSH, demonstrating that LBP had an effect on the liver injury caused by ethanol. This safety is likely due to the antioxidant properties of LBP, as evidenced by the slowdown in ethanol-induced L-02 cells for ROS and MDA production. SOD is the major enzymatic mechanism for protecting the body from oxygen-free radicals. If there is not enough SOD activity, antioxidant activity could not be exercised in due course, and radicals without oxygen assault the cells and cause cell harm. GSH-Px is a central enzyme that catalysis hyperoxide decomposition. The reduction reaction of GSH to hydrogen peroxide can be precisely catalyzed and cell membrane structure and functions are preserved. In this study, after 4 hours of ethanol treatment, the SOD activities and GSH – Px decreased significantly.

The SOD and GSH-Px behavior in each group increased after LBP intervention. There was a substantial difference between them and the injury group. Increased activities in SOD and GSH-Px suggested that LBP could increase the activity in cells of the active oxygen-scavenging enzyme, enhancing the body's antioxidant protection mechanism. Instead of accumulating in cells, oxygen-free radicals could therefore be eliminated in time. The peroxidation of cell lipids causes damage to the cell. The decrease in lipid per-oxidants and the increase in cell antioxidant capabilities will decrease cells' oxidative stress harm. By preserving the activity of antioxidants such as SOD and GSH-Px, LBPs show their antioxidant activity and thus reduce the level of active oxygen. Thus, LBP protects liver cells by increasing antioxidant enzyme activity and by inhibiting hepatocyte apoptosis and decreases the incidence of alcohol hepatic harm.

The Early Apoptotic Hepatocyte Prevention and Repair Groups, induced with ethanol, increased in that experiment. LBP had protective and repair effects on the early apoptotic cells after the stimulating factors were removed, but the later apoptosis cells eventually died. The LBP repair group's apoptotic rate was almost the same as that of the control group. The LBP's reparative effect was stronger than the preventive impact on ethanol-induced liver injury cells, suggesting LBP has greater capacity to restore hepatocytes than to prevent harm. Therefore, in alcoholic liver disease, LBP has defensive capabilities. The findings of this research help therapeutic substance use potentially. However, further analysis is needed of the basic mechanism.

This study showed that LBP could reduce the production of O2 by increasing antioxidant enzymes after alcoholic hepatic cell injury, reducing other oxygen-free production of radically-based radicals and improving the resistance to superoxide and free radicals. A large amount of oxygen-free radicals was reduced by the chain reaction which alleviated L-02 cell injuries and had a therapeutic effect.

Conclusion

Finally, our current research has shown clearly that LBP has a protective impact on liver cell injuries caused by ethanol. Cell apoptosis and oxidative stresses may be included in the potential process.

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Conflict of Interest: Nil

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