

Pioglitazone, quercetin and hydroxy citric acid effect on hepatic biomarkers in Non Alcoholic Steatohepatitis

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ABSTRACT

Background: Non alcoholic steatohepatitis (NASH), severe form of diseases belonging to the spectrum of the Non alcoholic fatty liver disease (NAFLD). It is an asymptomatic disease which leads to fibrosis and finally to cirrhosis, an end stage liver disease. **Objective:** To study the effect of pioglitazone, quercetin and hydroxy citric acid on hepatic biomarkers and various biochemical parameters in experimentally induced non alcoholic steatohepatitis (NASH). **Materials and Methods:** Male Wister rats were divided into 8 groups. The activities of alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and γ -Glutamyl Transferase (GGT) were assayed in serum. The levels of various other biochemical parameters such as serum albumin, total bilirubin, creatinine, urea, uric acid and glucose were also estimated in experimental NASH. **Results:** The NASH group produced severe liver injury by significantly increasing the serum levels of ALT, AST, GGT and LDH compared with that of the control. However, the experimental NASH rats treated with pioglitazone, with quercetin and with hydroxy citric acid showed an obvious decrease in ALT, AST, GGT and LDH levels when compared with that of NASH induced group. A significant increase in the levels of albumin, creatinine, urea, uric acid, glucose and total bilirubin was noticed in experimentally induced NASH group (group 2) when compared to rats in control group (group 1). **Conclusion:** It could be inferred from this study that, pioglitazone, quercetin and hydroxy citric acid may afford protection to the liver against NASH, as evidenced by the results of this study on the levels of various biochemical parameters such as glucose, urea, uric acid, creatinine and bilirubin. Whereas from the results of hepatic marker enzymes, it is evident that optimal protection was observed after quercetin treatment against experimental NASH whereas pioglitazone and hydroxy citric acid also confers protection to some extent against NASH.

Key words: Biochemical parameters, experimentally induced NASH, hydroxy citric acid, liver marker enzymes, non alcoholic steatohepatitis, pioglitazone, non alcoholic fatty liver disease, non alcoholic steatohepatitis, quercetin

INTRODUCTION

Noninvasive panels of serological markers have been developed to evaluate the presence of steatosis and hepatic necroinflammation to avoid liver biopsy. Avoiding liver biopsy is desirable because it has certain disadvantages as it is an invasive procedure, prone to sampling errors and suffers from inter-observer variability.^[1] Till date, no study

has demonstrated that a single biomarker or a panel of biomarkers can be used as an alternative method to liver biopsy to diagnose NASH. Since, the majority of NASH patients are asymptomatic, the specific investigation usually begins after detection of abnormal liver enzymes on routine evaluation.^[2]

Since, the pathogenesis of NASH involved interplay of three possible mechanisms^[3] such as hyper-insulinemia, lipotoxicity and oxidative stress, we have chosen three categories of drugs, pioglitazone as insulin sensitizer, quercetin as hepatoprotectant and antioxidant and hydroxy citric acid (HCA) as a lipid lowering agent and anti-obesity agent.

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Pioglitazone hydrochloride is a widely used drug in the treatment of insulin resistance diabetes.^[4] Quercetin is a plant-derived substance, or a phytochemical, which is known as a flavonoid.^[5,6] Emerging research suggests that quercetin may reduce the risk of upper respiratory tract infection during intense physical exercise, which is likely attributable to its antioxidant, anti-inflammatory and anti-pathogenic effects.^[6] The physiological and biochemical effects of (-)-HCA have been studied extensively for its unique regulatory effect on fatty acid synthesis, lipogenesis, appetite and weight loss.^[7] The derivatives of (-)-HCA have been incorporated into a wide range of pharmaceutical preparations in combination with other ingredients for the claimed purpose of enhancing weight loss, cardioprotection, correcting conditions of lipid abnormalities and endurance in exercise.^[8,9]

MATERIALS AND METHODS

The experimental model of NASH in rats by feeding high fat diet for 8 weeks^[10] and this model were used to conduct a comparative study of role of pioglitazone, quercetin and hydroxy citric acid on various parameters in experimental model of non alcoholic steatohepatitis. Male Wistar rats weighing approximately 150gm were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in control rooms with 12 h light/dark cycle. The animals received commercial rat diet, standard diet, high-fat diet and water *ad libitum* as per the experimental protocol. This study conformed to the guiding principles of Institutional Animal Ethical Committee (IAEC), Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Guide for the care and use of laboratory animals (IAEC Approval Number: 001/006/2010 and 01/007/2011).

The Male wistar rats selected for the study were divided into 8 groups as follows:^[11]

Group 1; Controls (n = 6): Control rats received regular standard diet for 8 weeks.

Group 2; NASH (n = 6): Rats induced with NASH by feeding high fat diet for 8 weeks.

Group 3; Pioglitazone Control (n = 6): Rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of Pioglitazone (4 mg/kg. b.wt) (0.5% methyl cellulose *w/v*) for further 4 weeks.

Group 4; Quercetin Control (n = 6): Rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of quercetin (20 mg/kg. b.wt) dissolved in 1% DMSO *v/v* for further 4 weeks.

Group 5; Hydroxy Citric Acid Control (n = 6): Rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of hydroxy citric acid (150 mg/kg. b.wt) for another 4 weeks.

Group 6; NASH + Pioglitazone (n = 6): Rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of Pioglitazone (4 mg/kg. b.wt) (0.5% methyl cellulose *w/v*) for another 4 weeks.

Group 7; NASH + Quercetin (n = 6): Rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of quercetin (20 mg/kg. b.wt) dissolved in 1% DMSO *v/v* for another 4 weeks.

Group 8; NASH + Hydroxy Citric Acid (n = 6): Rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of Hydroxy Citric Acid (150 mg/kg. b.wt) for another 4 weeks.

After the experimental period, the animals were sacrificed after 12 h of fasting by cervical decapitation and blood was collected, centrifuged for 5 min at 3000 rpm/min and serum was stored at -70°C from that various biochemical analysis were carried out. Alkaline phosphatase (ALP) was assayed by the method of King.^[12] The activity of aspartate transaminase (AST) was assayed by the method of Mohur.^[13] The activity of alanine transaminase (ALT) was assayed by the method of Mohur.^[13] The activity of lactate dehydrogenase (LDH) was measured by the method of Nieland.^[14] The Max Discovery™ γ -Glutamyl Transferase (GGT) Enzymatic Assay Kit uses an enzymatic reaction to measure enzyme levels in serum according to the model of Moss and Handerson.^[15] The levels of various other biochemical parameters such as serum albumin, total bilirubin, creatinine, urea, uric acid and glucose were estimated by using standard kits available commercially.

RESULTS

The effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum liver marker enzymes in experimental NASH were depicted in Table 1 and Figures 1-6. The experimental rats in NASH group (group 2) produced severe liver injury by significantly increasing the serum levels of ALT, AST, GGT and LDH compared with that of the control. Table 2 and Figures 7-12 depicts the effect of pioglitazone, quercetin and hydroxy citric acid on the levels of other biochemical parameters such as serum albumin, total bilirubin, creatinine, urea, uric acid and glucose in experimental NASH.

The experimental rats in NASH group (group 2) produced severe liver injury as evidenced by the significantly increased levels of ALT, AST, GGT and LDH in serum, compared with that of the control.

Table 1: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum liver marker enzymes in experimental NASH

Parameter	Group 1: Control	Group 2: NASH	Group 3: Pioglitazone control	Group 4: Quercetin control	Group 5: HCA control	Group 6: NASH+ Pioglitazone	Group 7: NASH+ Quercetin	Group 8: NASH+ HCA
ALP (IU/l)	119.1±8.4	121.6±10.1	109.6±7.4	104.5±7.3	118.4±9.1	118.7±9.5	120.3±8.6	126.4±8.2
ALT (IU/L)	55.5±3.4	187.4±11.9*	51.3±3.1	55.2±4.1	59.9±4.9	97.1±8.7 ^a	84.2±7.2 ^a	130.3±10.8 ^b
AST (IU/L)	68.9±3.8	107.4±8.9*	66.4±2.4	63.9±3.6	71.6±5.3	80.9±7.3 ^c	74.3±4.9 ^a	81.6±7.4 ^c
ALT: AST	0.80±0.07	1.7±0.02*	0.77±0.06	0.86±0.08	0.83±0.06	1.20±0.02 ^a	1.13±0.1 ^a	1.60±0.13 ^c
LDH (IU/L)	236.4±21.4	449.9±32.8*	189.4±11.6	185.3±11.4	237.4±11.6	308.3±19.6 ^a	278.6±24.5 ^a	218.2±21.9 ^a
GGT (IU/L)	29.2±1.4	59.9±3.8*	26.7±1.7	36.8±2.1	30.5±2.4	43.1±2.7 ^a	34.7±1.9 ^a	30.9±2.4 ^a

Values are expressed as mean±SEM for 6 animals in each group. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

Table 2: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of various biochemical parameters in experimental NASH

Parameter	Group 1: Control	Group 2: NASH	Group 3: Pioglitazone control	Group 4: Quercetin control	Group 5: HCA control	Group 6: NASH+ Pioglitazone	Group 7: NASH+ Quercetin	Group 8: NASH+ HCA
Albumin (g/dl)	2.5±0.2	4.58±0.3*	2.41±0.19	2.59±0.22	2.43±0.25	3.6±0.3 ^c	3.8±0.1 ^c	4.0±0.2
Total Bilirubin (mg/dl)	0.9±0.07	10.6±0.89*	0.81±0.07	0.63±0.05	0.64±0.04	2.6±0.01 ^a	1.1±0.02 ^a	8.5±0.41 ^c
Creatinine (mg/dl)	1.06±0.09	2.9±0.01*	1.89±0.17	1.7±0.19	1.4±0.13	1.4±0.01 ^a	1.5±0.01 ^a	1.7±0.01 ^a
Urea (mg/dl)	13.5±1.6	28.7±1.5*	17.6±1.6	16.4±1.3	14.3±1.2	24.1±1.4 ^c	19.4±1.6 ^a	20.6±1.9 ^b
Uric acid (mg/dl)	3.36±0.29	9.01±0.58*	3.45±0.31	3.67±0.32	3.81±0.33	5.18±0.43 ^a	5.13±0.38 ^a	4.35±0.25 ^a
Glucose (mg/dl)	107.8±8.1	160.5±9.2*	112.4±7.5	103.6±8.1	114.2±9.3	110.5±10.4 ^b	120.4±11.4 ^c	128.8±10.8 ^c

Values are expressed as mean±SEM for 6 animals in each group. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

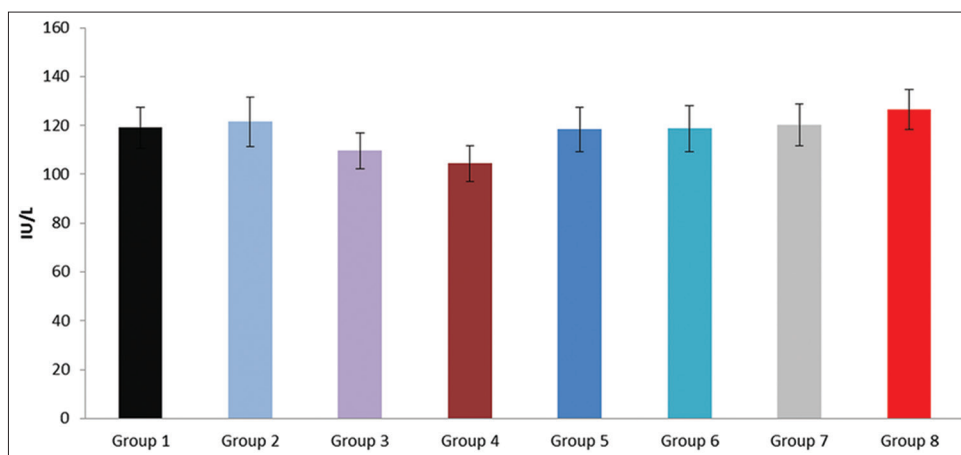


Figure 1: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum alkaline phosphatase (ALP) in experimental NASH

However, the experimental NASH rats treated with pioglitazone (group 6; NASH + pioglitazone), with quercetin (group 7; NASH + quercetin) and with hydroxy citric acid (group 8; NASH + HCA) showed an obvious decrease in ALT, AST, GGT and LDH levels when compared with that of NASH induced group (group 2). Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control), with quercetin (group 4; quercetin control) and with hydroxy citric acid (group 5; HCA control) does not show any significant effect on the liver marker enzymes compared to control group (group 1).

A significant increase in the levels of albumin, creatinine, urea, uric acid, glucose and total bilirubin was noticed in experimentally induced NASH group (group 2) when compared to rats in control group (group 1). The experimental NASH rats treated with pioglitazone (group 6; NASH + pioglitazone), with quercetin (group 7; NASH + quercetin) and with hydroxy citric acid (group 8; NASH + HCA) showed significant reduction in serum albumin, total bilirubin, creatinine, urea, uric acid and glucose levels when compared with that of NASH induced group (group 2). Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone

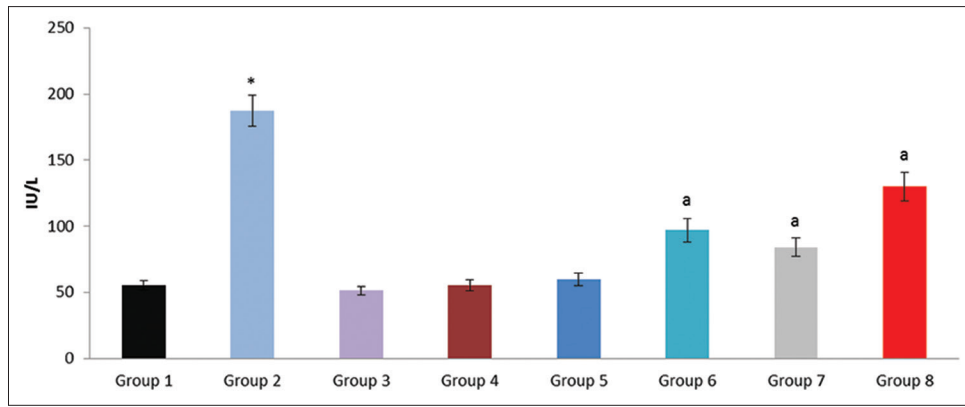


Figure 2: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum alanine transaminase (ALT) in experimental NASH. * $P < 0.001$ compared to control group; ^a $P < 0.001$ compared to NASH group; ^b $P < 0.01$ compared to NASH group; ^c $P < 0.05$ compared to NASH group

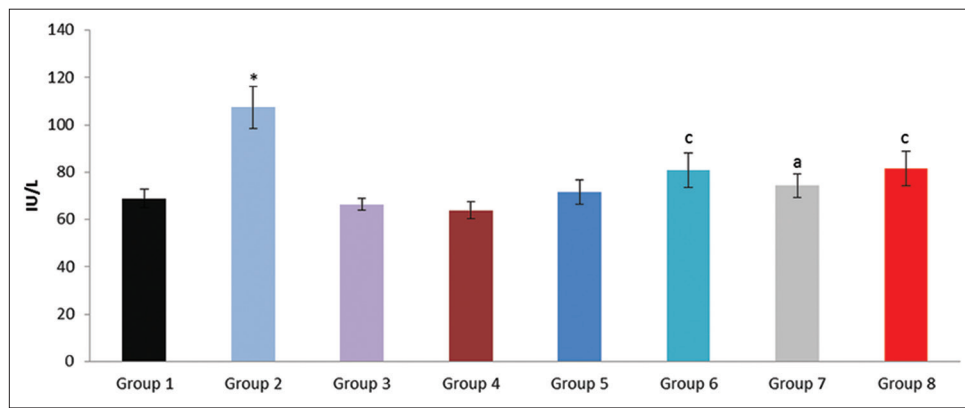


Figure 3: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum aspartate transaminase (AST) in experimental NASH. * $P < 0.001$ compared to control group; ^a $P < 0.001$ compared to NASH group; ^b $P < 0.01$ compared to NASH group; ^c $P < 0.05$ compared to NASH group

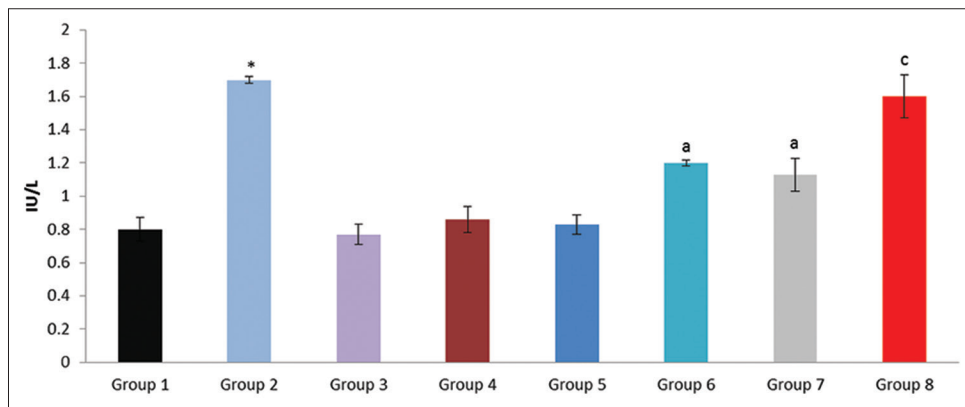


Figure 4: Effect of pioglitazone, quercetin and hydroxy citric acid on the ratio of serum alanine transaminase (ALT) and aspartate transaminase (AST) in experimental NASH. * $P < 0.001$ compared to control group; ^a $P < 0.001$ compared to NASH group; ^b $P < 0.01$ compared to NASH group; ^c $P < 0.05$ compared to NASH group

control), with quercetin (group 4; quercetin control) and with hydroxy citric acid (group 5; HCA control) does not show any significant effect on serum albumin, total bilirubin, creatinine, urea, uric acid and glucose compared to control group (group 1).

DISCUSSION

Laboratory tests that are routinely included in the evaluation of patients with suspected NASH include a serum panel of liver tests (alanine aminotransferase, ALT,

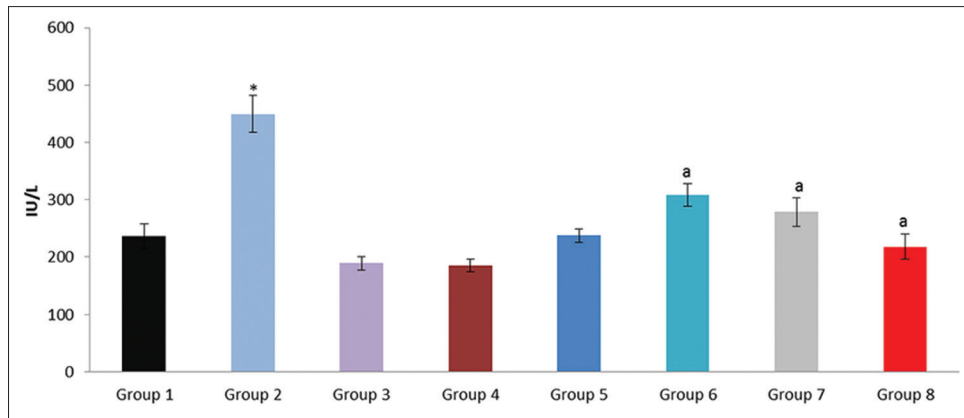


Figure 5: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum lactate dehydrogenase (LDH) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

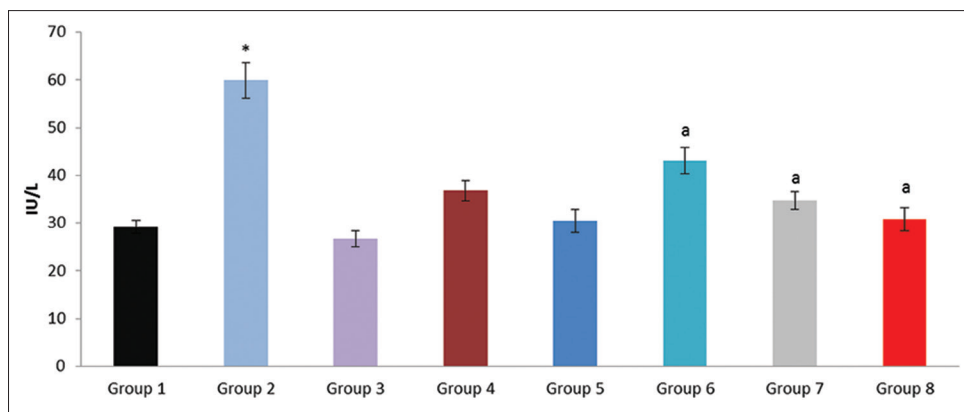


Figure 6: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum gamma glutamyl transferase (GGT) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

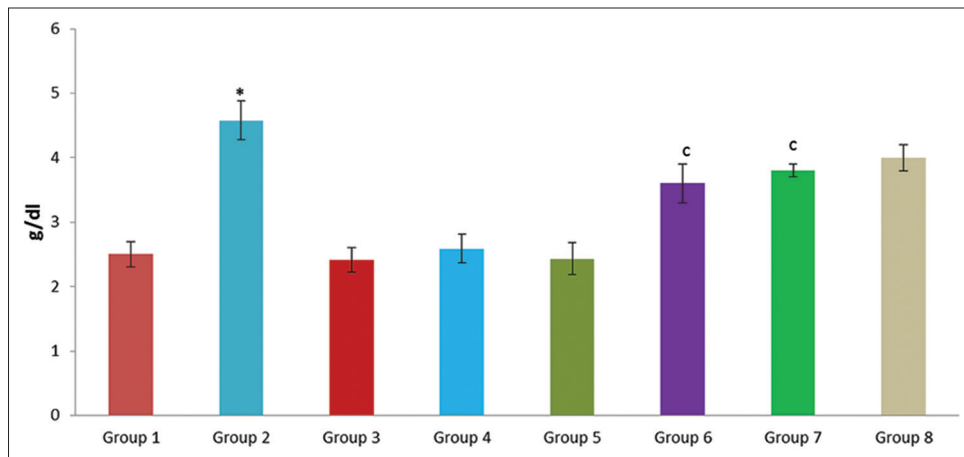


Figure 7: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum albumin in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

aspartate aminotransferase AST, alkaline phosphatase, ALP, gamma-glutamyl-transpeptidase, GGT) albumin, prothrombin time and complete blood counts. Serum aspartate aminotransferase (AST) and more commonly, alanine aminotransferase (ALT) show mild to moderate elevation in NASH patients. Therefore, the most

common laboratory abnormalities of patients with NAFLD are mild to moderate (twofold to threefold) elevations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), or both.^[16] However, as fibrosis advances, this ratio can reverse and lose its diagnostic value in assessing steatohepatitis. Other liver

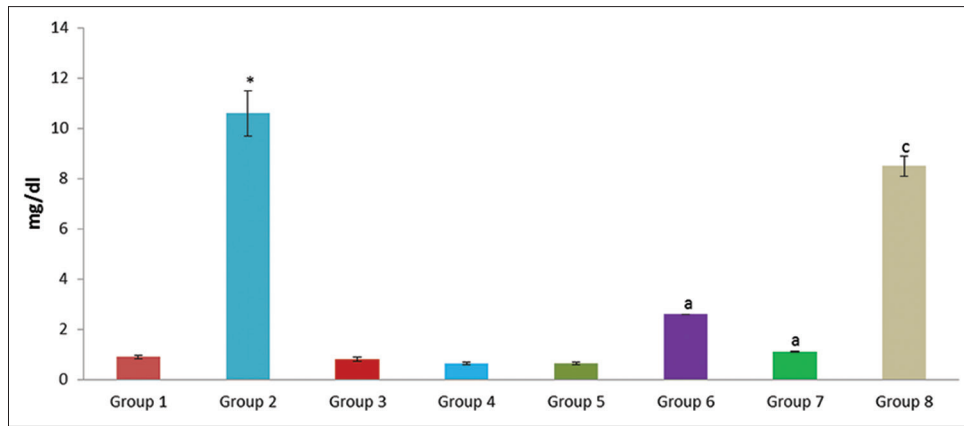


Figure 8: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum total bilirubin in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

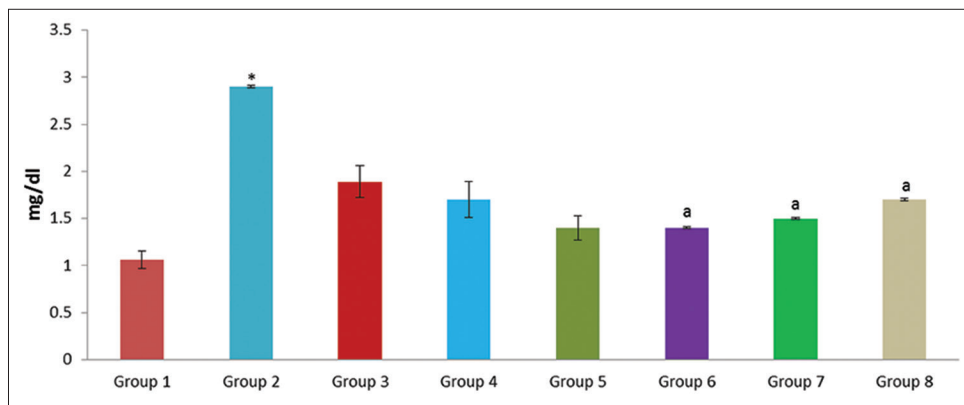


Figure 9: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum creatinine in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

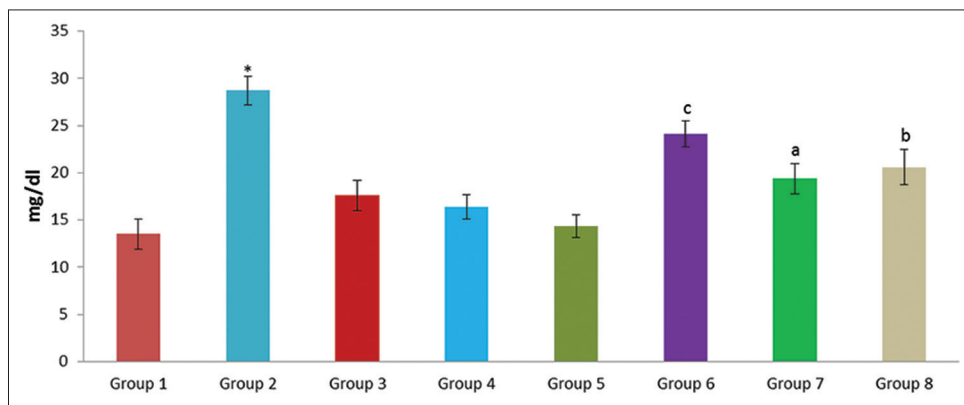


Figure 10: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum urea in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^bP<0.01 compared to NASH group; ^cP<0.05 compared to NASH group

enzymes (gamma-glutamyl transferase) may be elevated two to three times above the normal range.^[17]

Drugs like troglitazone (now withdrawn because of hepatotoxicity), rosiglitazone and pioglitazone proved to improve the serum aminotransferase levels.^[18] Thus, considering the markers of liver damage, GGT, ALT and AST were correlated to the severity of the hepatic damage

and also stated that amino transferase abnormalities probably occur at an earlier stage, different from GGT that would require a greater hepatic damage to be altered. The role of GGT, as a molecular marker for disease severity and diagnostics is still obscure in NASH. The findings of our present study were supported by various other studies report which showed a significant difference between GGT level and the severity of liver fibrosis.^[19]

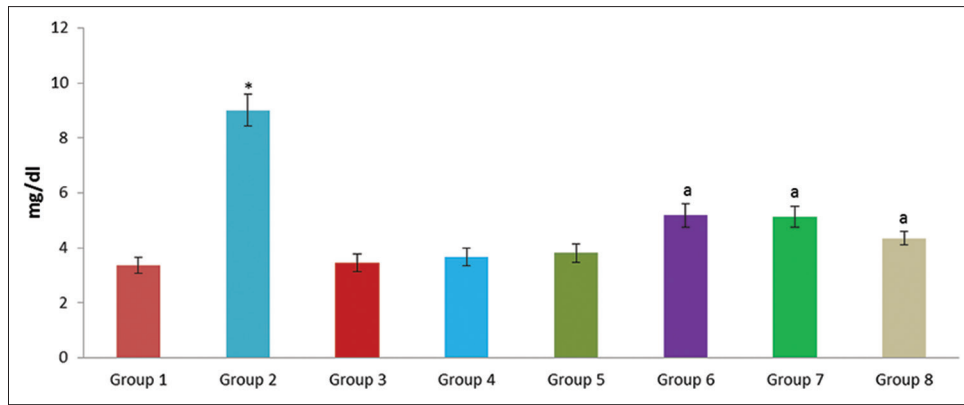


Figure 11: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum uric acid in experimental NASH. * $P < 0.001$ compared to control group; ^a $P < 0.001$ compared to NASH group; ^b $P < 0.01$ compared to NASH group; ^c $P < 0.05$ compared to NASH group

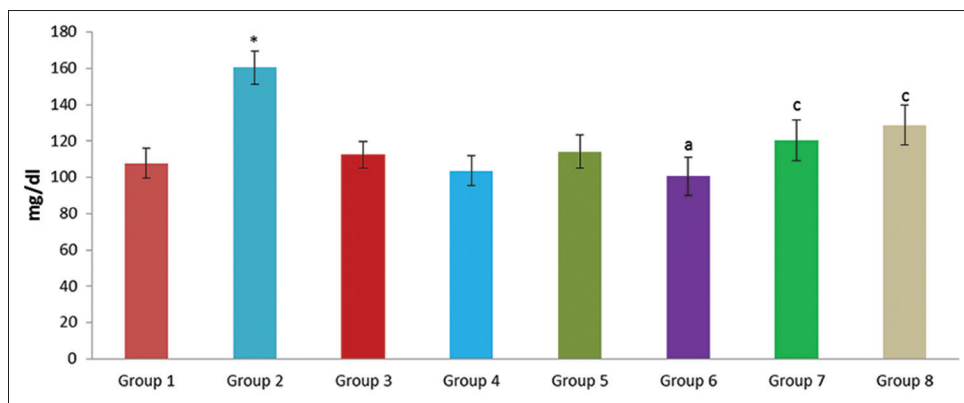


Figure 12: Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of blood glucose in experimental NASH. * $P < 0.001$ compared to control group; ^a $P < 0.001$ compared to NASH group; ^b $P < 0.01$ compared to NASH group; ^c $P < 0.05$ compared to NASH group

NASH is typically associated with an ALT level greater than the AST level^[16, 20] in the absence of cirrhosis. Elevation of aminotransferase levels were widely used to diagnose NASH but the estimation of amino transferase levels lacks adequate sensitivity to detect patients with or without NASH and are entirely nonspecific in predicting liver injury.^[21] Age >45 years, the presence of obesity or type 2 diabetes mellitus and ALT/AST ratio >1 have been identified as independent predictors of the liver fibrosis.^[17] The ratio between alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was predictive for the severity of the liver disease, with an ALT/AST ratio >1 suggesting cirrhosis or advanced fibrosis.^[17,22]

The degree of elevation of aminotransferases does not correlate with the severity of steatosis or fibrosis if the elevation is not higher than four times of the upper limit of normal.^[23] In the majority of cases, ALT/AST ratio is >1. Higher AST and ALT levels and ALT/AST ratio were significantly associated with NASH. The findings of this study were in accordance with the previous findings. Increased lactate dehydrogenase values were observed in patients with biopsy proven NASH. Also, increased levels of GGT and LDH were associated with

necro-inflammatory activity.^[24] The activity of GGT was an indicator of hepatic damage and used as sensitive marker in the diagnosis of hepatic diseases and as a marker of NAFLD in patients with metabolic syndrome.^[24,25] Serum levels of GGT were significantly enhanced by ethanol treated rats, along with higher concentrations of ethanol in blood and certain injury in rat liver.^[26]

To analyze the effect of pioglitazone, quercetin and hydroxy citric acid alone on the liver and on normal metabolic activities, we have chosen to have three drug control groups. Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control), with quercetin (group 4; quercetin control) and with hydroxy citric acid (group 5; HCA control) does not show any significant effect on the liver marker enzymes compared to control group (group 1). No metabolic alterations were observed in rats that were present in these three drug controls. All the three drugs did not produce any damage to the liver as evidenced by the levels of liver marker enzymes as shown in Table 1 and Figures 1-6.

However, the experimental NASH rats treated with pioglitazone (group 6; NASH + pioglitazone), with

quercetin (group 7; NASH + quercetin) and with hydroxy citric acid (group 8; NASH + HCA) showed an obvious decrease in ALT, AST, GGT and LDH levels when compared with that of NASH induced group (group 2) as shown in Table 1 and Figures 1-6.

Pioglitazone has been established as a widely used drug in the treatment of insulin resistance diabetes. Insulin resistance was believed to be a central mechanism involved in the development of hepatic steatosis. Pioglitazone was proved to improve sensitivity of insulin significantly, transaminases and liver histology.^[22,27] Rosiglitazone showed decrease in the activity of liver enzymes but was associated with many side effects.^[28] In our present study the experimental NASH treated with pioglitazone and hydroxy citric acid showed significant improvement and the present investigations are also in agreement with the above reports.

In quercetin-treated rats, the serum levels of liver marker enzymes were decreased significantly when compared with NASH group (group 2) reverting back to normal levels. These results may support the hepatoprotective effect of quercetin towards NASH.^[26] In support of our present findings, several studies proved the protective effect of quercetin on diabetes mellitus and improving insulin resistance.^[29,30,31] The hepatoprotective effect of quercetin on liver injury is well evident, which significantly inhibits the elevation of these enzymes levels in experimental NASH treated with quercetin by keeping the structural integrity of the liver.^[32]

There are several reports showing that the quantification of liver enzymes was useful for diagnosis of NASH. ALT, AST, GGT and LDH levels are fluctuated, in experimental NASH in the present study. The ratio between AST and ALT has also been found to have predictive value. Elevation of ALT activity in serum is the result of leakage from damaged cells and therefore reflects hepatocyte damage. ALT, AST, GGT and LDH levels are fluctuated in experimentally induced NASH group when compared to rats in control group. The ratio between ALT and AST has also been found to have predictive value. The protective effect of quercetin on experimental NASH showed more significant reduction of these liver enzymes in comparison with pioglitazone and hydroxy citric acid therapy.

The presence of hypoalbuminemia, prolonged prothrombin time and hyperbilirubinemia are common biochemical alterations suggest advanced NASH.^[33] These patients share a common clinical feature, obesity and potentially other features of metabolic syndrome: Hyperglycemia, dyslipidemia and hypertension.^[34] Several reports demonstrated the profibrinogenic nature of hyperglycemia and hyperinsulinemia.^[35,36] The significant association

between serum uric acid level and development of NASH suggest that high uric acid levels may play a causal role in the development of NASH.^[37,38] No metabolic alterations were observed in rats that were present in these three drug controls. All the three drugs did not produce any significant alterations in the levels of albumin, total bilirubin, creatinine, urea, uric acid and glucose as evidenced by the Table 2 and Figures 7-12.

However, The experimental NASH rats treated with pioglitazone (group 6; NASH + pioglitazone), with quercetin (group 7; NASH + quercetin) and with hydroxy citric acid (group 8; NASH + HCA) showed significant reduction in serum albumin, total bilirubin, creatinine, urea, uric acid and glucose levels when compared with that of NASH induced group (group 2) as shown in Table 2 and Figures 7-12. Pioglitazone improves insulin sensitivity and also reported the declined levels of serum liver enzymes.^[33] Treatment with quercetin also resulted in the reduction of the various biochemical parameters such as albumin, total bilirubin, creatinine, urea, uric acid and glucose and offers protection to the liver against NASH.^[39]

Hydroxy citric acid delays intestinal glucose absorption in rats, desirably decreasing the sugar levels and insulin responses^[40] and also HCA decreases the formation of glycation products^[41] suggesting the hepato-protective action. Oral treatment of HCA showed significant reduction against liver toxicity. These findings could be considered as a functional improvement of hepatocyte-induced elevated levels of urea, uric acid and creatinine. The significant reduction in the elevated levels of biochemical markers is an indication of stabilization of plasma membrane as well as repair of damage tissues.^[42] It can be concluded that the HCA possess good hepatoprotective activity against NASH by virtue of its antioxidant properties.

CONCLUSION

In the present study, by the observations on the levels of various biochemical parameters, it is evident that, the pioglitazone, quercetin and hydroxy citric acid may afford protection to the liver against NASH. Whereas from the results of hepatic marker enzymes, it is evident that optimal protection was observed after quercetin treatment against experimental NASH whereas pioglitazone and hydroxy citric acid also confers protection to some extent against NASH.

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