6. DISCUSSION

6.1. PHASE I: ESTABLISHMENT OF EXPERIMENTAL MODEL OF NASH

The goal of diagnostic procedures is to identify the patients with NASH before the onset of advanced fibrosis. To date, the pathophysiological pathways involved in liver damage and in the progression of pure fatty liver to NASH remain largely unknown. Currently, the most accepted theory to explain the pathogenesis of NASH is the so-called “two-hit” hypothesis (Day and James, 1998). According to this model, the development of hepatic steatosis constitutes the first hit, and cellular events leading to hepatic inflammation constitute the second hit. Experimental and clinical data have suggested a role for hepatocyte apoptosis in liver inflammation and tissue damage, regeneration of parenchyma, and fibrosis (Jaeschke et al., 2002; Rust and Gores, 2001; Jaeschke et al., 2003). In this regard, a reduction in hepatocyte apoptosis has been shown to result in decreased liver fibrosis in animal models of cholestasis (Canbay et al., 2004).

Liver Biopsy is required not only to confirm the diagnosis but also provide important prognostic information. Liver biopsy is now considered the “gold standard” for the assessment of liver fibrosis, which is an invasive procedure associated with severe pain and many complications and risks. Moreover, the prognosis of patients with NASH appears to be dictated by the presence and extent of fibrosis present on liver biopsy.
Thus, at present an invasive liver biopsy is the only reliable way to diagnose the presence of NASH and assess the severity of liver damage presents (Wieckowska., 2007).

Obtaining liver biopsy of a patient with suspected NASH to confirm the presence or absence of NASH is a very difficult task since liver biopsy is an invasive procedure and since NASH is an asymptomatic disease patients will not give consent to proceed with the liver biopsy and this becomes the major obstacle for the clinicians and researchers to conduct studies in NASH pertaining to its diagnosis and treatment. Also, the progress in the understanding and treatment of NASH has been hindered by the lack of a practical experimental model that reproduces the key features of the disease in human beings. This emphasizes the importance of the establishment of the experiment model of NASH to conduct the studies related to the diagnosis and treatment of NASH.

Keeping this as an aim, we conducted Phase – I studies to establish an experimental model of NASH in rats which mimics the features of human NASH.

6.1.1. PHASE – I: EXPERIMENTAL PROTOCOL TO CREATE THE MODEL OF NASH IN RATS

Experimental NASH was established according to the model of Rivera et al (2006) with slight modifications. Male Wistar rats, which were individually housed and fed either a standard diet with protein 20%; fat 5%; carbohydrates 5%; fiber 5% and a high-fat diet with 20% of energy
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derived from protein; 15% from corn oil; 50% from sucrose; 5% from fiber. The standard diet has the same fat content as the average “normal” diet available commercially. The overall compositions of the standard and high fat diets are shown in Table 1.

The animals were broadly divided into three major groups (n=18) as shown below to establish the experimental model of NASH in rats: These 3 major groups were sub-grouped as shown below:

6.1.1.1. **Group A: Dose & Duration = 4 weeks (n=18)**
- **Group A1; Control (n=6):** Rats fed with normal rat pellet diet *ad libitum* (available commercially) for 4 weeks.
- **Group A2; Standard Diet (n=6):** Rats fed with standard diet *ad libitum* for 4 weeks.
- **Group A3; High Fat Diet (n=6):** Rats fed with high-fat diet *ad libitum* for 4 weeks.

6.1.1.2. **Group B: Dose & Duration = 8 weeks (n=18)**
- **Group B1; Control (n=6):** Rats fed with normal rat pellet diet *ad libitum* (available commercially) for 8 weeks.
- **Group B2; Standard Diet (n=6):** Rats fed with standard diet *ad libitum* for 8 weeks.
- **Group B3; High Fat Diet (n=6):** Rats fed with high-fat diet *ad libitum* for 8 weeks.

6.1.1.3. **Group C: Dose & Duration = 12 weeks (n=18)**
- **Group C1; Control (n=6):** Rats fed with normal rat pellet diet *ad libitum* (available commercially) for 12 weeks.
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- **Group C2; Standard Diet (n=6):** Rats fed with standard diet *ad libitum* for 12 weeks.

- **Group C3; High Fat Diet (n=6):** Rats fed with high-fat diet *ad libitum* for 12 weeks.

After the experimental period (4th, 8th, 12th week), liver tissues of all the experimental groups were dissected out and fixed in 10% buffered neutral formalin solution for histopathological studies for the assessment of the development of NASH. The results of the histopathological studies conducted to create experimental model of NASH in rats have been shown in Figures 2 (A-C); 3 (D-F) and 4 (G-I).

6.1.2. EFFECTS OF STANDARD AND HIGH-FAT DIETS FOR 4, 8 & 12 WEEKS

6.1.2.1. HISTOPATHOLOGICAL STUDY OF PHASE – I

Liver biopsy remains a gold standard against the other methods used to diagnose and confirm the presence or absence of NASH. Histological analysis of liver tissue specimens was carried out and the photomicrographs of the histological analysis of the liver tissues were shown in Fig. 2(A-C). The rats that were fed with normal rat feed for 4 weeks (group A1) (Fig. 2A) and rats that were fed with standard diet for 4 weeks (group A2) (Fig. 2B), showed normal architecture of liver tissue on histopathological evaluation. Whereas the rats that fed with high fat diet for 4 weeks (group A3) (Fig. 2C), showed slightly enlarged hepatocytes with feathery degeneration of cytoplasm (early change). Liver histological analysis of the rats that fed with normal rat feed, standard diet and high fat
diet for 8 weeks, were shown in Fig. 3(D-F). The rats that were fed with normal rat feed for 8 weeks (group B1) (Fig. 3D) and rats that were fed with standard diet for 8 weeks (group B2) (Fig. 3E), showed normal architecture of liver tissue on histopathological evaluation. Whereas the rats that were fed with high fat diet for 8 weeks (Fig. 3F) showed diffused fatty infiltration of hepatocytes (steatosis) with mono nuclear inflammatory infiltrate (inflammation), confirming the development of NASH. Liver histological analysis of the rats that fed with normal rat feed, standard diet and high fat diet for 12 weeks, were shown in Fig. 4(G-I). The rats that were fed with normal rat feed for 12 weeks (group C1) (Fig. 4G) and the rats that were fed with standard diet for 12 weeks (Fig. 4H), showed normal normal hepatocytes. Whereas the rats that were fed with high fat diet for 12 weeks (Fig. 4I) showed hepatic macrovesicular steatosis, obvious fibrosis, larger lipid accumulation (fatty cysts & larger fatty vacuoles).

The progress in the understanding and treatment of NASH has been hindered by the lack of a practical experimental model that reproduces the key features of the disease in human beings. In the present study, our findings demonstrated that rats fed with high-fat diet ad libitum for 8 weeks as evidenced by Fig.3 (F), showed diffused fatty infiltration of hepatocytes (steatosis) with mono nuclear inflammatory infiltrate (inflammation), confirming the development of NASH and this observation concurred with an earlier report (Lieber et al., 2002).
Thus, this rat model reproduces the key features of human NASH and provides a realistic experimental model for elucidating its treatment. The present study clearly demonstrated that the ingestion of the high-fat diet for 8 weeks produces all the prominent characteristics of NASH and the principal histological features of NASH, including steatosis, inflammation, which mimics the NASH in humans.

Few experimental models of NASH were available in literature to conduct research studies in NASH but they fail to reproduce the features of NASH in humans since the diet used to produce NASH in rats lacks the essential nutrients like choline and methionine thereby producing many other nutritional disorders, which is highly uncommon feature of NASH in humans (Enriquez et al., 1999; Raucy et al., 1991; Irizar et al., 1995; Weltman et al., 1996). Keeping this important feature in our mind, we have incorporated all the essential nutrients in our high-fat diet, which is used to create an experimental model of NASH in rats that mimics the NASH in humans without leading to any nutritional disorders. Our model is based on the model proposed by the Rivera et al (2006) with major modifications. The major modifications in the preparation of the standard and high-fat diet include the addition of minerals in the form of NaCl, addition of fibre which is essential to maintain normal health in human beings, addition of fructose and addition of cholesterol. The basic difference between the standard diet and high-fat diet includes:

1. Standard diet contains 50 g/kg of sucrose, whereas High-fat diet contains 500 g/kg of sucrose
2. Standard diet contains 2.5 g/kg of corn-oil, where as High-fat diet contains 150 g/kg of corn-oil

3. Standard diet contains 50 g/kg of fructose, where as High-fat diet contains 5 g/kg of fructose

In the present study, the presence of large amounts of sucrose in high-fat diet (10 folds increase, compared to the standard diet) stimulates the de-novo production of the lipids (lipogenesis) and there by leads to the hepatic steatosis and finally leading to the steatohepatitis (accumulation of fat in liver coupled with the inflammation of the liver), without the presence of alcohol (Yamashita et al., 2001; Towle., 2005; Uyeda and Repa., 2006; Michael K Pickens et al., 2009). It is very obvious and a well known fact that the excess amounts of cholesterol and corn oil in high-fat diet, compared to standard diet contributes to the accumulation of fat in liver.

So, the presence of excessive amounts of sucrose, corn-oil, cholesterol and decreased amounts of fructose in high-fat diet, when compared to the composition of the standard diet contributes to the accumulation of fat in liver and there by leading to the inflammation, producing NASH.

6.1.2.2. CONCLUSION OF PHASE – I STUDIES:

A high-fat diet (HFD) is used to create a model of NASH and NASH has been successfully developed in the rats fed with high fat diet for 8 weeks. Thus, this model mimics the most common features of NASH in humans and provides an ideal tool to study the role of events involved in
the pathogenesis of NASH and to define any future experimental therapy, which ameliorates the degree of liver injury.

6.2. PHASE II: COMPARATIVE STUDY OF PROTECTIVE ROLE OF PIOGLITAZONE, QUERCITIN AND HYDROXY CITRIC ACID

Currently, no specific therapies for NASH exist. Experimental approaches under evaluation in patients with NASH include antioxidants, such as vitamin E, selenium, and betaine. These medications act by reducing the oxidative stress that appears to increase inside the liver in patients with NASH. Whether these substances actually help to treat the disease is not known, but the results of clinical trials should become available in the next few years.

Another experimental approach to treating NASH is the use of newer anti diabetic medications—even in persons without diabetes. Most patients with NASH have insulin resistance, meaning that the insulin normally present in the bloodstream is less effective for them in controlling blood glucose and fatty acids in the blood than it is for people who do not have NASH. The newer antidiabetic medications make the body more sensitive to insulin and may help reduce liver injury in patients with NASH. Studies of these medications—including metformin, rosiglitazone, and pioglitazone—are being sponsored by the National Institutes of Health and should answer the question of whether these medications are beneficial in NASH.

Many strategies have been tried to treat NAFLD. The link between NAFLD and insulin resistance has been well established and
documented in the literature (Malhi and Gores., 2008; Cohen., 2011). Two types of drugs, which are widely termed as ‘insulin sensitisers’, have been tried (Gumieniczek., 2003) viz. biguanides (metformin) and the glitazones (rosiglitazone and pioglitazone) have been tried for the treatment of NASH (Riera-Guardia and Rothenbacher., 2008; Ratziu et al., 2010). Thiazolidinediones, such as pioglitazone is an oral antidiabetic agent that acts primarily by decreasing insulin resistance, are synthetic ligands for peroxisome proliferator-activated receptors (PPARs) (Semple et al., 2006). Pioglitazone hydrochloride is a widely used drug in the treatment of insulin resistance diabetes (Jagdish kakadiya and Nehal shah., 2011).

Quercetin is a plant-derived substance, or a phytochemical, which is known as a flavonoid (Molina et al., 2003; Alexandra and Bentz., 2009). Flavonoids are compounds found in fruits and vegetables; to date, more than 4,000 have been identified (Ververidis Filippos et al, 2007). The flavonoids have generated scientific interest because of their potential beneficial effects on human health, including antioxidant, anti-inflammatory, antitumor, and antiviral activities (Sakanashi et al., 2008). 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 (Sexton and Jarow., 1997; Alexandra and Bentz., 2009). The scope of the present study is expected to adequately fulfill the aim and help in identifying safe and effective flavone compounds to treat NASH.
For the past several years, small and complex molecules have been isolated from the various species of *Garcinia*, which include xanthones and xanthone derivatives (Rama Rao et al., 1980; Preuss et al., 2005). However, the isolation of (-)-hydroxycitric acid ((-)-HCA) from a few species of *Garcinia* and its biological properties have attracted the attention of biochemists and health practitioners (Minami et al., 1994). 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 (Minami et al., 1994; Shara et al., 2004). 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 (Rama Rao et al., 1980; Bennet and Lee., 1989; Minami et al., 1994).

6.2.1. STRATEGY AND CONCEPTUAL FRAME WORK ADOPTED IN CHOOSING THE DRUGS

Since the pathogenesis of NASH involved interplay of 3 possible mechanisms as shown in Fig. 1 (Cusi K., 2009) such as hyper-insulinemia, lipotoxicity and oxidative stress, we have chosen 3 categories of drugs, pioglitazone as insulin sensitizer, quercetin as hepatoprotectant & antioxidant and hydroxy citric acid as a lipid lowering agent & anti-obesity agent.
6.2.2. EXPERIMENTAL MODEL ADOPTED TO INDUCE NASH

The experimental model of NASH in rats by feeding high fat diet for 8 weeks (Surapaneni Krishna Mohan et al., 2012) and this model was 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.

6.2.3. HISTOLOGICAL STUDY

Histopathological evaluation, which remains the sole method of distinguishing disease progression and regression of NASH was measured after the treatment of pioglitazone, quercetin, and hydroxyl citric acid. The photomicrographs are shown in Fig. 5 (A-H). The rats in group 1 (control) that were fed with standard diet for 8 weeks (Fig. 5A), represented normal architecture of rat liver tissue on histopathological evaluation. The rats in group 2 (NASH induced) that were fed with high fat diet for 8 weeks showed diffused fatty infiltration of hepatocytes (steatosis) with mono nuclear inflammatory infiltrate (inflammation) (Fig. 5B), confirming the induction of NASH.

Histological examination of livers from rats fed with high fat diet for 8 weeks demonstrated substantial fatty infiltration (steatosis) with inflammatory changes (hepatitis) (together termed as steatohepatitis), the characteristic features of NASH. The macrophages are massively recruited into the liver upon toxic injury and may differentiate into fibrocytes (Musso et al., 2003; Niedermeier et al., 2009). NASH histopathology has been reported to have panacinar or periportal
steatosis, rare ballooning and portal tract expansion by chronic inflammation or fibrosis (Brunt., 2009). Neutrophilic cells in lobular inflammatory infiltrate are a distinguishing feature of steatohepatitis. Histologically, fat deposition is typically macro vesicular and inflammation of steatohepatitis is predominantly lobular. Neutrophilic cells in lobular inflammatory infiltrate are a distinguishing feature of steatohepatitis and differentiate it from other chronic hepatitis (Das and Kar., 2005). Inflammation consists of a mixed inflammatory cell infiltrate, composed of lymphocytes, some eosinophils and occasionally, a few neutrophils (Hubscher, 2006; Brunt., 2005).

To analyze the effect of pioglitazone, quercetin and hydroxy citric acid alone on the liver, we have chosen to have three drug control groups. Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control) (Fig. 5C), with quercetin (group 4; quercetin control) (Fig. 5D) and with hydroxy citric acid (group 5; HCA control) (Fig. 5E) showed normal hepatocytes. No pathological changes 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 histological studies (Fig. 5C, 5D and 5E).

The rats that were present in experimental NASH treated with pioglitazone (group 6; NASH+pioglitazone) (Fig. 5F) showed no fatty degeneration. Pioglitazone therapy was associated with a reduction in these histologic predictors of progressive disease. Therefore, long-term pioglitazone therapy may arrest or reverse the progression of NASH and
improve its clinical outcome. Treatment with pioglitazone was associated with a reduction in steatosis, injury lobular inflammation (Aithal et al., 2008). Our findings were supported by few other studies which showed that pioglitazone had significant improvement in other important histologic features of nonalcoholic steatohepatitis (Powell et al., 1990; Bacon et al., 1994).

In those rats present in group 7, which were treated with quercetin (NASH+quercetin) (Fig. 5G) hepatocytes appear normal and no obvious fatty infiltration and inflammation were seen. Quercetin treated group showed normal hepatocytes and no obvious fatty & inflammatory changes are seen which may be due to the anti-inflammatory and lipid lowering effect of quercetin (Mari Hamalainen et al., 2007; Alexandra and Bentz, 2009).

There observed local hepatocyte necrosis with inflammatory collections in the rats present in experimental NASH treated with hydroxy citric acid (group 8; NASH+HCA) (Fig. 5H). The treatment with HCA reduced the fatty infiltration in liver but the inflammation was not reverted back completely as evidenced in Fig. 5H. The reduction in fatty infiltration is due to the antiobesity effect and hypocholesterolemic action of the hydroxy citric acid (Heymsfield et al., 1998; Mattes and Bormann, 2000; Sakariah et al., 2002; Deore et al., 2011).

The key histologic components of nonalcoholic fatty liver disease quantify the severity of steatosis and inflammation (Kleiner et al., 2005). Histologic examination of livers from high fat diet consumed rats
demonstrated substantial steatosis with inflammatory changes (Rohit Kohli et al., 2010). A decrease in the severity of these components (especially hepatic steatosis) may also decrease with progression of fibrosis to cirrhosis.

6.2.4. SCANNING ELECTRON MICROSCOPICAL STUDIES (SEM)

Scanning Electron Microscopy (SEM) photomicrographs are shown in Fig. 6 (A-H). The rats in group 1 (control) that were fed with standard diet for 8 weeks (Fig. 6A), represented the epithelium and layers of the liver with normal architecture. The rats in group 2 (NASH induced) that were fed with high fat diet for 8 weeks showed accumulation of fat on liver tissues (steatosis), with cellular destruction showing pathological changes with mono nuclear inflammatory infiltrate (inflammation) (Fig. 6B), confirming the induction of NASH.

Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control) (Fig. 6C), with quercetin (group 4; quercetin control) (Fig. 6D) and with hydroxy citric acid (group 5; HCA control) (Fig. 6E) showed the epithelium and layers of the liver with normal architecture and no pathological changes were observed in rats that were present in these three drug controls.

The rats that were present in experimental NASH treated with pioglitazone (group 6; NASH+pioglitazone) (Fig. 6F) showed marked reduction in the size of fat accumulation. In group 7 rats that were treated with quercetin (NASH+quercetin) (Fig. 6G) hepatocytes appear normal and no obvious fatty infiltration and inflammation were seen. The rats that
were present in experimental NASH treated with hydroxy citric acid (group 8; NASH+HCA) (Fig. 6H) there observed local hepatocyte necrosis with inflammatory collections but showed reduction in the size of fat accumulation.

SEM studies revealed accumulation of fat on liver tissues, with cellular destruction showing pathological changes in experimental NASH group, where as in pioglitazone & hydroxy citric acid treated against NASH showed reduced deposition of fat in liver tissues. Quercetin showed protective role with no pathological inflammatory changes in liver tissues which may be due to hepato protective & antioxidant activity. The results of the histopathological studies were supported by the scanning electron microscopy (SEM) studies.

The histopathological evidence of experimental NASH in animals indicated that progressive disease was associated with the presence of diffused fatty infiltration of hepatocytes with mono nuclear inflammatory infiltrate whereas, quercetin treated groups showed normal hepatocytes and no obvious fatty & inflammatory changes are seen. The severity of experimental NASH was reduced after treatment with pioglitazone and hydroxy citric acid. The results of the histopathological studies were supported by the scanning electron microscopy (SEM) studies.

The severity of NASH was reduced by co-treatment with pioglitazone, hydroxy citric acid and quercetin which are clearly evident in histological & SEM studies. Quercetin showed more protection when it is compared with pioglitazone & hydroxy citric acid against NASH, so this
drug can be considered for further advanced studies to understand the mechanism of its protective action.

6.2.5. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON SERUM LIVER MARKER ENZYMES IN EXPERIMENTAL NASH

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 (Ratziu et al., 2005). 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 (Clark., 2006).

The effect of pioglitazone, quercetin and hydroxy citric acid on the levels of serum liver marker enzymes in experimental NASH were depicted in Table - 2 and Fig. 7 (A-F). 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.

Laboratory tests that are routinely included in the evaluation of patients with suspected NASH include a serum panel of liver tests (alanine aminotransferase, ALT, 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—and often the only—laboratory abnormalities of patients with NAFLD are mild to moderate (twofold to threefold) elevations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), or both (Powell et al., 1990). However, as fibrosis advances, this ratio can reverse and lose its diagnostic value in assessing steatohepatitis. Other liver enzymes (gamma-glutamyl transferase) may be elevated two to three times above the normal range (Angulo et al., 1999).

Drugs like troglitazone (now withdrawn because of hepatotoxicity), rosiglitazone and pioglitazone proved to improve the serum aminotransferase levels (Angulo., 2005). 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 was supported by Sakugawa., (2003) report which showed a significant difference between GGT level and the severity of liver fibrosis.

NASH is typically associated with an ALT level greater than the AST level (Lee., 1989; Powell et al., 1990) 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 (Adler and Schffner., 1979). 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 (Angulo et al., 1999). 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 (Oh et al, 2008; Angulo et al., 1999).

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 (Sanyal et al, 2002). 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 (Linda et al., 2009). 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 (Linda et al., 2009; Banderas et al., 2012). 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 (Choi et al., 2006).

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 - 2 and Fig. 7 (A-F).

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 - 2 and Fig. 7 (A-F).

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 (Neuschwander-Tetri et al, 2003; Oh et al., 2008). Rosiglitazone showed decrease in the activity of liver enzymes but was associated with many side effects (Ratziu et al., 2010). 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 (Choi et al., 2006). In support of our present findings, several studies proved the protective effect of quercetin on diabetes mellitus and improving insulin resistance (Coskun et al., 2005; Rivera et al., 2008; Kobori et al, 2009). 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 (Xi Chen., 2010).

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.

Thus, it could be inferred from the observations on the levels of ALT, AST, GGT and LDH 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.
6.2.6. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON VARIOUS BIOCHEMICAL PARAMETERS IN EXPERIMENTAL NASH

Table - 3 and Fig. 8 (A-E) 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. A significant increase in the levels of albumin, creatinine, urea, uric acid, glucose & total bilirubin was noticed in experimentally induced NASH group (group 2) when compared to rats in control group (group 1).

The presence of hypoalbuminemia, prolonged prothrombin time, and hyperbilirubinemia are common biochemical alterations suggest advanced NASH (Collantes et al., 2004). These patients share a common clinical feature, obesity, and potentially other features of metabolic syndrome: hyperglycemia, dyslipidemia, and hypertension (Fierbinteanu-Braticevici et al., 2010).

Several reports demonstrated the profibrinogenic nature of hyperglycemia and hyperinsulinemia (Munoz et al., 2009; Paradise et al., 2001). When compared to patients without NASH to those with NASH, high ALT and AST, glucose, serum liver enzymes, creatinine, urea and uric acid were observed in NASH patients. 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. Two potential reasons could explain the mechanism by which high serum uric acid levels
participates in the development of NAFLD. The first reason being uric acid acts as a strong oxidant in the environment of metabolic syndrome (Hayden and Tyagi., 2004). Recent studies have suggested that the elevation of serum uric acid as a novel risk factor for the development of metabolic diseases, including type 2 diabetes mellitus (Dehghan et al., 2008). These results also suggested the level of serum uric acid as an independent factor which predicts the development of NASH (Dehghan et al., 2008). The second explanation being the generation of increased oxidative stress by virtue of increased serum uric acid levels through xanthine oxidoreductase reaction. Therefore, increased uric acid generation may result in increased oxidative stress to the liver (Harrison et al., 2002; Berry and Hare., 2004).

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 significant alterations in the levels of albumin, total bilirubin, creatinine, urea, uric acid and glucose as evidenced by the Table - 3 and Fig. 8 (A-E).

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 - 3 and Fig. 8 (A-E). Pioglitazone improves insulin sensitivity and also reported the declined levels of serum liver enzymes (Collantes et al., 2004). 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 (Czerny., 2000).

Hydroxy citric acid delays intestinal glucose absorption in rats, desirably flattening sugar and insulin responses (Sullivan et al., 1974) and also HCA decreases the formation of glycation products (Bousova et al., 2009) suggesting the hepato protective action. Garcinol shows strong antioxidant activity (Padhye et al., 2009) and hepato protective activity. Hence antioxidants play an important role in preventing hepatotoxicity (Harish et al., 2006). 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 (Chattopadhyay et al., 1992). It can be concluded that the HCA possess good hepatoprotective activity against NASH by virtue of its antioxidant properties.
Thus, it could be inferred from the above observations on the levels of biochemical parameters such as glucose, urea, uric acid, creatinine and bilirubin, pioglitazone, quercetin and hydroxy citric acid may afford protection to the liver against NASH.

6.2.7. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON SERUM LIPID PROFILE AND LIPOPROTEINS IN EXPERIMENTAL NASH

Table - 4 and Fig. 9 (A-F) shows the effect of pioglitazone, quercetin and hydroxy citric acid on the lipid profile levels in experimental NASH induced rats. The levels of serum lipid profile parameters such as total cholesterol, free cholesterol, esterified cholesterol, phospholipids, triglycerides and free fatty acids levels were raised significantly in experimental NASH induced rats (group 2) when compared to control group (group 1). The levels of Lipoproteins such as HDL, LDL, VLDL, HDL:LDL, TC:HDL were presented in Table 5 and Fig. 10 (A-E). Raised levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol are pointers to insulin resistance. The disease progression was directly proportional to the lipoprotein levels, showed by significant increase of lipoprotein levels in experimentally induced NASH group (group 2) when compared to control rats (group 1).

It was observed that NASH is associated with a more atherogenic lipid profile, including hyper triglyceridemia, a higher plasma concentration of very low-density lipoprotein (VLDL) and LDL that are larger in size, and with lower levels of high-density lipoprotein (HDL) (Malaguarnera et al.,
Presence of increased circulating and/or hepatic saturated fatty acids might promote the development and progression of liver damages activating apoptosis (Shimabukuro et al., 1998; Targher et al., 2005). Moreover, in the liver, the increase of fatty acids synthesis associated with the reduction of their delivery from hepatocytes by VLDL because of degradation of apolipoprotein B100 causes the unbalance of hepatic fat turnover resulting in steatosis (Sakata et al., 2001). Fatty acids are a source of oxidative stress and damage of mitochondria with increased beta oxidation and raising levels of reactive oxygen species (De Almeida et al., 2002). The amplification of fatty acid esterification pathway and formation of triglycerides could be implicated in hepatic insulin resistance (Teoman Uysal et al., 1997).

NASH is characterized by the accumulation of triglycerides, which are formed from the esterification of free fatty acids (FFA) and glycerol within the hepatocyte. FFAs arise in the liver from three distinct sources viz. lipolysis (the hydrolysis of FFA and glycerol from triglyceride) within adipose tissue, dietary sources, and de novo lipogenesis. In contrast, FFA may be utilized either through beta-oxidation, re-esterification to triglycerides and storage as lipid droplets, or packaged and exported as very low density lipoprotein (VLDL). Hence, hepatic fat accumulation can occur as a result of increased fat synthesis, increased fat delivery, decreased fat export, and/or decreased fat oxidation (Postic et al., 2008) raised serum urate, triglyceride, low-density lipoprotein (LDL) cholesterol
and low levels of high-density lipoprotein (HDL) cholesterol are pointers to insulin resistance.

The present study revealed a significant dyslipidemia (lower HDL-c, higher total cholesterol, LDL-c and triglycerides) in experimental NASH rats. Cholesterol in univariate logistic regression analysis was a good predictor of NAFLD; however, LDL-c was a good predictor in both uni- and multivariate analysis. Association of dyslipidemia with NAFLD in this study comes in agreement with other studies (Browning et al., 2004; Sharabi and Eldad., 2000).

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) does not show any significant effect on levels of total cholesterol, esterified cholesterol, phospholipids, free fatty acids but showed significant reduction in free cholesterol and triglyceride levels when compared to controls (group 1) and this could be due to the advent of pioglitazone, a hypoglycemic drug that modulates the levels of lipids (Doggrell., 2008). On the other hand, rats fed with standard diet simultaneously with quercetin (group 4; quercetin control) does not show any significant effect on all the lipid profile parameters compared to control group (group 1). Whereas, rats fed with standard diet simultaneously with hydroxy citric acid (group 5; HCA control) does not show any significant effect on levels of total cholesterol, esterified cholesterol, triglycerides, free fatty acids but showed
significant reduction in the levels of free cholesterol and phospholipids when compared to controls (group 1) and this may be due to the lipid lowering action of the HCA (Heymsfield et al., 1998; Mattes and Bormann., 2000).

Also, rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control) and with quercetin (group 4; quercetin control) do not show any significant effect on levels of lipoproteins when compared to controls (group 1). On the other hand, rats fed with standard diet simultaneously with hydroxy citric acid (group 5; HCA control) does not show any significant effect on levels of LDL and VLDL but showed significant increase in the levels of HDL when compared to controls (group 1) and this could be due to the lipid lowering and hypo-cholesterolemic action of the HCA (Heymsfield et al., 1998; Mattes and Bormann., 2000).

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 marked reduction in levels of lipid profile parameters when compared with that of NASH induced group (group 2).

Protective effect of pioglitazone, quercetin and hydroxy citric acid was noticed by decreasing the lipoprotein levels towards normalcy in group 6 (NASH+pioglitazone), group 7 (NASH+quercetin) and group 8 (NASH+HCA), when compared to experimentally induced NASH group (group 2).
We found that pioglitazone was effective in reducing either serum total cholesterol or LDL values. Patients with insulin resistance have impaired responses by muscle, adipose tissue, and the liver to insulin, causing compensatory increases in pancreatic insulin secretion to keep glucose levels within the normal narrow range. Chronic hyperinsulinemia causes triglyceride to accumulate in hepatocytes by favoring the formation of triglyceride instead of mitochondrial beta-oxidation, yet possibly impairing the secretion of triglyceride into the circulation. Compounding this dysfunctional metabolic handling of fat in the liver is the continued release of free fatty acids by peripheral adipose tissue in the fed state because of insulin resistance at the level of adipocytes (Gisela Wilcox, 2005; Gideon et al., 2008).

Lipid peroxidation and oxidant stress have been proposed as an important link between the accumulation of fat and subsequent liver injury (Day and James, 1998). Quercetin could improve the outcome of NASH since it reduces lipid levels, limits oxidative stress, and modulates inflammatory responses. An improvement in lipid profile with a better insulin sensitivity and lower values of fasting plasma glucose were reported in the present study suggesting the fact that quercetin supplementation induces regression of NASH. In addition, this flavonoid can also reduce the number of adipocytes, either by decreasing adipogenesis or increasing apoptosis. Kobori et al., (2011) reported that chronic dietary intake of quercetin reduced body weight gain, as well as visceral and liver fat accumulation, and improved systemic parameters.
related to metabolic syndrome (hyperglycemia, hyperinsulinemia and dyslipidemia), probably by decreasing oxidative stress. In the present study, quercetin exhibited significant hypolipidemic and hypocholesterolemic activities. The elevated level of LDL-C significantly reduced among rats with NASH following treatment with quercetin. This might be due to the antioxidant property of quercetin and also due to the increased level of HDL-C, which is capable of inhibiting the LDL-C peroxidation and retarding the LDL-C accumulation. Remarkably, serum HDL-C level increased more than 50% in quercetin treated experimental rats (Viswanadha Vijaya Padma et al., 2012). HDL-C as a multifunctional lipoprotein, possess antioxidant and anti-inflammatory activities (Alireza Rezazadeh et al., 2012).

(-)-HCA was shown to be a potent inhibitor of ATP citrate lyase, which catalyzes the extra mitochondrial cleavage of citrate to oxaloacetate and acetyl-CoA. The inhibition of this reaction limits the availability of acetyl-CoA units required for fatty acid synthesis and lipogenesis during a lipogenic diet, that is, a diet high in carbohydrates (Watson et al., 1969). Extensive animal studies indicated that (-)-HCA suppresses the fatty acid synthesis, lipogenesis, food intake, and induced weight loss (Preuss et al., 2004). In vitro studies revealed the inhibitions of fatty acid synthesis and lipogenesis from various precursors. Experiments with (-)-HCA have demonstrated that citrate cleavage enzyme is an obligatory enzyme in lipogenesis from pyruvate and that the lipogenic system of rabbit adipose tissue resembles that of a ruminant in that it is adapted to utilize acetate
rather than glucose (Sullivan et al., 1974; Asghar et al., 2007). This enzyme catalyzes the conversion of citrate and coenzyme A to acetyl coenzyme A (acetyl Co-A) and oxaloacetate. Acetyl Co-A is a critical building block in the biosynthesis of fatty acids, cholesterol and lipids, as well as the neurotransmitter acetylcholine. Thus, the primary mechanism of HCA is believed to be involved in the inhibition of fat synthesis (Kovacs et al., 2006).

Research has shown that the conversion of carbohydrates into fat is prevented by (-)-HCA, a more important function of this incredible nutrient is its ability to increase the carnitine palmitoyl transferase - 1 (CPT-1) activity by decreasing the pool of acetyl-CoA, thus reducing the level of malonyl-CoA and raising the activity of CPT 1 (Jena et al., 2002). HCA is also suggested to have an effect on plasma cholesterol, via increased plasma high-density lipoprotein cholesterol (HDL-C) and so may also protect low density lipoprotein (LDL) from oxidation (Ji-Eun Kim et al., 2011). In the present study HCA supplementation significantly reduced plasma triglyceride levels and observed increased plasma HDL-Cholesterol.

The preservation of the near normal activities of the lipid profile parameters and lipoproteins upon treatment with pioglitazone, quercetin and hydroxy citric acid attributes the hepatoprotective effect of these drugs against NASH.
6.2.8. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON LIPID PEROXIDATION AND ANTIOXIDANTS IN EXPERIMENTAL NASH

Oxidative stress is one of the key mechanisms responsible for liver damage and disease progression in NASH. Non enzymatic antioxidant such as GSH and antioxidant enzymes such as catalase, SOD, GPx, GR, and GST were decreased significantly in experimental NASH rats (group 2), compared to that of controls (group 1) as shown in Table 6 and Fig. 11 (A-G). The levels of lipid peroxidation products (MDA) have been significantly increased in experimentally induced NASH group (group 2) compared to the control group (group 1).

Increased intra hepatic levels of fatty acids provide a source of oxidative stress. It has been proposed that the vulnerable fatty liver is injured by reactive oxygen species (ROS) generated from microsomal, mitochondrial, and/or other hepatocellular pro-oxidant pathways. Increased lipid peroxidation has been demonstrated in both animal models of fatty liver (Leclerq et al., 2000; George et al., 2003; Mehmet Koruk et al., 2004; Lieber., 2004) and patients with NASH. Increased levels of oxidative stress were observed in NASH as compared with patients with steatosis alone (Yang et al., 2000; Videla et al., 2004a; Videla et al., 2004b).

FFAs are the likely source of oxidative stress within the liver in these patients. NASH patients have increased lipolysis and increased delivery of FFAs to the liver (Marchesini et al., 2001; Sanyal et al., 2001).
The products of FFA oxidation (hydrogen peroxide, superoxide, and lipid peroxides) are capable of generating oxidative stress and subsequent lipid peroxidation (Plaa and Witschi., 1976; Sie., 1991; Yun-Zhong Fang et al., 2002; Olga et al., 2003).

In response to oxidative stress, there is usually an increased synthesis of antioxidants and ROS scavengers. However, changes in the activities of the serum antioxidant enzymes and their relationships to oxidative stress have inadequately been studied in patients with NASH. The body protects itself from oxygen free radical toxicity by enzymatic antioxidant mechanisms (eg: glutathione peroxidase, GSH-Px; glutathione reductase, GR; superoxide dismutase, SOD; and catalase) and by non-enzymatic antioxidants (eg, vitamins, uric acid, albumin, bilirubin, and many others) (Cotgreave et al., 1988; Yun-Zhong Fang et al., 2002; Olga et al., 2003). Antioxidant enzymes reduce the levels of lipid peroxides as well as hydrogen peroxide and are important in preventing lipid peroxidation and maintaining the structure and function of biologic membranes (Olga et al., 2003; Devasagayam et al., 2004). SOD catalyses the dismutation of peroxide to hydrogen peroxide and GSH-Px catalyses the oxidation of glutathione (Yim et al., 1990; Mullineaux and Creissen., 1997; Yin et al., 2000; Strange et al., 2000; Olga et al., 2003).

Evidence of lipid peroxidation in the form of increased MDA production, a marker of oxidative stress, has been noted in previous studies and serum levels of MDA have been correlated with the severity of chronic hepatitis (Paradis et al., 1997; Yadav et al., 2002). In the present
study, serum MDA levels were significantly increased in experimental NASH, indicating increased oxidative stress. The defense against free radical-mediated injury includes enzymatic deactivation and direct reaction with free radicals (DiMascio et al., 1991). SOD, the first line of defense against oxygen derived free radicals, converts superoxide anion into H\textsubscript{2}O\textsubscript{2}, forming as neutral products O\textsubscript{2} and H\textsubscript{2}O. GSH-Px catalyses reductive destruction of hydrogen and lipid hydroperoxides, using glutathione as an electron donor (Harris., 1992).

Oxidative stress due to increased ROS production plays a key role in the pathogenesis of NASH. Hepatocytes are continuously exposed to ROS and are protected from oxidative injury by a range of antioxidant pathways (Zhang et al., 2000). The state of oxidative stress exist when there is imbalance between pro-oxidant and antioxidant chemical species. There is insufficient knowledge about antioxidant defence mechanisms, particularly the enzymatic components, in the pathogenesis of NASH. The balance between oxidative stress and antioxidant defense mechanisms may be impaired by depletion of enzymatic antioxidants and decreased serum levels of MDA and NO in patients with NASH. The present study demonstrated that failure of antioxidant defense mechanisms against oxidative stress may be an important factor in the pathogenesis of NASH.

Vendeniale et al., (2001) reported decreased glutathione in NASH patients. Therefore, patients with NASH have an impaired ability to produce sufficient antioxidants. This may be related to the recently observed decrease in three genes involved in ROS sequestration (Cu/Zn
superoxide dismutase, glutathione peroxidase, and catalase) in cirrhosis secondary to NASH (Sreekumar et al., 2000). GST, a membrane bound enzyme plays on important role in glutathione metabolism. In oxidative stress the level of oxidized glutathione increased and hepatic GST is induced. This then converts oxidized glutathione to reduced glutathione. Therefore GST has an important role in antioxidant defense system at the cellular level and is a valuable marker of oxidative stress in NASH (Irie et al., 2012).

Oxidative stress has long been recognized as a key mechanism responsible for liver damage and disease progression in NAFLD and also in NASH. (Oliveira et al., 2002; Roskams et al., 2003; Mehmet Koruk et al., 2004;). Day (2002) demonstrated the enhanced oxidative stress occurs in the liver of patients with NASH as well as animal models of NASH (Wanless et al., 2004). Several oxidation pathways may play a role in the overproduction of reactive oxygen species (ROS) in NASH including mitochondrial, peroxisomal, cytochrome P-450, myeloperoxidase, and nitric oxide synthase. Each of these pathways may generate different oxidation products that could be potentially quantified. Most groups have used either method to measure systemic levels of stable lipid byproducts of ROS activity such as lipid peroxides and thiobarbituric acid-reacting substance or “total antioxidant status” with mixed result (Chalasani et al., 2004; Mehmet Koruk et al., 2004; Horoz et al., 2005; Bonnefont-Rousselot et al., 2006).
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) does not show any significant effect on the levels of lipid peroxidation products (MDA) and non-enzymatic (GSH) and enzymatic (catalase, SOD, GPx, GR and GST) antioxidants when compared to control group (group 1). On the other hand, rats fed with standard diet simultaneously with hydroxy citric acid (group 5; HCA control) does not show any significant effect on the levels of lipid peroxidation products (MDA) and GSH, GPx, GR and GST but showed significant decrease in the levels of catalase and SOD when compared to controls (group 1) this could be due to the antioxidant property of the HCA (Devasagayam et al., 2006). No significant 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 lipid peroxidation products and antioxidants as evidenced by the Table 6 and Fig. 11 (A-G).

Malondialdehyde (MDA), lipid peroxidation product is an index of lipid peroxidation. 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 malondialdehyde (MDA) levels when compared with that of NASH induced group (group 2).
However, The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) showed marked increase in the levels of GSH, catalase and SOD but does not show a significant effect on the levels of GPx, GR and GST levels when compared to the experimentally induced NASH group (group 2). Pioglitazone showed limited positive effect on the antioxidant parameters (Somi et al., 2009).

Similarly, experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) showed marked increase in the levels of GSH and catalase but does not show a significant effect on the levels of SOD, GPx, GR and GST levels when compared to the experimentally induced NASH group (group 2). Hydroxy citric acid also showed a limited positive effect on the the antioxidant parameters (Devasagayam et al., 2006) and this could be attributed to the limited antioxidant properties of the HCA. Hydroxy citric acid may improve nonalcoholic steatohepatitis because it protects cellular structures against damage from oxygen free radicals and reactive products of lipid peroxidation. Previous reports suggested that the involvement of oxidative stress in the pathogenesis of NASH, suggests that antioxidants might have beneficial effects in the treatment of NASH patients (Sanyal et al., 2001; Sanyal et al., 2004; Baskol et al., 2007).

On contrary to these two drugs viz. pioglitazone and hydroxy citric acid, the experimental NASH rats treated with quercetin (group 7; NASH+quercetin) showed significant increase in the levels of antioxidants viz. GSH, catalase, SOD, GPx, GR and GST when compared with that of NASH induced group (group 2) showing maximum protective effect
against NASH. Quercetin demonstrates these protective effects on liver damage by increasing the antioxidant (enzymatic and non enzymatic) activity and decreasing prooxidant effect (Choi et al., 2003; Amalia et al., 2007; Kamaraj et al., 2007). It has been documented that the structure of quercetin plays an important role in its antioxidant property. The o-dihydroxy structure in the B-ring of quercetin has been recognized to accord higher stability to the radical form there by enabling it to participate in the delocalization of electrons (Viswanadha Vijaya Padma et al., 2012). Quercetin offers protective effect against NASH by attenuating lipid peroxidation, by scavenging free radicals that were generated by the excessive oxidative stress and by increasing the levels of glutathione and enhancing the activity of antioxidant enzymes, which in turn detoxify free radicals (Choi et al., 2003; Amalia et al., 2007; Kamaraj et al., 2007).

Thus, by the results of the present study with respect to the levels of lipid peroxidation products and antioxidants - it can be concluded that quercetin offers maximum protection against NASH by showing positive effect on all antioxidant parameters viz. GSH, catalase, SOD, GPx, GR, GST and by decreasing the lipid peroxidation unlike the action of pioglitazone and hydroxy citric acid.

6.2.9. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON ECM COMPONENTS IN EXPERIMENTAL NASH

The imbalanced production of pro- and anti-inflammatory adipokines secreted from fat contributes to the pathogenesis of NASH.
Leptin has been proved to be a good predictor of the disease with median levels of NASH. The levels of hyaluronic acid and leptin were increased significantly in experimentally induced NASH group (group 2) compared to controls (group 1); whereas adiponectin, an important adipokine decreased significantly in experimentally induced NASH group compared to controls (group 1). The pattern of hyaluronic acid, leptin and adiponectin were depicted in Table 7 and Fig. 12 (A-C).

Adiponectin is an important adipokine secreted specifically by adipocytes that circulates at relatively high levels in the blood stream. It is produced outside the liver and appeared to protect against liver damage (Tilg and Hotamisligil., 2006). These molecules reveal modulatory functions in systemic and hepatic inflammation (Hotamisligil and Spiegelman., 1994; Agheli et al., 1998; Friedman et al., 1998; Spiegelman and Flier., 2001). Leptin is a hormone secreted above all by the fat as a product of the obese gene. It is involved in immunity, bone metabolism, energy balance and body weight regulation (Zhang et al.,1994; Halaas et al., 1997).

In obese or NASH subjects, serum leptin level is significantly increased. Conversely, the leptin receptor (Ob-Re) level is down regulated, thus favoring a condition of leptin resistance (Kamada et al., 2008). The close relationship of leptin with adipose tissue and fat stores of the body suggests its involvement in the etiology and pathogenesis of NAFLD (Huang et al., 2008). Leptin has a pro-inflammatory role and is considered to be an essential mediator of liver fibrosis (Tsochatzis et al., 2009).
Adiponectin is an antidiabetic and antiatherogenic acting polypeptide which is strongly correlated with systemic insulin sensitivity in humans (Berg et al., 2002; Pellme et al., 2003; Tschritter et al., 2003). Adiponectin increases fatty acid beta oxidation in muscle (Fruebis et al., 2001), improves post absorptive insulin mediated suppression of hepatic glucose output by enhancing hepatic insulin action (Berg et al., 2002) and decreases lipid accumulation in macrophages. Beyond its metabolic effects, adiponectin also has direct anti-inflammatory effects (Yokota et al., 2002). Moreover, adiponectin attenuates oxidative stress, pro-inflammatory cytokine production and ameliorates liver fibrosis via suppression of activated hepatic stellate cell function. Therefore, low levels of circulating adiponectin may be associated with the development of NASH in patients with steatosis and in the progression of NASH towards cirrhosis.

Adiponectin levels correlate significantly with serum bile acids and thus accompany disease progression. This increase is due to less clearance of adiponectin in the liver of patients with cirrhosis. However, a recent study has shown that the serum adiponectin concentration was negatively associated with higher levels of fibrosis and that low adiponectin levels are an independent risk factor for advanced fibrosis in patients with NAFLD (Savvidou et al., 2009). Thus, in interpreting adiponectin levels in patients with NAFLD, one has to take into account the stage of the disease: if characteristically low in early stages, it will increase with the progression of the disease to actually be elevated in
patients with cirrhosis. The decreased level of serum adiponectin represents an independent risk factor for (NAFLD) and liver dysfunctions in humans. Interestingly, in the present study serum leptin levels were significantly positively correlated with each of TC, TG, LDL-C, non essential free fatty acids (NEFFA) and significantly negatively correlated with HDL. These findings are in concordance with those of Couilard et al., (1997), who reported that the associations between plasma leptin and lipid concentrations were dependent of the degree of obesity. Also, Bugianesi et al., (2005) revealed that serum adiponectin was significantly negatively correlated with each of TC, TG, LDL-C, NEFFA and significantly positively correlated with HDL. Adiponectin secreted by visceral adipocytes correlates strongly with insulin sensitivity (Berg et al., 2002).

The increase in serum adiponectin levels during pioglitazone therapy may be mediated through peroxisome proliferator-activated receptor-mediated transcriptional activation of the adiponectin gene (Iwaki et al., 2003) and this action may account for some of the beneficial effects of the pioglitazone (Pfützner et al., 2005; Anannya Banga., 2009). In contrast, serum leptin levels increased significantly in patients receiving pioglitazone, which probably reflects the weight gain seen in this group. Although leptin may play a role in the development of fibrosis in NASH (Marra., 2002) this may occur only in the presence of other cofactors such as alcohol or hepatitis C (Blendis et al., 2006; Filippidis et al., 2006).

Leptin signals "fullness" post prandially and also promotes insulin sensitivity. Despite elevated plasma levels of leptin, patients with NASH
Discussion

often do not experience normal satiety, and they also have insulin resistance. Thus, hyperinsulinemia and defective leptin signaling are conspicuous at early stages of NASH. It has also been shown that leptin is clearly related to hepatic stellate cell activation (Ikejima et al., 2002). It is believed that leptin has a lipostatic function: when the quantity of fat stored in the adipocytes increases, leptin is released into the bloodstream. Chitturi et al., (2002) found that leptin promotes insulin resistance, contributes both to oxidative stress and to enhanced secretion of inflammatory cytokines and may play a role in causing fibrosis (Loffreda et al., 1998; Bouloumie et al., 1999).

Fibrosis is a dynamic process that may result in increased circulating levels of extracellular matrix (ECM) components. Several groups have used this reasoning to develop different blood tests using individual or a composite of ECM components. Suzuki et al., (2005) determined the reliability of serum hyaluronic acid (HA) to predict the severity of hepatic fibrosis in patients with histologically confirmed NAFLD. Fibrosis assessment is crucial in NASH because it represents an advanced stage of liver injury. Several studies evaluated certain matrix components, such as transforming growth factor β, hyaluronic acid, tissue inhibitors of metalloproteinases, and others (Oh et al., 2008).

Hyaluronic acid (HA) is an extracellular matrix protein, often associated with a various inflammatory diseases. It also promotes insulin resistance, contributes both to oxidative stress and to enhanced secretion of inflammatory cytokines (Loffreda et al., 1998; Bouloumie et al., 1999;
Chitturi et al., 2002) and may play a role in causing fibrosis. Hyaluronic acid (HA) and tissue inhibitor of metalloproteinase-1 (TIMP-1) are reliable markers of liver fibrosis and are closely linked to the proinflammatory status (Miele et al., 2009).

Haukeland et al., (2006) reported hyaluronic acid is one of the important inflammatory markers, shown to be elevated in patients with NASH. Evaluation of the HA levels was found to be useful for predicting severe fibrosis in patients with NASH (Suzuki et al., 2005). Our findings in the present study were in concordance with the studies reported by Promrat et al., (2004).

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) does not show any significant effect on the levels of extracellular matrix components viz. hyaluronic acid, leptin and adiponectin compared to control group (group 1). On the other hand, rats fed with standard diet simultaneously with hydroxy citric acid (group 5; HCA control) does not show any significant effect on the levels of leptin and adiponectin but showed significant increase in hyaluronic acid levels when compared to controls (group 1). No significant metabolic alterations in extra cellular matrix 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 ECM components as evidenced by the Table 7 and Fig. 12 (A-C).

The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) showed significant decrease in the levels of hyaluronic acid and significant increase in the levels of adiponectin levels when compared to experimentally induced NASH group (group 2) but does not show any effect on the levels of leptin, compared to experimentally induced NASH group (group 2). Gastaldelli et al., (2010) showed that the reduction in hepatic steatosis that is observed post pioglitazone treatment and is inversely correlated with the increase in plasma adiponectin. Adiponectin has been proposed to be a major insulin-sensitizing adipokine (Yamauchi et al., 2001; Berg et al., 2001; Combs et al., 2001) and is a plausible candidate for one of the adipokines that may mediate the pioglitazone induced amelioration of insulin resistance (Yamauchi et al., 2001; Berg et al., 2001; Combs et al., 2001).

Pioglitazone can improve blood glucose and lipid levels in a TNFα-independent manner (Wellen et al., 2004). Thiazolidinediones have been shown to up-regulate adiponectin expression in white adipose tissue and plasma adiponectin levels, and these up-regulations have been proposed to be a major mechanism of the thiazolidinedione-induced amelioration of insulin resistance. In this study, we addressed the important question of whether pioglitazone induced up-regulation of plasma adiponectin levels is causally involved in the insulin sensitizing actions of pioglitazone or not. Also, we have successfully demonstrated the amelioration of insulin
resistance induced by pioglitazone (Fruebis et al., 2001; Kubota et al., 2002; Maeda et al., 2002).

The experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) does not show any effect on all the three extracellular components (hyaluronic acid, leptin and adiponectin). The imbalanced production of pro- and anti-inflammatory adipokines secreted from fat contributes to the pathogenesis of NASH but hydroxy citric acid does not show any effect on the decreased levels of adiponectin. This clearly indicates that hydroxy citric acid offers fewer protections against NASH.

On contrary to these two drugs viz. pioglitazone and hydroxy citric acid, the experimental NASH rats treated with quercetin (group 7; NASH+quercetin) showed significant decrease in the levels of hyaluronic acid and leptin compared with that of NASH induced group (group 2) and significant decrease in adiponectin levels compared with that of NASH induced group (group 2), conferring maximum protection against NASH, when compared to the other two drugs. Quercetin may increase adiponectin levels possibly by improving the secretion of adipose tissue adiponectin, which is known to inhibit hepatic fatty acid synthesis, gluconeogenesis and de novo lipogenesis and improve insulin resistance. Adiponectin offers protection to the liver through the inhibition of steatogenesis and fibrogenesis at multiple levels viz. attenuation of oxidative stress, production of pro-inflammatory cytokines (Kamada et al., 2003; Xu et al., 2003; Masaki et al., 2004; Antoniades et al., 2009).
Thus, it could be inferred that quercetin may offer maximum protection against NASH by virtue of increasing the levels of adiponectin significantly, when compared to pioglitazone and hydroxy citric acid.

6.2.10. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON INFLAMMATORY MARKERS IN EXPERIMENTAL NASH

Inflammatory mediators have also been investigated as potential diagnostic tools. NASH was associated with an increase in tumor necrosis factor alpha (TNF-α) and MPO levels and the imbalance may play an important role in the development of NASH. The pattern of inflammatory markers has been depicted in the Table 8 and Fig. 13 (A-B). A significant increase in the levels of inflammatory markers such as tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO) was noticed in experimental NASH rats (group 2) as compared to control group (group 1). The enhanced levels of TNF-α in NASH in turn activates specific redox sensitive kinases (Yuan et al., 2001). These activated specific redox sensitive kinases up regulate the pro-inflammatory pathways resulting in the enhanced insulin resistance which is believed to play a key role in the pathogenesis of NASH (Yuan et al., 2001).

A chronic low-grade inflammatory state characteristic of patients with metabolic syndrome has been extensively associated with the development of steatosis as well as liver damage in NASH (Dieh et al., 2001; Dieh et al., 2004). Inflammatory mediators have also been investigated as potential diagnostic tools. Moreover, the TNF-α levels
were also observed to correlate with the severity of inflammation and fibrosis (Abiru et al., 2006; Feldstein et al., 2004; Hui et al., 2004; Li et al., 2003; Xu et al., 2003). The adipose tissue produces several bioactive mediators, such as leptin, adiponectin and TNF-α. Obese patients with NASH will have enhanced expression of TNF-α mRNA, whereas enhanced expression of TNF-α mRNA was not observed in obese patients without NASH (Yin et al., 1999; Crespo et al., 2001) which is concordant with our present findings.

Lipid peroxidation products initiate chemical modification of many biological molecules and also they participate in signal transduction of many inflammatory responses with up-regulation of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1. TNF-α in turn induces NADPH oxidase that leads to inflammation. The generation of excess reactive oxygen species, in addition to triggering lipid peroxidation of cellular membranes, leads to the release of tumor necrosis factor-alpha (TNF-alpha) via hepatocytes, kupffer cells, and adipose tissue (Kern et al., 1995).

The release of TNF-alpha activates specific redox sensitive kinases that can up regulate pro-inflammatory pathways and enhance insulin resistance (Yuan et al., 2001). HDL-C, as an anti-inflammatory agent, restricts expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and inhibits activation of nuclear factor kappa B (NF-kB) by interrupting a sphingosine kinase signaling pathway (Bindu et al., 2011; Saemann et al., 2010). In addition, expression of the transforming growth
factor (TGF)-β2, a cytokine with anti-inflammatory properties, is regulated by HDL-C (Saemann et al., 2010).

Cytokine production is increased in nonalcoholic steatohepatitis and is believed to play a role in its pathogenesis. In the liver, tumor necrosis factor-alpha can contribute to oxidative stress (Tilg and Diehl., 2000; Tilg and Hotamisligil., 2006) and may contribute to insulin resistance through activation of the inhibitor of kappa kinase beta (Yuan et al., 2001).

TNF-α and adiponectin suppress each other’s production and are also able to antagonise each other’s action (Yokota et al., 2002). Therefore, reduced adiponectin mRNA expression might be partially due to these suppressive effects of elevated TNF-alpha expression in NASH (Crespo et al., 2001; Tilg and Diehl., 2000). The present study showed increased liver TNF-alpha mRNA expression in subjects with NASH. Among the proinflammatory molecules, TNF-α has been proposed to be the key link between obesity and insulin resistance.

TNF-α is usually over-expressed in adipose tissues of obese animals and humans, and obese mice lacking TNF-α and/or its receptor demonstrate protection against development of insulin resistance. Evidence seem to show that in humans chronic activation of proinflammatory pathways of insulin targetted cells can result in obesity/steatosis-related insulin resistance. The influence of circulating and hepatic levels of proinflammatory cytokines TNF-α, IL-6, and C-reactive protein (CRP) in patients with NAFLD and their correlation with disease severity are well studied. Patients with NASH have generally
significantly higher levels of serum TNF-α and IL-6 than seen in patients with simple steatosis (Borst et al., 2004; Lorenzo et al., 2008).

Madhavan et al., (2006) suggested that the cytokine TNF-α can be inhibited by quercetin, which may be of clinical significance in host defense mechanisms against various infections. Quercetin-induced suppression of TNF-α can result in the stimulation of anti-inflammatory cytokines via inhibiting the activation of NF-κβ, and therefore, we anticipate that quercetin can be widely used as an anti-TNF-α therapy.

MPO possesses potent antimicrobial activity and is an indicator of neutrophilic degranulation and was reported to play a crucial role in tissue injury (Klebanoff., 2005). Inflammation and oxidative stress are considered critical factors in the progression of nonalcoholic fatty liver disease (Sander S Rensen et al., 2009; Schults et al., 2012). Myeloperoxidase (MPO) is an important neutrophil enzyme that can generate aggressive oxidants (Nauseef WM et al., 1998). One of the principal molecules released after recruitment and activation of phagocytes is myeloperoxidase (MPO), an important enzyme involved in the generation of reactive oxygen species (Klebanoff., 2005).

In the presence of physiological chloride concentrations, MPO reacts with hydrogen peroxide (H₂O₂, formed by the respiratory burst) to catalyze formation of hypochlorous acid/ hypochlorite and other oxidizing species (Nauseef WM et al., 1998; Klebanoff., 2005). These oxidants may contribute to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates, including DNA, lipids, and
protein amino groups (Davies et al., 2008). In the absence of physiological chloride concentrations, the MPO-H$_2$O$_2$ system can also form reactive nitrogen species (Hazen et al., 1999) that may initiate lipid peroxidation. Interestingly, macrophages are known to generate high amounts of reactive oxygen and nitrogen species. Therefore, MPO-containing macrophages/ Kupffer cells could play a role in NASH (Mantovaniet al., 2007). MPO activity might be a driving factor underlying the progression of human NASH (Malle et al., 2007; Sander S Rensen et al., 2009).

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 inflammatory markers compared to control group (group 1). All the three drugs did not produce any significant alterations in the levels of inflammatory markers as evidenced by the Table 8 and Fig. 13 (A-B).

The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) and rats treated with quercetin (group 7; NASH+quercetin) showed significant decrease in the levels of TNF-α and MPO. Pioglitazone stimulate maturation of visceral fat, and hence change the adipocytokine profile secreted by adipose tissue. Pioglitazone leads to an increase in adiponectin levels, which counteracts pro-inflammatory cytokines such as tumor necrosis factor-α (TNF α) and promotes beta
oxidation of fatty acids via adenosine monophosphate-activated protein kinase (AMP-K) activation (Coletta et al., 2009; Miyazaki et al., 2004). The increase in beta oxidation, Immature adipose tissue in conjunction with a reduction in de novo lipogenesis, decreases gluconeogenesis (Lutchman et al., 2006; Miyazaki et al., 2004). Quercetin is an effective inhibitor of human myeloperoxidase (MPO) activity, both with purified enzyme and in a system using stimulated human neutrophils. Moreover, quercetin is directly able to scavenge hypochlorous acid (HOCl), a chlorinated species generated by the, MPO/H2O2/Cl– system. (Pincemail et al., 1988).

In this present study, quercetin showed protective effect against NASH by significant reduction in inflammatory markers due to its anti-inflammatory activity. Quercetin inhibits TNF-α and IL-6, simultaneously induce IL-10 release, and thus evoke the antiinflammatory effect (Comalada et al., 2006). Quercetin also inhibits STAT-1 and NF-κB and thus possesses the anti-inflammatory effect (Mari Ham¨ ainen et al., 2007). Quercetin’s anti-inflammatory activity may also have a beneficial effect on the muscle damage experienced by athletes after intense exercise (Harwood., 2007). It has been demonstrated that quercetin can inhibit nuclear factor-kappa B (NF-κB) (Harwood., 2007), a chemical in the body which has been shown to play a central role in regulating the immune response to inflammation. Cell culture studies and in vivo (animal) studies have provided evidence supporting quercetin’s anti-inflammatory effects (Harwood., 2007).
Whereas, the experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) does not show any significant effect on the levels of inflammatory markers viz. TNF-α and MPO. Hydroxy citric acid which is a phytoconstituent present in the *Garcinia Indica* or in general *Garcinia species*, and is a proven anti obesity agent with the lipid lowering actions (Heymsfield et al., 1998; Mattes et al., 2000; Sakariah et al., 2002). It’s been well established as a lipid lowering agent and Hypocholesterolemic agent (Heymsfield et al., 1998; Mattes and Bormann., 2000; Sakariah et al., 2002; Deore et al., 2011). Recent research showed that it also possesses little anti-inflammatory activity, apart from these two properties (Khatib et al., 2010). But, in the present study the experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) does not show any significant effect on the levels of inflammatory markers, showing that HCA does not possess any anti-inflammatory action. Due to this, HCA can confer very less protection against NASH, since inflammation of the liver (hepatitis) is one of the principle and key features of NASH.

Quercetin and pioglitazone offers protection against NASH by ameliorating the inflammation (hepatitis), a principle and key feature of NASH. This effect on inflammation could be due to anti-inflammatory properties of pioglitazone and quercetin, whereas hydroxy citric acid offers very little protection against NASH since it does not possess any anti-inflammatory action.
6.2.11. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON PHOSPHOLIPIDS IN EXPERIMENTAL NASH

Effect of pioglitazone, quercetin and hydroxy citric Acid on the levels of different phospholipids in experimental NASH was shown in Table 9. NASH induced group (group 2) showed significant decrease in the levels of phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl inositol along with the significant increase in phosphatidyl glycerol when compared to the control group (group 1).

The separation of phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol by thin layer chromatography (TLC) analysis was showed in Fig. 14. The changes in the levels of these phospholipids were distinct in the livers of the experimentally induced NASH group (group 2) when compared to the controls (group 1).

The distribution of phospholipids in NAFLD have shown the importance of the phosphatidyl choline (PC) / phosphatidyl ethanolamine (PE) ratio on liver disease progression from steatosis to steatohepatitis and its implication as a key regulator of cell membrane integrity (Li et al., 2006). An alteration in lipid homeostasis leads to the initiation of inflammation that is associated in the progression of NASH (Rouzer et al., 2007). Changes in the liver content of PC and PE due to the depletion of choline and methionine along with the relative depletion of PC or the decreased PC/PE ratio results in a loss of membrane integrity and
influences liver damage (Rouzer et al., 2007). Changes in the content of the phospholipids will always precede the inflammatory process (Li et al., 2006). Steatohepatitis is known to be associated with alterations in metabolism of phospholipids in liver (Puri et al., 2007).

Insulin resistance is also associated with increased intracellular content of fatty acids and their metabolites, including ceramide (Holland et al., 2007; Mari et al., 2007). In addition, cytokines such as TNF-α and IL-6, which participates in the pathogenesis of NASH, elicit changes in the metabolism of sphingolipids and stimulate the production of bioactive lipids, such as ceramide, which acts as second messengers (Malagarie-Cazenave et al., 2002; Malagarie-Cazenave et al., 2004). In our present study, we found an elevation of hepatic levels of these two lipids Irrespective of the defect in the cytokines. This is in accordance with a very recent study that reported a trend of increased hepatic content of sphingomyelin in patients with NASH (Puri et al., 2007).

The mechanisms and biological implications of these changes to the pathogenesis of NASH are still unclear. There is a possibility of hepatocytes to increase the synthesis of sphingomyelin, a membrane phospholipid containing choline analogous to PC, to compensate for the loss of glycerophospholipids. Such synthesis of sphingomyelin probably occurs through increased de novo synthesis of ceramide, possibly due to the enhanced incorporation of free fatty acids. The role of ceramide in hepatocellular death and, in general, as a bioactive lipid with pro-apoptotic properties were reported in the literature (Mari et al., 2007; Fernandez-
Checa et al., 2005) and it is tempting to speculate that the increased content of ceramide contributes to the liver injury in NASH.

Significant reduction of phosphatidyl choline (PC), and phosphatidyl ethanolamine (PE) fractions were marked in the subjects with NASH (Puri et al., 2009). Increased free cholesterol levels are often associated with increased PC synthesis (Tabas., 2000). Few studies showed depletion of PC despite increasing FC content in NAFLD. Arachidonic acid (20:4n-6) is released from membrane phospholipids by the action of an enzyme, phospholipase-A2, from phosphatidylglycerol bisphosphate by the action of another enzyme, phospholipase-C through di acyl glycerol (DAG) and is rapidly converted into proinflammatory prostaglandins, thromboxanes, and leukotrienes by cyclooxygenase (Di Marzo et al., 1995). Increased utilization of arachidonic acid may also contribute to the observed decrease in its levels in NAFLD. If true, the modulation of phospholipases and cyclooxygenase may provide another mechanism to control the inflammatory pathways in NASH. Decreased levels of arachidonic acid, PC and also key n-3 fatty acids and an increase in the n-6: n-3 fatty acid ratio could play a role in the pathogenesis of NASH. Decrease in PC in NASH suggests that oxidative stress may contribute to these changes (Colles et al., 2000; Aiyar et al., 2007).

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 protective effect normalizing the levels of phosphatidyl glycerol, phosphatidyl ethanolamine,
phosphatidyl choline and phosphatidyl inositol back to normal, compared to the control group (group 1) as shown in Table 9 as evidenced by the separation of phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol by thin layer chromatography (TLC) analysis in Fig. 14.

Pioglitazone treatment showed the significant increase in phosphatidyl inositol, phosphatidyl choline and phosphatidyl ethanolamine when compared to the NASH group (group 2) and reverted the levels back to near normal. But the phosphatidyl glycerol levels were significantly reduced when compared to NASH group (group 2) and the levels of the same were enhanced than the normal levels when compared to controls.

In the present study, the experimentally induced NASH rats co-treated with quercetin showed significantly increased levels of the phosphatidyl inositol, phosphatidyl choline and phosphatidyl ethanolamine when compared to the NASH group (group 2) and reverted the levels back to near normal. Quercetin significantly reduced the phosphatidyl glycerol levels were when compared to NASH group (group 2) and the levels of the same were reverted back to normal when compared to controls. Quercetin exerts this protective effect of by virtue of its action in reducing the tissue cholesterol, triglycerides, accumulated lipid peroxides and ceramide thereby restoring the profile of phospholipids (Ricardo et al., 2001; Babenko and Shakhova., 2008; Krishnaveni et al., 2010).

Hydroxy citric acid is patented as an anti-obesity drug (Heymsfield et al., 1998; Mattes and Bormann., 2000; Sakariah et al., 2002; Deore et
The experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) showed protective effect normalizing the levels of phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol back to normal, compared to the control group (group 1). This protective effect of hydroxy citric acid could be due to its Hypocholesterolemic and lipid lowering action (Heymsfield et al., 1998; Mattes and Bormann., 2000). (-)-HCA exerts its anti-obesity effect by inhibiting ATP-citrate lyase, consequently inhibiting the cleavage of citrate to oxaloacetate and acetyl-CoA, a key molecule, which plays a critical role in energy storage as fat (Cheema-Dhadli., 1973; Márquez et al., 2012). Under these circumstances, instead of fat synthesis, the energy is diverted to the production of glycogen in the liver and muscles. This depresses the production of fatty acids and cholesterol successive induction of the content of the phospholipids (Mahendran and Shyamala Devi., 2001; Márquez et al., 2012). These phospholipids being active components of very low density lipoproteins (VLDL), increases the synthesis of VLDL. This lipoprotein which is synthesized in liver, transports fats synthesized in liver to adipose tissue ameliorating the fatty liver condition.

Thus, it could be inferred that hydroxy citric acid and quercetin showed protection against NASH by altering the levels of phospholipids reverting them back to the normal. Pioglitazone also exerts its protection against NASH to some extent by increasing the levels of phosphatidyl inositol, choline and ethanol amine.
6.2.12. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON VEGF mRNA

Quantitative real-time polymerase chain reaction (RT-PCR) analysis of vascular endothelial growth factor (VEGF) messenger RNA (VEGF mRNA) was analyzed in all the groups and was represented in Fig. 15. High expression of VEGF mRNA in hepatic cells was observed in experimentally induced NASH group (group 2) when compared to the expression of VEGF mRNA in control group (Lane 2; group 1).

Vascular endothelial growth factor (VEGF) is an inducer of angiogenesis, which is a hallmark of various ischemic and inflammatory diseases. *In vitro* study revealed that leptin exerted a proangiogenic activity in the presence of VEGF. VEGF plays an important role in the development of liver fibrosis and hepatocarcinogenesis in NASH (Kitade et al., 2006). VEGF levels were significantly elevated in patients with simple steatosis and borderline significantly elevated in NASH patients compared to the serum levels of healthy control subjects (Coulon et al., 2012).

Mild increase in the expression of VEGF mRNA in experimental NASH treated with pioglitazone (Lane 4; group 6; NASH+pioglitazone) was observed when compared with the NASH induced group (Lane 2; group 2).

Pioglitazone restores the blood flow recovery and capillary density in ischemic muscle and that this process is associated with increased expression of VEGF (Federico Biscetti et al., 2009). VEGF mRNA expression in liver tissue were decreased by pioglitazone compared to
normal group which is concordant with the previous study in muscles. By contrast, the high-dose pioglitazone suppress vascular endothelial growth factor (VEGF)–induced endothelial cell proliferation via suppressing VEGF receptor 1 (Flt-1) and 2 (Flk/KDR) expression in vitro (Murata et al., 2000). These studies will give supportive evidence for the present findings of pioglitazone on growth factors decreased levels in liver tissue.

Hydroxy citric acid showed mild increase in the expression of VEGF mRNA in experimental NASH treated with hydroxy citric acid (Lane 5; group 8; NASH+HCA) when compared with the NASH induced group (Lane 2; group 2).

Whereas, Quercetin showed very mild increase in the expression of VEGF mRNA in experimental NASH treated with quercetin (Lane 3; group 7; NASH+quercetin) when compared with the NASH induced group (Lane 2; group 2).

The VEGF induction was dependent on quercetin-mediated hypoxia-inducible factor-1 (HIF-1) activation. Quercetin delayed HIF-1alpha protein disappearance, which occurred by inhibiting HIF-prolyl hydroxylase (HPH), the key enzyme for HIF-1alpha hydroxylation and subsequent von Hippel Lindau-dependent HIF-1alpha degradation (Jeon et al., 2007). Our data suggest that the clinical effect of quercetin may be partly attributed to the activation of an angiogenic pathway HIF-1-VEGF via inhibiting HPH and the chelating moieties of quercetin were required for inhibiting HPH.

Quercetin activates an angiogenic pathway, hypoxia inducible factor (HIF)-1-vascular endothelial growth factor, by inhibiting HIF-prolyl
hydroxylase: a structural analysis of quercetin for inhibiting HIF-prolyl hydroxylase. Quercetin, a flavonol found in several varieties of berries, at concentrations up to 70–80 mg/kg dry wt, was found to inhibit growth and stimulate apoptosis of cancer cells (Jeon et al., 2007).

By virtue of our findings, it could be concluded that the drug quercetin showed an effective inhibition of VEGF mRNA expression and perhaps only smaller inhibition of VEGF mRNA level seen in hydroxy citric acid and pioglitazone treated rats. This study showed the therapeutic value of quercetin, pioglitazone and hydroxy citric acid.

6.2.13. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON CYP2E1

Detection of Cytochrome P450 2E1 (CYP2E1) cenzyme levels in liver by immunoblot analysis was depicted in Fig. 16. CYP2E1 catalytic activity was increased in experimentally induced NASH group (group 2) compared to control group (group 1) as evidenced in Fig. 16.

A common denominator in the pathogenesis of insulin resistance and nonalcoholic steatohepatitis is increased oxidative stress. Hepatic induction of the pro-oxidant enzyme CYP2E1 occurs in both NAFLD and type 2 diabetes. Insulin resistance and increased cytochrome P450 2E1 (CYP2E1) expression are both associated with and mechanistically implicated in the development of nonalcoholic fatty liver disease. Insulin resistance and CYP2E1 expression may be interrelated through the ability of CYP2E1-induced oxidant stress to impair hepatic insulin signaling (Tirosh et al., 2001). Up-regulation of microsomal proteins viz. CYP2E1
and CYP4A have been observed in patients with NASH and constitute the most important factors in the oxidation of FFAs. The CYP2E1 activity is directly related to the hepatic steatosis (Emery et al., 2003). It has been well documented that alcohol-mediated up regulation of cytochrome P450 2E1 (CYP2E1) may initiate lipid peroxidation by the production of reactive oxygen species (Ekström and Ingelman-Sundberg, 1989; Ronis et al., 1996; Lieber, 1997). CYP2E1 plays a key role in the pathogenesis of liver injury by virtue of its capacity to generated reactive oxygen species and lipid peroxides (Dai et al., 1993). CYP2E1 over expression occurs in animals and humans with nonalcoholic steatohepatitis (Estebauer, 1996; Kono et al., 1999). The significant increased activity of CYP2E1 in our present study in experimentally induced NASH is concordance with these studies (Estebauer, 1996; Kono et al., 1999).

Oxidative stress mediated by over expression of CYP2E1 has been shown to promote liver injury in both alcoholic and non alcoholic fatty liver disease (Tietz, 1976; Bell et al., 1993). It is hypothesized that CYP2E1-induced oxidative stress may act to sensitize hepatocytes to death. The present study supported this hypothesis by demonstrating over expression of CYP2E1 in NASH induced rat hepatocytes. CYP2E1 catalyze “leaky” redox cycles, which can also produce ROS during fatty acid metabolism in the endoplasmic reticulum or even in the absence of substrate (Obertson et al., 2001; Schattenberg et al., 2005). In our present study, the activity of CYP2E1 was significantly increased in experimentally induced NASH,
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which in concordance with the previous report (Weltman et al., 1996; Weltman et al., 1998).

Immunoblot analysis revealed the low levels of CYP2E1 in the experimental NASH treated with pioglitazone, quercetin and hydroxy citric acid NASH animals (Fig. 16).

Mild decrease in the levels of CYP2E1 level was observed in experimental NASH treated with pioglitazone (group 6; NASH+pioglitazone) compared to NASH group (group 2). This could be due to the reduction of insulin resistance in pioglitazone treated rats and this observation of our present study was supported by the other studies (Ersoy et al., 2008). Insulin resistance and CYP2E1 expression may be interrelated through the ability of CYP2E1-induced oxidant stress to impair hepatic insulin signaling (Tirosh et al., 2001). Hydroxy citric acid also showed mild decrease in the levels of CYP2E1 level in experimental NASH treated with hydroxy citric acid (group 8; NASH+HCA). This effect could be attributed to the little antioxidant property of the hydroxy citric acid (Devasagayam et al., 2006).

On contrary to the action of pioglitazone and hydroxy citric acid, quercetin showed an approximate 2-fold decrease in the level of CYP2E1 levels in experimental NASH treated with quercetin (group 7; NASH+quercetin) compared to NASH group (group 2). The present findings of our study were supported by various other studies on quercetin’s ability to reduce CYP2E1 levels (Tang et al., 2012).
This significant effect of quercetin of the levels of CYP2E1 could be attributed to the powerful antioxidant property of the quercetin, which achieves this effect by reducing the lipid peroxidation and, increasing activities of the antioxidant system oxidative stress (Alexandra and Bentz., 2009; Davis., 2009a; Davis., 2009b). Quercetin’s chemical structure enables it to scavenge oxygen-centered free radicals, the reactive molecules in the body that participate in oxidative reactions that cause cell damage (Harwood., 2007; Alexandra and Bentz., 2009).

Thus it can be inferred that being a powerful antioxidant, quercetin offers absolute protection to liver against NASH by reducing the levels of CYP2E1 and thereby reducing CYP2E1 mediated oxidative stress, which is believed to be the one of the key factor in the pathogenesis of NASH. On the other hand, pioglitazone and quercetin exerted limited effect on the levels of CYP2E1.

6.2.14. EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON CK-18

Liver tissue lysates were analyzed by immuno histochemistry for the expression of CK-18 (Fig. 18) and then quantification was carried out (Fig. 19). There observed no expression of CK-18 cells in hepatic tissue of the rats in control group (group 1) (Fig. 18A). Greater expression of CK-18 cells was observed in experimentally induced NASH group (group 2) reflecting the liver damage.

The understanding of the role played by hepatocyte apoptosis, oxidative stress and insulin resistance in the pathophysiology of liver injury
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has enabled the development of promising biomarkers of NASH, such as caspase-cleaved cytokeratin 18 fragments or numerous adipokines. Wieckowska et al., (2006) first researcher who demonstrated the correlation of the CK18 fragments in serum with the degree of inflammation in NASH. CK18 M30 fragments have recently been shown as a biomarker to diagnose NASH by a number of studies (Feldstein et al., 2009; Wieckowska et al, 2006) and its correlation with the severity of NASH.

A central consequence of the apoptotic process is the activation of the effector caspases (mainly caspase 3) which cleave a number of different substrates inside the cell including cytokeratin 18 (CK-18) (Danial and Korsmeyer., 2004). CK-18 is the major intermediate filament protein in the liver, resulting in the characteristic morphologic changes of apoptosis (Danial and Korsmeyer, 2004). CK-18 fragments were significantly elevated in the NAFLD patients as compared to controls and plasma levels correlated with the expression levels in the liver (Wieckowska et al., 2006). Caspase-generated CK-18 fragments released in NAFLD will serve as an indicator of hepatic inflammation (Vos et al., 2008; Younossi et al., 2008). Few studies proposed that the determination of CK-18 fragments in blood accurately identifies the presence of NASH on liver biopsy (Ariel et al., 2009).

CK-18 fragments reflect the increased apoptotic rate as a consequence of the hepatic inflammatory response, may therefore distinguish NASH from simple steatosis (Yilmaz et al., 2007). Elevated CK-
18 fragments have been identified in NAFLD patients with normal amino transferase levels (Yilmaz et al., 2009).

The circulating levels of cytokeratin-18 (CK18) fragments have been investigated in our present study, to establish CK-18 as a novel biomarker to diagnose and confirm the presence of NASH. Our findings in the present study showed greater expression of CK-18 cells was observed in experimentally induced NASH group (group 2) reflecting the liver damage and this observation was in concordance with the previous research reports (Wieckowska et al., 2006; Vos et al., 2008; Younossi et al., 2008; Ariel et al., 2009).

After the completion of CK-18 studies in NASH, we wanted to know whether the CK-18 levels have also been expressed in alcoholic liver disease (ALD) or not, since ALD and NASH closely resemble each other except the involvement of alcohol in NASH. We have planned to do this comparison with ALD to move further steps to establish CK-18 as novel biomarker to diagnose NASH. In order to establish CK-18 as a novel biomarker to diagnose NASH, it must be expressed only in NASH but not in ALD, a close associate to NASH. Surprisingly, CK-18 was also the over expressed in ALD compared to control rats, but the percentage of decrease was less compared to NASH as evidenced by Fig. 18F and Fig. 19.

The liver tissues of the rats in experimental NASH treated with pioglitazone group (group 6; NASH+pioglitazone) showed less expression of CK-18 cells than that of the NASH group (group 2) (Fig. 18C). The
reduction in CK-18 expression after treating with pioglitazone may be due to its effect on TNF-α level in NASH. Pioglitazone decreases the TNF-α and interleukin-6 (IL-6) levels through ability of PPARγ to inhibit inflammatory response (Mark J Czaja., 2009). The reduction in TNF-α and interleukin-6 (IL-6) possesses beneficial proliferative and hepatoprotective effects. So, the decreased levels of TNF-α as a result of pioglitazone treatment, inhibits proliferation of the liver tissue that resulted in the reduced expression of CK-18 (Mark J Czaja., 2009).

The liver tissues of the rats in experimental NASH treated with hydroxy citric acid group (group 8; NASH+hydroxy citric acid) showed moderate expression of CK-18 cells than that of the NASH group (group 2) (Fig. 18D). Hydroxy citric acid is a very well known and patented to use as a hypocholesterolemic agent (Mattes and Bormann., 2000; Sakariah et al., 2002; Deore et al., 2011) and it possess a very little anti-inflammatory activity (Khatib et al., 2010). Also, it showed a very limited role in reducing the enhanced TNF-α levels in NASH. This can be attributed to the moderate expression of CK-18 in liver tissues of HCA treated rats.

Whereas, the liver tissues of the rats in experimental NASH treated with quercetin (group 7; NASH+quercetin) showed very mild expression of CK-18 cells than that of the NASH group (group 2) (Fig. 18E), showing maximum protection against NASH. Quercetin is very effective in reducing the over expressed CK-18 levels in NASH when compared to that of pioglitazone and hydroxy citric acid. This action of quercetin could be due to its anti-inflammatory and antioxidant properties (Harwood., 2007;
Sakanashi et al., 2008; Alexandra and Bentz., 2009). Quercetin was very effective in reducing the elevated TNF-α levels, reducing the lipid peroxidation products as well as in scavenging the free radicals, thereby preventing cell damage and apoptosis to a greater extent, which resulted in the very mild expression of the CK-18 in the liver tissues of the rats treated with quercetin.

By the results of the above studies on CK-18, it can be inferred that the over expressed CK-18 levels in NASH were markedly reduced by treatment with pioglitazone, quercetin and HCA suggesting their role to prevent cell death. The maximum significant protection was shown against NASH by quercetin compared to pioglitazone and HCA. The over expression of CK-18 was also observed in ALD compared to control rats, but the percentage of decrease was less compared to NASH.

6.2.15. “TPSA” LEVELS IN NASH AND COMPARISON WITH ALD

By the results of CK-18 studies in NASH and ALD, it has been observed that CK-18 has been over expressed in both ALD and NASH and hence the usefulness of the CK-18 as a novel biomarker to diagnose NASH. This led us in search of identification of another novel biomarker to diagnose NASH, which is expressed in NASH but not in the healthy individuals and ALD as well.

Keeping this as a key objective, we have conducted studies on the expression of tissue polypeptide specific antigen (TPSA) in NASH and ALD, compared to controls.
Tissue Polypeptide Specific antigen (TPSA) has recently been proposed as diagnostic marker of apoptosis in NASH. The levels of TPSA estimated by ELISA in control (group 1), experimentally induced NASH (group 2) and also compared with alcoholic liver disease (ALD) was depicted in Fig. 20. Significantly increased levels of TPSA were observed in experimentally induced NASH group (group 2) when compared to the controls (group 1). Whereas, TPSA was not expressed in alcoholic liver disease (ALD) giving a hope that it may be useful as a marker to diagnose NASH and more studies to be conducted on usefulness of TPSA as a maker to diagnose NASH.

Tissue polypeptide-specific antigen (TPSA), a serological mirror of keratin 18 has been widely used as a marker for various cancers (Tarantino et al., 2007). Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH (Fierbinteanu et al., 2010). The studies conducted to evaluate the relationship between liver diseases and serum TPSA levels showed a significant relation between high TPSA and high AST-ALT levels (Putzki et al., 1998; Leandro et al., 1989; Yeo et al., 2000). The correlation between TPSA and transaminase levels as markers of hepatocyte lysis were reported in literature (Yeo et al., 2000; Cetin et al., 2003).

The liver cytokines were valuable tools for understanding the cellular origin of neoplasms and the pathogenesis of liver diseases (Stumptner et al., 2000). The levels of TPSA were found to be high in patients with cirrhosis (Moraglio et al., 1994) and our findings of TPSA in
NASH in this present study were in concordance with this report.

Till date, liver biopsy remains the gold standard to diagnose nonalcoholic steatohepatitis (NASH). Plasma cytokeratin 18 (CK-18) fragment levels correlate with the magnitude of hepatocyte apoptosis and independently predict the presence of NASH (Ariel et al., 2009), but the CK-18 was expressed in ALD also. Our findings of this study suggested that non-invasive monitoring of hepatocyte apoptosis in blood of patients with NAFLD is a novel and reliable tool to diagnose NASH in patients with suspected NAFLD supporting its potential usefulness in clinical practice as a noninvasive NASH biomarker.

Serum TPSA, a fragment of CK-18 routinely used as a marker for malignant and non malignant liver diseases (Qonazalez-quintela et al., 2009). Increase in hepatocyte cell death by apoptosis is typically present in humans with NASH as well as animal models of NASH but absent in those with NAFLD (Feldstein et al., 2003). Activation of the effector caspases (mainly caspase 3) which cleave a number of different substrates inside the cell including cytokeratin 18 (CK-18), the major intermediate filament protein in the liver, resulting in the characteristic morphologic changes of apoptosis was the central consequence of the apoptotic process (Danial and Korsmeyer., 2004). Caspase generated CK-18 fragments were also tested in the livers as well as in plasma of patients undergoing a liver biopsy for suspected NAFLD and the CK-18 fragments were significantly elevated in the NAFLD patients as compared to controls and plasma levels correlated with the expression levels in the
liver (Wieckowska et al., 2006). Increased apoptosis lead to increased production of CK-18 which in turn leads to increased TPSA levels. Since several studies reported TPSA as marker of hepatocyte lysis, the present investigation also taken into an account and showed positive reports and TPSA was not expressed in ALD.

Thus, it can be inferred that the above results of TPSA in NASH and ALD generates a hope to evaluate TPSA as a novel biomarker to diagnose NASH. These findings will also be beneficial for future studies on the diagnosis and management of NASH.