Biochemical, Histopathological and NMR Metabolomite Profiling of Hepatoprotective Drug in Mouse Alcoholic Liver Disease Model

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Abstract

The main objective of this study was to determine the effect of hepatoprotective marketed liver tonic on mouse model of alcohol induced liver disease (ALD) by adopting NMR metabolomics technique. The hepatoprotective activity was studied in experimental animals in which 5% and 10% of alcohol was mixed with drinking water for 6 weeks in two different groups. The two treatment group received hepatoprotective cadihep syrup 0.1 and 0.15 ml/ kg, p.o respectively along with the 5% and 10% v/v alcohol which was mixed with drinking water for 6 weeks. The effect of the drug was determined by measuring the biochemical estimations like AST, ALT, Bilirubin (Conjugate and Unconjugate) and ALP levels. Further, serum was used to perform the NMR metabolomics studies along with the histopathological observation. Treatment with cadihep syrup significantly reduced the elevated levels of AST, ALT, Bilirubin (Conjugate and Unconjugate) and ALP in the alcohol induced groups. The NMR results exhibited the presence of high alcohol and acetate levels in the alcohol treated groups when compared with the normal group. A significant decrease in these metabolites was observed when compared with the alcohol treated group. A comparative histological study of liver treated with cadihep syrup showed the normal architecture when compared with the alcoholic induced group. The present study suggests that cadihep syrup possess significant hepatoprotective effect against alcohol induced liver disease. The current study focuseson early detection by NMR estimations.

Keywords: *Alcoholic Liver Disease (ALD), Cadihep, Hepatoprotective, NMR, Metabolomics.*

1. Introduction

Alcoholic Liver Disease (ALD) poses a significant global health challenge, with alcohol abuse contributing to 2.5 million deaths annually [1]. The progression from alcoholic steatosis to hepatitis and cirrhosis is closely linked to the duration and quantity of alcohol consumption, with genetic factors playing a crucial role. Around 90% of alcoholics develop alcoholic steatosis, 25% progress to alcoholic hepatitis, 15% develop cirrhosis, and 10% face hepatocellular carcinoma [2].

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The severity of ALD manifestations ranges from asymptomatic alcoholic steatosis to the life-threatening complications of cirrhosis, including ascites, hepatic encephalopathy, and hepatorenal syndrome [3]. Laboratory tests, particularly the AST/ALT ratio, BMI, and gender, aid in detection, but non-invasive methods such as metabolomics, specifically NMR spectroscopy, are gaining traction for early diagnosis and effective monitoring [4].

In addressing the diagnostic challenges of ALD, this study emphasizes the potential of NMR metabolomics. Metabolomics, a rapidly advancing field, quantifies metabolite concentrations to identify markers for early detection. NMR spectroscopy, among various techniques like GC-MS and LC-MS, stands out for its reproducibility and minimal sample handling requirements [5]. By elucidating the metabolic changes associated with alcohol-induced liver diseases, this research aims to contribute to the development of non-invasive diagnostic tools crucial for timely intervention and improved therapeutic outcomes in ALD.

Methods

Animal selection and housing:

Adult Swiss mice of either sex weighing 20-30 g were used and divided into five groups of six animals each. The animals were acclimatized for few days under laboratory conditions.

They were housed in polypropylene cages and maintained at (27 ‡ 2) °C under 12 h dark / light cycle. They were fed with standard mice feed (Gold Mohur Lipton India Ltd.) water and ad libitum was provided. Ethical clearance for use of animals was obtained from the Institutional Animal Ethical Committee (01/KLECOPH/18 - 08/ September- 2018) prior to beginning of the project work.

Experimental design and estimation procedures:

The proposed work carried out in Swiss albino mice weighing around 20-30 g. The induction of alcohol liver disease is caused by chronic alcohol feeding to mice. In this model the normal mice are fed ad libitum with water. Group Il and Group III mixed with 5% and 10% (v/v) alcohol and given for 6 weeks. Group IV and Group V mixed with 5% and 10% (v/v) alcohol and the treatment was given by marketed poly herb drug Cadihep syrup for the Group IV syrup was given orally after 3 days (dose 0.1 ml/kg,p.o.). Group V received orally after 3 days (dose 0.15 ml/kg,p.o.).

Dose preparation

For the experiment the marketed hepatoprotective liver tonic Cadihep syrup is used and dose was calculated according to the animal dose calculation based on body surface area i.e., conversion of human dose to animal dose.

Collection of blood, and tissue

After the last treatment, blood samples were obtained by retro-orbital or cardiac puncture method under light anaesthesia using heparinised micro capillaries for the biochemical estimation. After withdrawing of blood, animals were sacrificed by carotid bleeding and midline abdominal incision was performed, and liver tissue dissected out was blotted out from blood, washed with saline and stored in 10% formalin and proceeded for histopathology to evaluate the details of hepatic architecture in each group. The same sample was used to perform the NMR metabolomics study.

PARAMETERS

Food consumption

Average food intake of each group has been measured daily till the end of the study from which food intake per animal was calculated. At the end of study daily data of individual animal has been converted into weekly consumption. The differences were calculated by subtracting the value of week 0 from the values of subsequent weeks and the graph was plotted.

Water consumption

Average water consumption of normal group has been measured daily till the end of the study and was evaluated using the same procedure as food consumption.

Alcohol consumption

Average alcohol consumption of all other groups has been measured daily till the end of the study and was calculated using the same procedure as food consumption.

Body weight measurement

Weekly body weight was measured of each mice from all the groups .Mean was calculated and the average weight per group for week 0 was taken as 0. The change in weight between was calculated by subtracting the average weight of week 0 from subsequent weeks.

Liver weight

Animals were sacrificed and liver weight was taken at the end of the study of every animal from each group.

Relative liver weight

It was calculated by taking the percentage of liver weight to body weight of each animal and expressed as gram percentage.

Biochemical estimations:

Liver Function Tests such as Estimation of serum alanine transaminase (ALT), Estimation of Serum Aspartate amino Transferase (AST), Alkaline phosphate (ALP), Estimation of Serum Bilirubin were carried out and reported in result section.

NMR Estimation:

Procedure: 'H-NMR spectra for all serum samples were obtained on JEOL advanced ECZ400S spectrometer operating at 400 MHz proton frequency using 5 mm THS Broad band direct probe. 200uL serum taken in cleaned dried 5 mm NMR tube and 300u deuterium oxide added to the same NMR tube to make total volume of the sample 500 uL. Deuterium oxide served as the filed-frequency lock. One-dimensional Carr-Purcell-Meiboom-Gill (CPMG) beat succession was utilized for obtaining 1H-NMR spectra. CPMG takes out the wide flags from protein, lipids and macromolecules. All spectra were recorded with 16 time space information focuses, 15 ppm ghastly width, number of sweeps 256, sham output 4, unwinding deferral of 5 s and steady beneficiary increase esteem 60, tau interim 0.172ms. Delta 5.0.5.1 NMR software was used for phase and baseline correction and for getting integral (area under the curve) value. Assignment of the metabolites were done using metabolomics data base**.**

Histopathological study:

The liver tissue that carried out histopathological studies using haematoxylin and eosin staining (H&E), all the slides were observed for changes in histopathological characteristics and photographs were taken (20x and 40x) and reported in result section.

Statistical analysis:

The results are calculated by Mean ± S.E.M. The statistical significance analyzed by using 1-way ANOVA pursued by Bonferroni's test, data was calculated by utilizing Graph pad prism programming 5.0 version.

Results:

General Observations:

Food consumption: Food consumption in mice was observed for the duration of 6 weeks for all the groups, from the beginning of the treatment period. The results are presented as change in the food consumption of animals at the beginning with subsequent weeks. The normal group showed significant changes in food consumption when compared to the alcoholic group. The alcoholic group showed no significance changes in food consumption starting from one week compared to normal group with 5% and 10% alcoholic control group. In normal group the food consumption was different from the alcoholic treated group. Then again both alcoholic and treatment groups showed no significant difference in the food consumption compared to all the groups (Figure 1).

Water consumption: Water consumption in mice observed for the duration of 6 weeks, from the beginning to end of the treatment period. Water was provided only in a normal group as this group compared with alcoholic and treatment groups.
Values are expressed as Mean ± SEM (n=6) change in average food consumption per group from beginning to the subsequent weeks. The statistical significance was analysed by using one-way ANOVA followed by Bonferroni test .Where *p* value is not significant when compared to alcoholic and treatment groups.

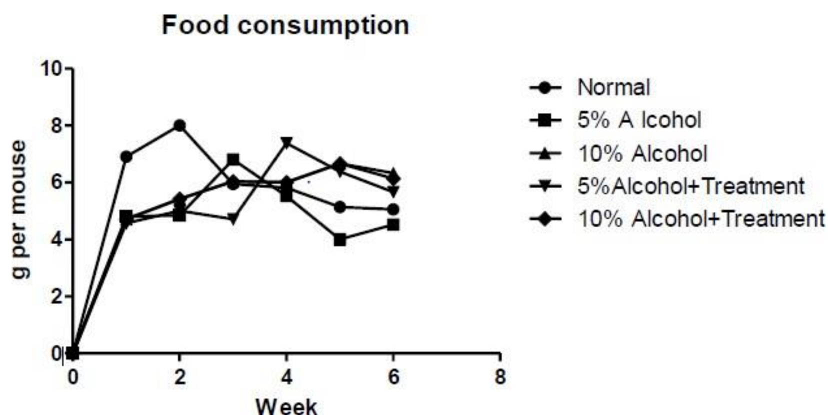

Figure 1. Food consumption pattern of mice

Alcohol consumption: Alcohol consumption in mice was observed for 6 weeks, from the beginning to end of the treatment group. The consumed alcohol measured daily and noted. (Figure 1.1). Values are expressed as change as Mean ± SEM (n=6) in average food consumption per group from beginning to the subsequent weeks. The statistical significance was analyzed by using one-way ANOVA followed by Bonferroni test. Where *p* has not shown any significant value when compared with alcohol and treatment groups.

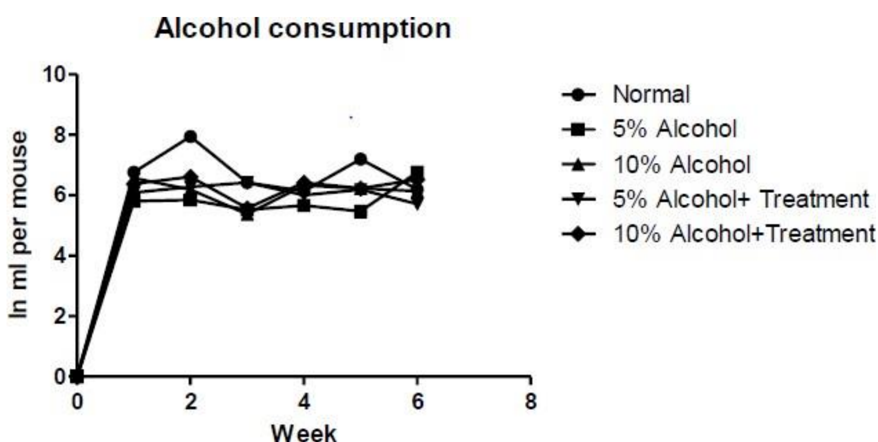

Figure 1.1. Alcohol consumption pattern of mice

Body weight: Changes in body weight in mice was observed for the duration of 6 weeks, from the beginning to end of the treatment period. The results are presented as change in the body weight of animals. The normal and 5% alcoholic groups (29.19±0.60) were almost same as normal (25.60±0.36)and there were significant increase in the group with 10% alcoholic control (31.87±0.76) and the significant decrease in the treatments groups were (27.88±0.69 and 30.45±0.76) observed. Where ***p*<0.01 ****p*<0.001 when compared with normal group. Where *p* is not significant when compared with treatment groups. Values are expressed as Mean ± SEM (n=6) by one way ANOVA followed by Bonferroni test. Where ***p*<0.01 ****p*<0.001 when compared with normal group (Figure 1.2).

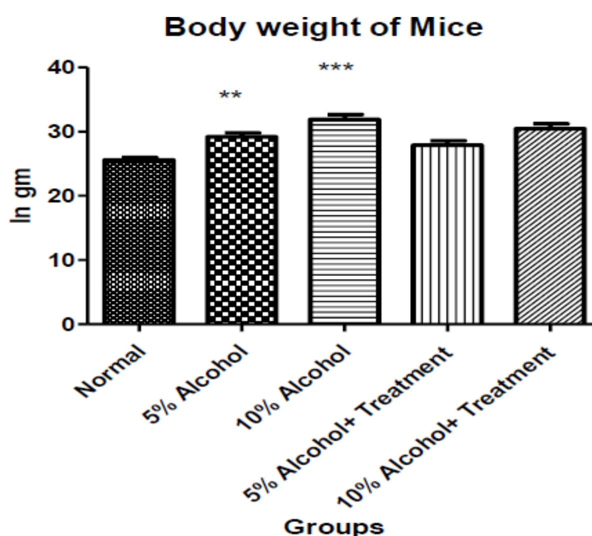

Figure 1.2. Body weight of the mice

Liver weight: The alcohol consumed group significantly increase in the liver weight (1.17±0.08 and 1.17±0.13) when contrasted with the normal group (1.08±0.08).The treatment group (Cadihep syrup) significantly decreased when compared to alcoholic and normal group (1.3±0.15 and 1.4±0.14).Where ***p*<0.01 when compared to 10% Alcohol. Values are expressed as Mean ± SEM (n=6) by one-way ANOVA followed by Bonferroni test. Where *p* is not significant compared to the 5% alcoholic group and treatment groups. Where ***p*<0.01 when compared to 10% Alcohol with normal group (Figure 1.3).

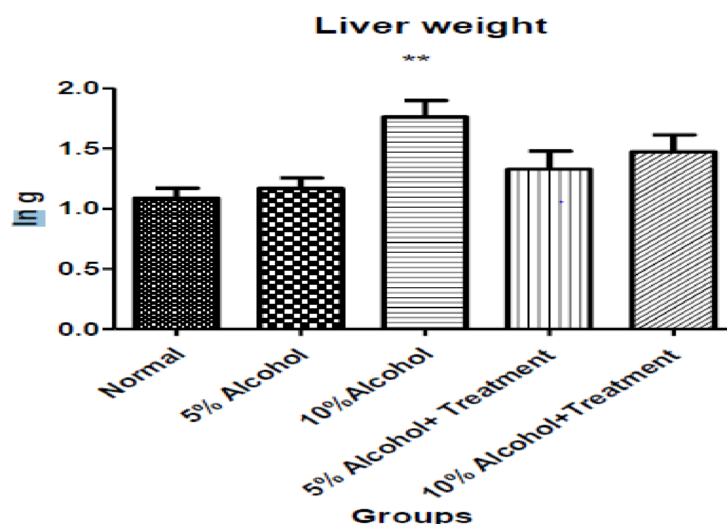

Figure 1.3. Liver weight of the mice

Relative liver weight: The alcoholic group animals showed increase in relative liver weight (3.912±0.245 and 4.562±0.469**)** compare to the normal group (3.413±0.243). The treatment group significantly decreased the relative liver weight when compare to alcoholic group (3.990±0.371 and 4.368±0.225).Where *p* is not significant compared to the all group. (Figure -1.4)

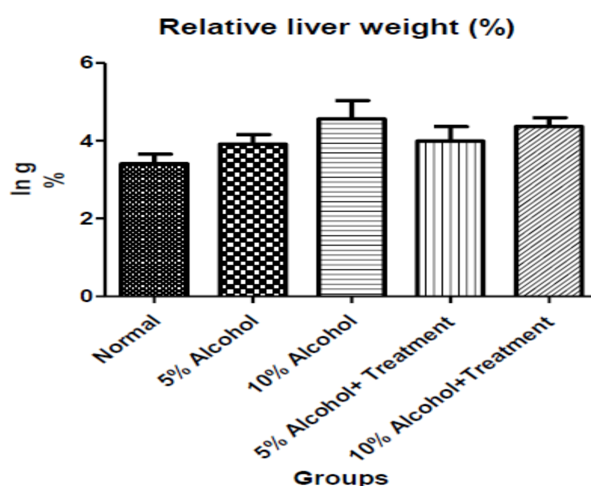

Figure 1.4. Relative liver weight of the mice

Biochemical estimations:

Estimation of liver function test: The significant increase in the alcoholic group in ALP (IU/L), ALT (IU/L), Total bilirubin (BIT) levels, AST (IU/L), conjugate and unconjugate bilirubin levels and (mg/dl) when compared to the normal group. Even on the other hand the treatment group have significantly decreased the all parameters compared to alcoholic group.
Conjugate Bilirubin: The bilirubin conjugate levels were significantly increased in alcoholic group (0.053±0.006 and 0.125±0.012) when differentiated with normal group (0.043±0.005). In the treatment group the levels were decreased when compared to the alcoholic groups (0.053±0.004 and 0.096±0.014)**.** Where *p* is not significant compared to the 5% alcoholic group and treatment group. Where***p<0.001 when compared to the 10% alcohol group and normal group (Figure 1.5).

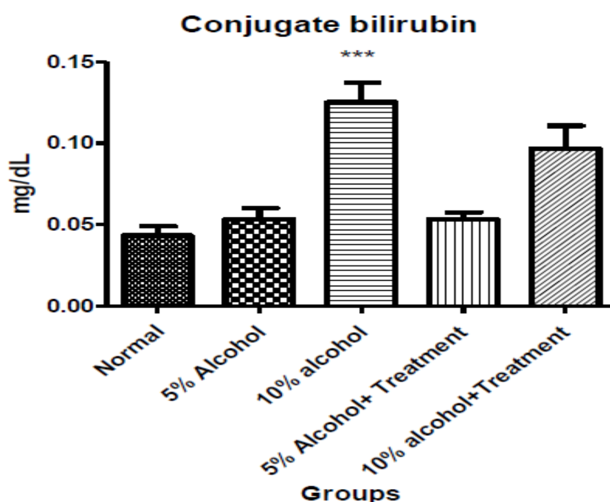

Figure 1.5. Conjugate bilirubin levels of mice

Unconjugate Bilirubin: The bilirubin unconjugate levels were significantly increased in alcoholic group (0.070±0.005 and 0.096±0.009) when differentiated to the normal group (0.060±0.005). In the treatment batch the levels were decreased when compared to the alcoholic groups (0.078±0.007 and 0.070±0.009)**.**Where *p<0.1 when normal is compared with the 10% alcoholic group (Figure 1.6). Values are demonstrated as Mean±SEM (n=6) by one-way ANOVA followed by Bonferroni test. Where *p<0.1 when normal is compared with the 10% alcoholic group.

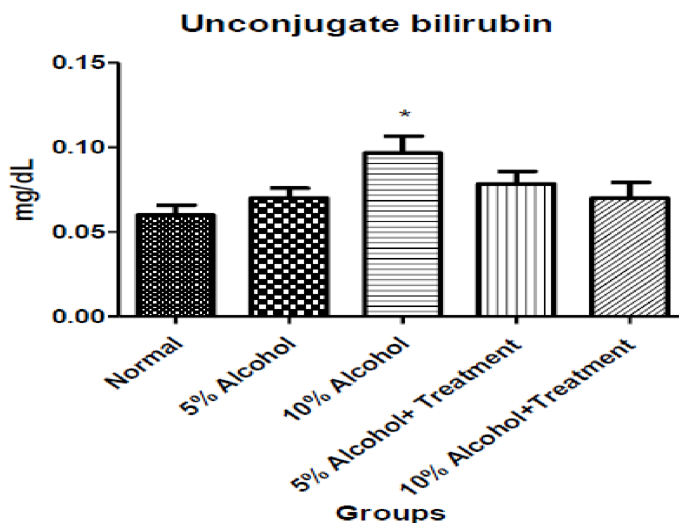

Figure 1.6. Unconjugate bilirubin levels of mice

Total bilirubin: These levels were found to be significantly increased in alcoholic group (0.118±0.004 and 0.181±0.009) when compared to the normal group (0.100±0.006). In the treatment group the levels were decreased when compared to the alcoholic groups (0.076±0.019 and 0.150±0.006).Where p is not significant compared to the 5% alcoholic and treatment group. Where ***p<0.001 when compared to the 10% Alcohol group and normal group (Figure 1.7).

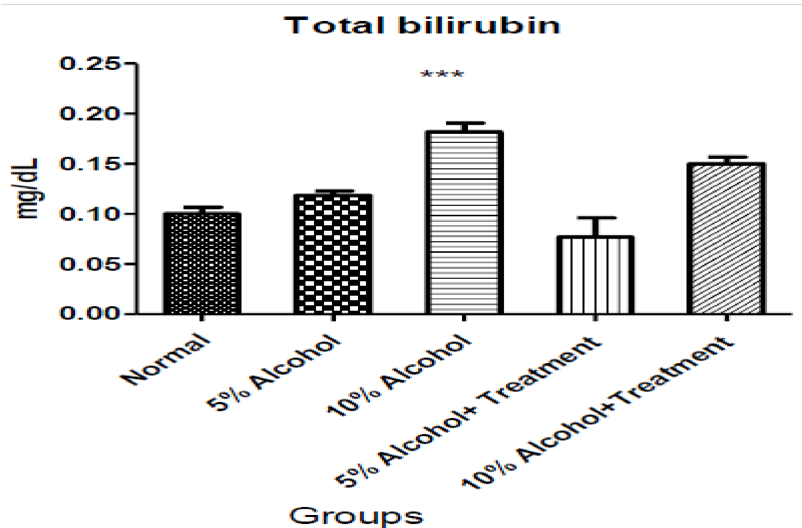

Figure 1.7. Total bilirubin levels of mice

SGOT or AST levels: AST levels were observed significantly increased in alcoholic group when (103.3±5.796 and 238.1±38.59) compared to the normal group (29.81±2.019).In the treatment group the levels were decreased when compared to alcoholic groups (91.80±4.49 and 199.2±23.34). Where *p* is not significant compared to the 5% alcoholic and treatment group. Where ***p<0.001 when compared to the 10% Alcohol group and normal group (Figure 1.8). Values are expressed as Mean±SEM (n=6) by one way ANOVA followed by Bonferroni test.Where*p* is not significant compared to the 5% alcoholic and treatment group. Where ***p<0.001 when compared to the 10% Alcoholic and normal group.

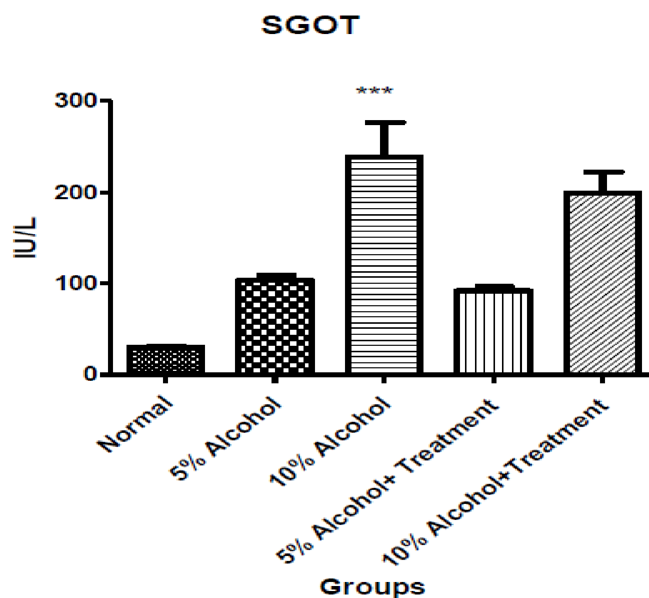

Figure 1.8. SGOT or AST levels of mice

SGPT or ALT levels: ALT levels were observed to be significantly increased in alcoholic group (84.28±9.269 and 114.9±5.347) when compared to the normal group (30.55±1.932). In the treatment group the levels were decreased when compared to the alcoholic groups (77.20±9.298 and 114.0±8.664).Where **p<0.01 when compared with 5% and ***p<0.001 when compared 10% with normal group (Figure 1.9).

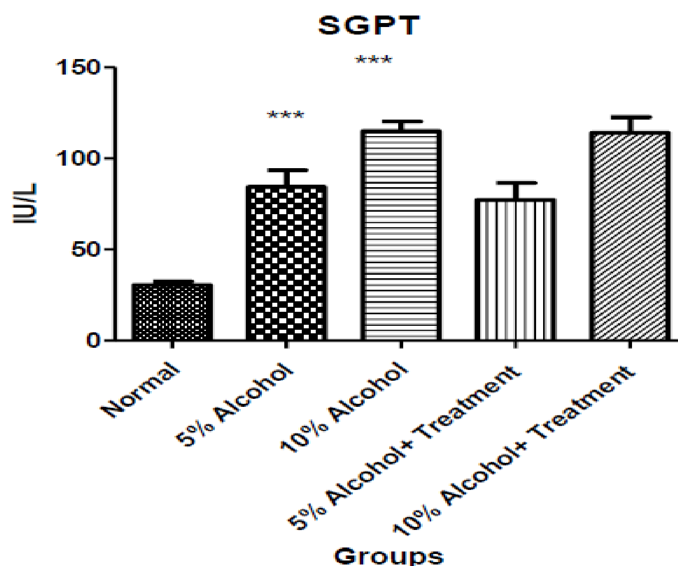

Figure 1.9. SGPT or ALT levels of mice

ALP levels: These levels were found to be significantly increased in alcoholic group (53.99±4.735 and 136.5±8.421) when compared to the normal group (46.00±4.286). In the treatment group the levels were decreased when compared to the alcoholic groups (50.88±7.119 and 120.0±13.84).Where ****p*<0.001 compared with the 10% Alcohol group (Figure 2.0). Values are shown in terms of Mean±SEM (n=6) by one-way ANOVA followed by Bonferroni test.Where *p* is not significant with 5% alcohol and treatment groups. Where ****p*<0.001 compared with the 10% Alcoholic with normal group.

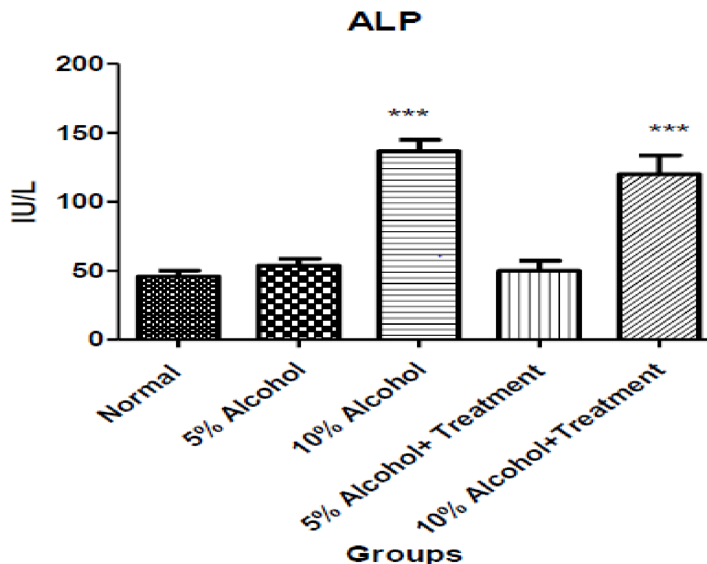

Figure 2. ALP levels of mice

NMR Result: 1H NMR spectra of serum from mice fed with alcohol. The 1H NMR spectra shows the presence of alcohol in the serum along with alcohol metabolism metabolites like acetate, sugar molecules. From the spectra it is also clear that there are many small metabolites like citrate, glutamine and branched amino acids are identified (Figure 2.1 – Figure 2.5).

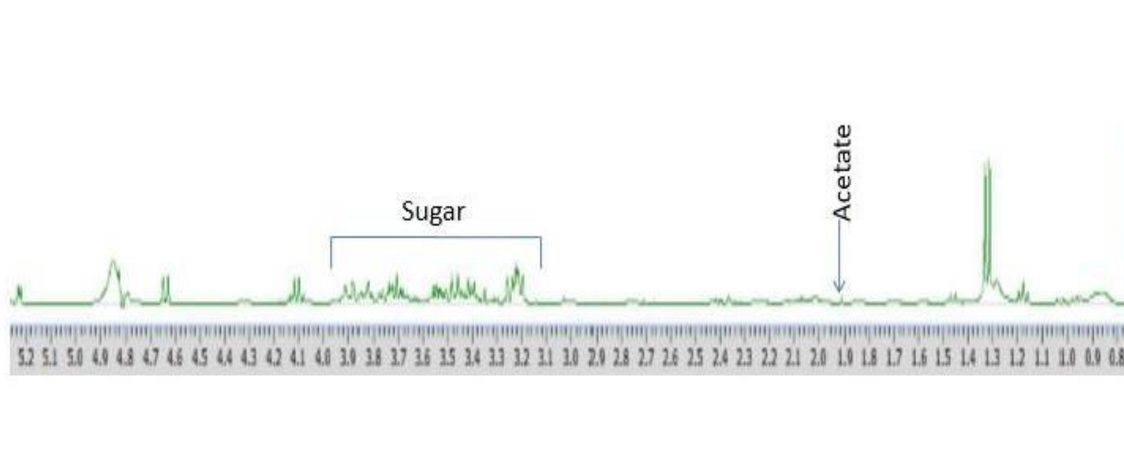

Figure 2.1. Normal Serum: The1H NMR spectra shows the existence of metabolites sugar and acetate molecules.

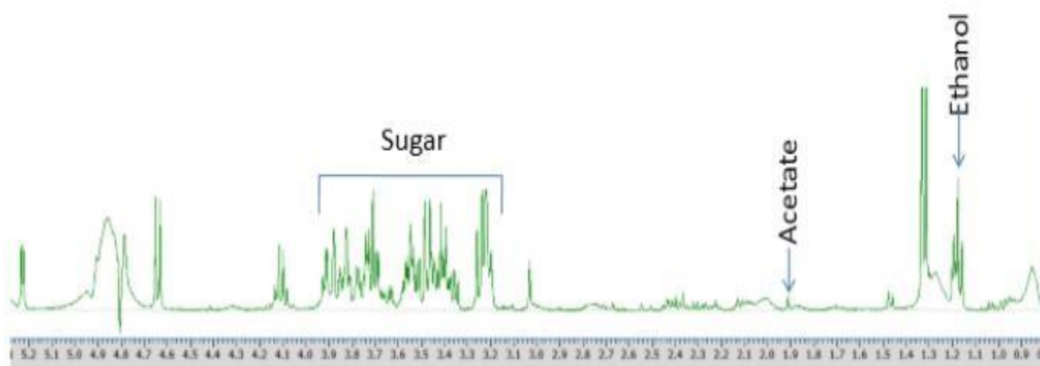

Figure 2.2. 5% Alcohol:1H-NMR spectra presents the alcohol metabolism along with acetate and sugar molecules. In this group the raise in acetate level compare to the normal group. The small metabolites were also present.

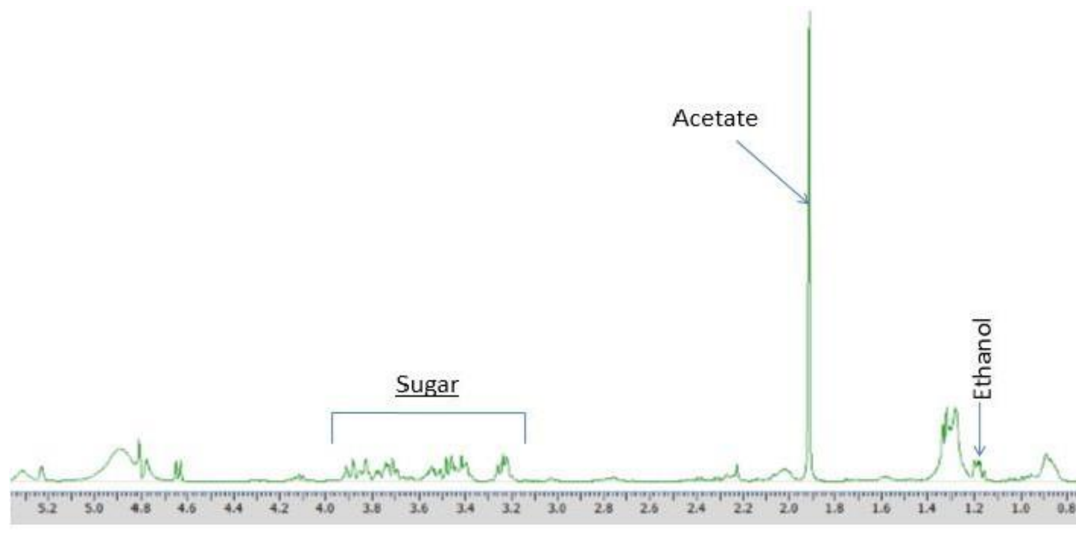

Figure 2.3. -10% Alcohol: 1H spectra of serum from mice fed with 10% alcohol

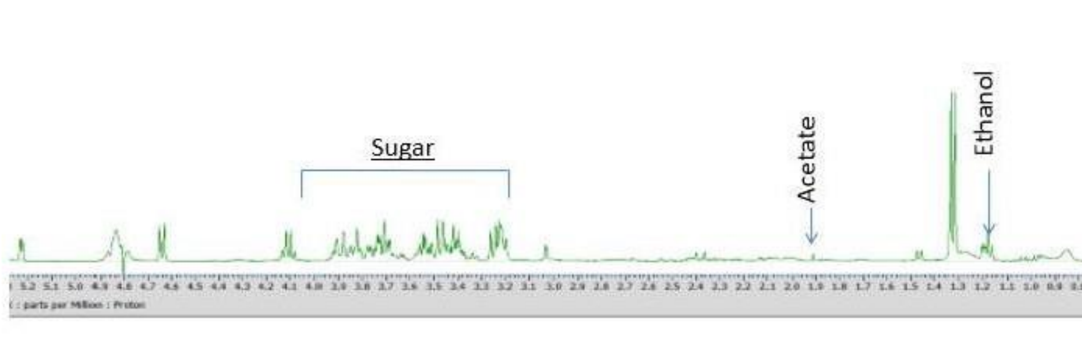

Figure 2.4. -5% Alcohol+ Treatment:1H spectra of serum from mice fed with 5% Alcohol+ treatment.

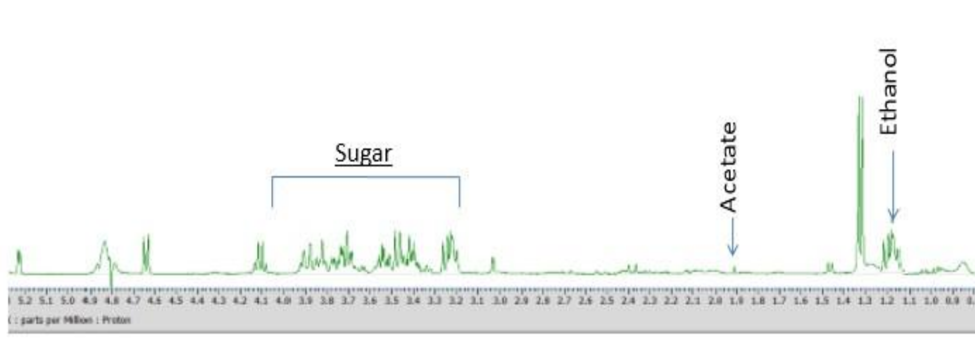

Figure 2.5. 10% Alcohol +Treatment:1H spectra of serum from mice fed with 10% Alcohol+ treatment.

Histopathological results:

Normal group:

In a case of group, central vein and sinusoidal congestion was seen but hepatic globular structure looks normal and there were no pathological changes (Figure 2.7).

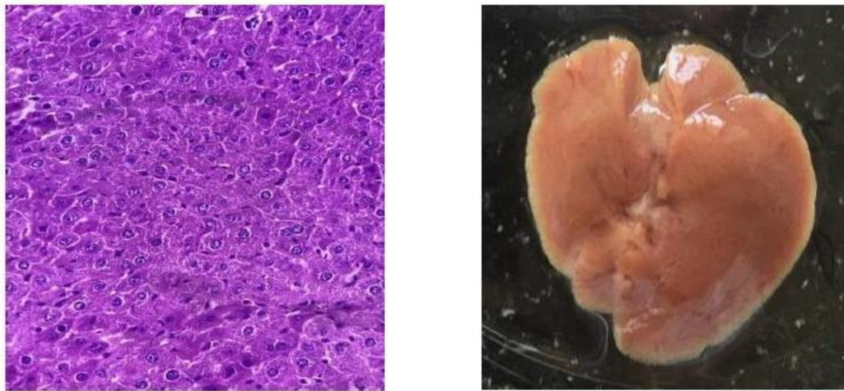

Figure 2.7. Normal Smouse liver (10x): Aspect of a mouse liver showing hepatocytes, Globular structure look like normal.

5% Alcoholic group: In this group the central vein and sinusoidal congestion was seen with fatty liver is characterized by the fat accumulation in the pericentral zone (Figure 2.8).

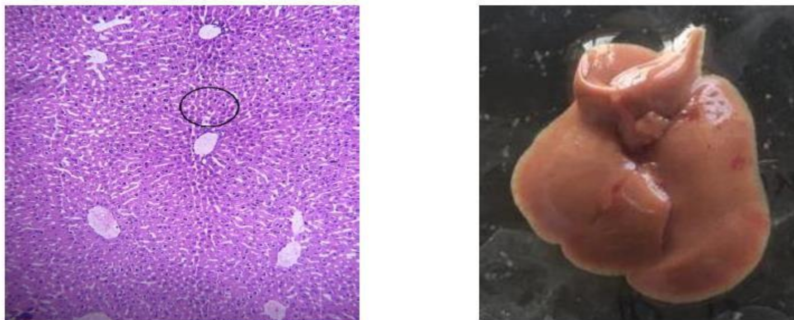

Figure 2.8. -5%Alcohol group (10x): Hepatocytes show vacuoles in the cytoplasm. Fatty change almost around the Central vein with inflammatory cells and infiltrate consisting of plasma cells with histocytes.

10% Alcoholic group: In this group the parenchymal inflammation occurs and fat accumulation in pericentral zone and ballooning hepatocytes (Figure 2.9).

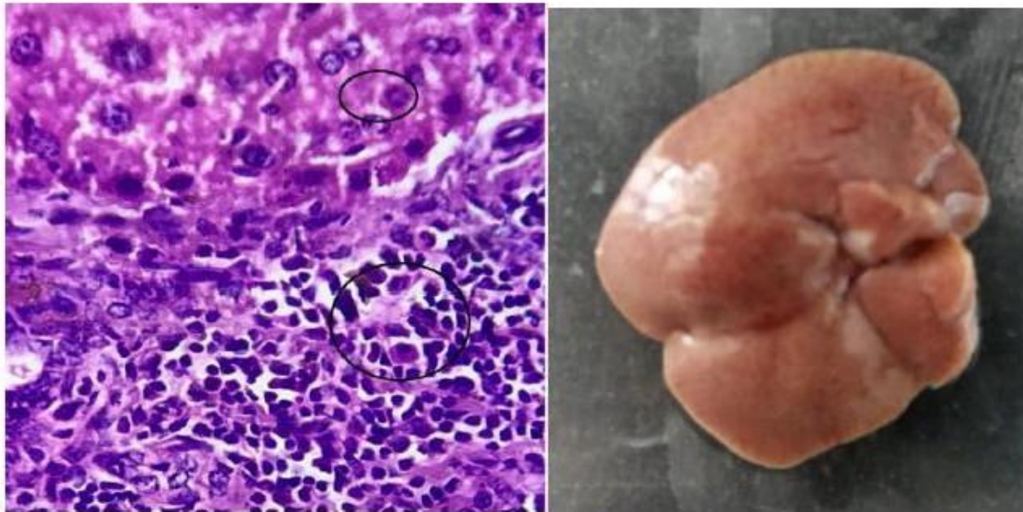

Figure 2.9. -10% Alcohol group (40x): The portal path have a dense infiltrate inflammatory cells. Majorly the hepatic fat globules are present. Bridging fibrosis forming a nodule.

5% Alcoholic + Treatment group: There is a significance decrease in the fat accumulation and after the treatment it just like a normal pathological condition. (Figure 3).

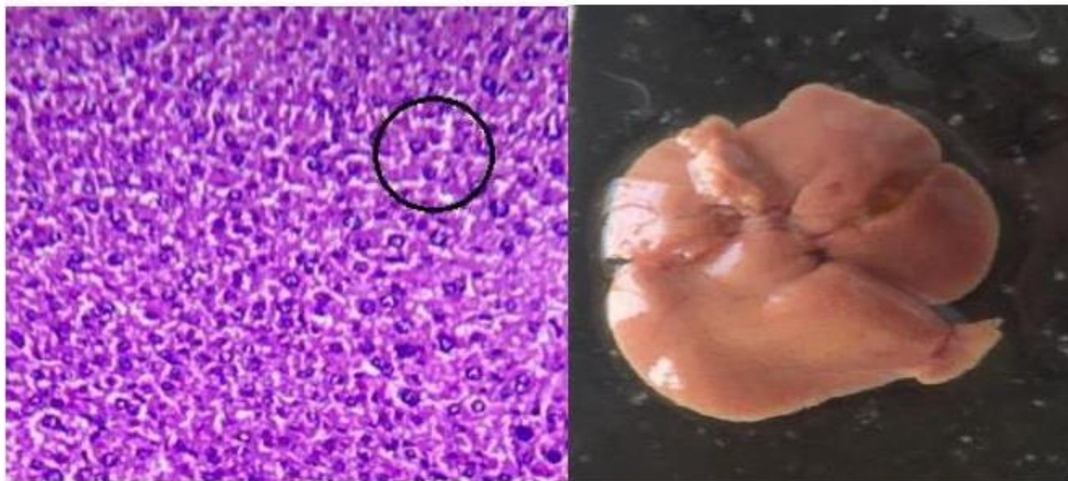

Figure 3. -5% Alcohol+ Treatment (20x): The fatty globules have gone and the liver showing central vein, hepatic artery and portal triad.

10% Alcoholic + Treatment group: There is a less decrease in the inflammation well. Very spores are present. (Figure 3.1).

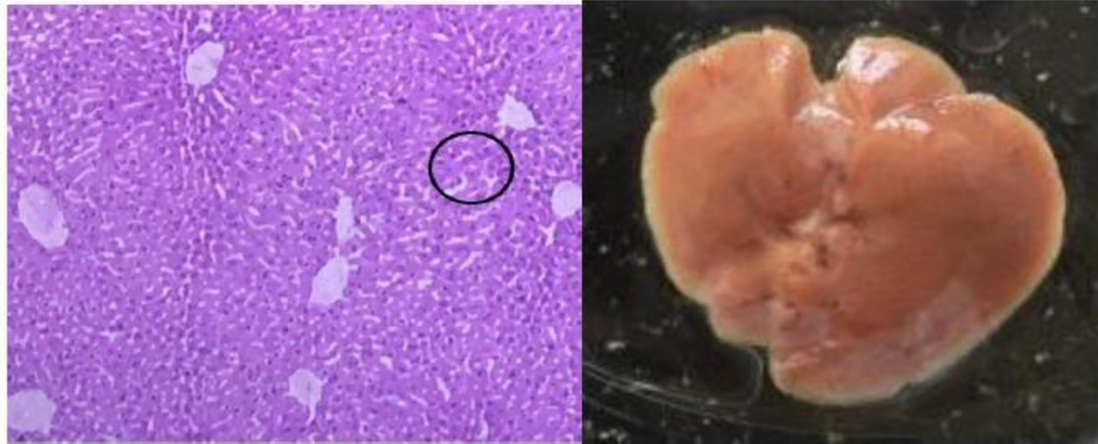

Figure 3.1. 10% Alcohol + Treatment (20x): Very spores are present and the inflammatory cells are present. The marked circle shows the hepatocytes ballooning.

Discussion

The previous studies shown that the blockage of triglycerides in the plasma due to the fat deposition. Multiple conditions causing in increase of cholesterol levels equally increases triglycerides enzymes that are sensitive to cytotomic injury .These are serum glutamic pyruvic transaminase (SGPT) now called Alanine amino transferase (ALT) and serum glutamic oxaloacetic transferase (SGOT) that is well known as Asparatate amino transferase (AST). They both are available in liver with high concentrations. The results of this study revealed that, inducing of alcohol to the mice causes the liver damage based upon the quantity of alcohol taken. Smaller quantity of alcohol intake produces less hepatic changes that can be reversible. If more quantity of alcohol is taken the liver damage results stage by stage hepatic changes leading to the cirrhosis, which is the last stage of ALD and it is not reversible. The hepatic changes or the liver damage were identified by the increase in the serum levels of AST, ALP, Bilirubin and these values are compared with the normal and treatment groups [4].

The onset of the disease starts with the impairment of liver cells with altered functional transition, causing membrane permeability as well the expelling of enzymes into the extracellular space. When there is membrane destruction or necrosis on hepatocytes the enzymes rushes into the circulations and this confirms the hepatic damage. In the present study increased levels of marker enzymes that are observed in alcohol treated group II, III. Whereas the decreased levels of enzymes were seen in the treatment group IV and V.

In the metabolism of lipids, a carbohydrates and proteins liver plays a major role. ALD shows a splendid growth in triglyceride, total cholesterol and LDL levels and declining the HDL levels. Cholesterol levels are increased due to the raise in the esterification of fatty acids β-oxidation and excretion of cellular lipids. The collection of triglyceride in the liver may occur due to the exhaustion of lysosomal lipase and VLDL secretion. Antioxidant enzymes destructs the cells therefore antioxidant activity and inhibition of free radical production are necessary in preventing in the ALD. The body has influence on defence mechanism to avoid and neutralise free radicals. This is regulated by the antioxidants such as Glutathione, Superoxide dismutase and catalyse. ROS is detoxified from anti-oxidant system consists both enzymatic and non-enzymatic anti-oxidants [6].

The imbalance between ROS production and anti-oxidant defence mechanism causes the oxidative stress resulting in hepatic changes. When the disease progress, the hyperbilirubinemia reflects the pathology of liver and helps in measuring the binding as well conjugation with excretory capacity of liver cells. Another cause of impairment of the liver cells is lipid peroxidation which occurs

between the amount of free radicals and antioxidant in the body. When the hepatic changes occur, the amount of oxidants is more than the amount of anti-oxidants. These extra oxidants can be bound with body's crucial compounds involving double bonds of membrane and causes the destruction to the liver cells. As Lipid peroxidation progress, number of degraded products affects the cell membrane of the liver. In the present study it is observed that alcohol induced liver damage is increased in all test groups through liver function test when compared with normal control groups. CYP2E1 enzyme was the major contributor for the increased serum levels and liver damage. The increased enzyme activity occurs due to induced alcohol. In this study, the treatment with Cadihep has significantly decreased levels of all liver function tests when compared to alcoholic group.

From the study, it was found that the effect of alcohol inducing in mice resulted in increased biochemical and histopathological changes. The histopathological changes incorporate expanded entrance vein endotoxin fat and kupffer cell, TNF-α creation and decrease in the coursing adiponectin. It also results in inflammation, ballooning hepatocyte, scarring of liver cells and spotty necrosis in alcoholic induced group. When the treatment is given using Cadihep syrup it reduces the action of all the histopathological changes.

Previous studies show that liver biopsy method was majorly adopted for detecting of ALD. Recent studies have revealed that metabolomics is new technique for detection of ALD. Metabolomics is a new technique that deals with the study of molecular phenotype and cellular metabolism in terms of small metabolites and it is useful tool for the clinicians in the diagnostic purpose in ALD. In the present study NMR technique is used. Through the NMR technique the altered level in sugar and acetate was found. It is also observed that small metabolites like citrate, glutamine and branched amino acids also shows increased levels resulting in conformation of ALD.

Glutamine is metabolically linked with ammonium detoxification in the body by the hepatocytes. Altered levels of glutamine reflect increased hepatic dysfunction and destruction of liver cells. The lower level of glutamine with higher level of glutamate indicates the shift in the production in the glutamate and releasing of ammonium by impairing the ammonium detoxification ability. In the hepatocyte injury there is altered glucose signal intensity leading to alteration in the glycolysis and glucose production in the liver.

Thus it is concluded that the use of histology, liver function test and NMR in detecting of ALD where the NMR technique has a potential similarity to other techniques in the early detection of ALD. The NMR method helps in precise discovery of changes in liver cells and in assessment of compounds, motioning of little metabolites, which are in charge of lipidomic changes in the liver. Consequently, NMR method is helpful in understanding the instrument of beginning periods of ALD [5].

Conclusion

In this research, the present models are expected to establish the alcoholic liver disease by closely mimicking the liver injury which increases the possibility of liver injury complications. The present study revealed that the hepatoprotective activity of marketed liver tonic Cadihep showed the effect of decreased levels of liver enzyme marker in the mouse alcoholic liver disease model.

The observation in the result suggests that the Cadihep syrup has reduced the levels of elevated serum marker levels ALT, AST, ALP, Total bilirubin and conjugate and unconjugate bilirubin in the treatment group but in case of alcoholic induced model there is an increased level of all biochemical estimations when compared to the normal groups. The histopathology features have changed when normal is compared with the alcoholic groups. The 5% treatment group almost recovered like a normal group. But in the 10% treatment group it has not shown significance decrease in the biochemical estimations as well in the histopathology studies. In the NMR estimation the acetic acid derivation levels are expanded in the alcoholic groups, when contrasted and the ordinary and treatment gathering. From spectra with alcohol

metabolism the acetate and sugar molecules were present and the small molecules like citrate, glutamine and branched amino acids are present and these metabolites are compared with the normal groups.

Universally there is no accepted therapy to treat or hold the progression of ALD but many potential therapies that target at least one of these potential mechanisms are being examined. Alcohol can damage the liver through various mechanisms so one therapy will not be sufficient to treat ALD. Dietary care, life style modification and pharmacologic intervention together may help in effective therapy.

Clinically ALD is diagnosed by measuring liver enzymes like AST, ALP, ALT, Total and direct bilirubin and liver biopsy is a gold standard method. However multiple biopsies are required that are invasive due to high heterogeneity of liver. Even though imaging modalities like CT scan and MRI available these techniques are preferred at end of the diagnosis process. The early detection of ALD is important for ALD prevention and control. The NMR technique involved in detection of disease includes small metabolites like glutamine, citrate, and branched amino acids. The NMR data are supported by blood chemistry and histology data of our study indicating that NMR spectroscopy has great potential for diagnosis of ALD .

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