CHAPTER 6

DISCUSSION
Liver is the largest organ in the human body and is crucial in maintenance of good health. It is involved in almost all biochemical pathways that allow growth, protection from diseases, synthesis and supply of nutrients etc. The liver performs more than 500 vital functions of metabolic importance (Naruse et al., 2007). Liver is constantly exposed to several environmental and chemical insults to which it gives a specific response. Chronic exposure to such insults leads to inflammation and compromise in liver function (Sahu, 2007). Several drugs and/or chemicals are known to cause hepatotoxicity (Bigoniya et al., 2009). Several adverse effects of liver protective drugs in allopathic medical practices have prompted the untiring search for herbs and nutritional supplements for management of various liver disorders. Use of number of medicinal plants and their formulations are common for the treatment of liver diseases in ethno-medical practices and in traditional medicine system (Dange, 2010; Kumar et al., 2011; Shaik et al., 2012). Modern medicine has little to suggest for improvement of hepatic diseases and it is essentially the plant based preparations which are employed for the treatment of liver disorders (Chatterjee, 2000). Approximately 80% of the world population depends on the use of traditional medicine system which is predominantly based on plant materials (WHO, 2002). It is estimated that local health traditions, mostly in rural and tribal villages of India, use about 7,500 plants. Out of these, the real medicinal value of over 4,000 plants is either little known or yet to be known to the mainstream population. The classical systems of medicine such as Ayurveda, Siddha, Unani, Tibetan, and Amchi use about 1,200 plants (Pandey, 2011). Various herbs and plant extracts have significant hepatoprotective activity as indicated from studies in animal models (Malhotra and Singh, 2006; Chaudhary et al., 2010). Nutritional supplements with omega-3 fatty acids and diet rich in carbohydrates, fats and proteins with adequate calories and helps in regeneration of liver cell for patients with liver diseases (Drevon, 2009; Shukla and Kumar, 2013)

Studies in the present thesis have indicated hepatoprotective activities of herbal and nutritional supplement(s), and the combinatorial hepatoprotective effects of best performing herbal and nutritional supplements against acetaminophen and alcohol induced liver damage.
6.1 Guduchi Satwa (*T. cordifolia*, *T. sinensis* and Neem-giloe)

6.1.1 Organoleptic Characteristics of Three *Tinospora* forms

There are very few reports available for organoleptic characteristics of *T. cordifolia* and Neem-giloe satwa (Sharma et al., 2012; Patil and Chaudhary, 2013; Sharma et al., 2013a; Chavan et al., 2014; Sharma et al., 2015). As per classics, the taste of Guduchi satwa from *T. cordifolia* is *swadu* (palatable/pleasant) (Navare, 2011) but some recent reports describe Guduchi satwa from *T. cordifolia* as slight bitter (Sharma et al., 2013a; Sharma et al., 2015) and Guduchi satwa from Neem-guduchi as tasteless (Sharma et al., 2012; Sharma et al., 2015). However, in present study, satwa from *T. cordifolia* and *T. sinensis* was found to be tasteless while that from Neem-giloe was found to be bitter in taste. In *Rasa Yoga Sagara* (Sharma, 2004), the colour of satwa is mentioned as ‘*Shubhrakhandnibha*’ (clear white like sugar cubes) (Sharma, 2004) and *Yoga Ratnakara* reveals it as ‘*Shankhanibha*’ (clear white like conch shell) (Shastri, 2002) but some texts mention the colour of satwa as greenish white (Reddy, 2005) or greyish white (Hiremath, 2005) and the colour of satwa from Neem-guduchi as pale (Sharma et al., 2012) to clear white (Sharma et al., 2015). In the present work, colour of the satwa prepared from *T. cordifolia*, *T. sinensis* and Neem-giloe were found to be grey, greyish white and yellowish white respectively, which resembles the recent reports. The texture and taste of satwa also depends on the age of the plant material used for satwa preparation. A recent study by Patil and Chaudhary (2013) have examined stem pieces of different thickness and maturity of *T. cordifolia* to find best size of the stem to achieve maximum yield of satwa. A yield of 0.48% -0.1% satwa from *T. cordifolia* was reported from fresh stem (Mehra and Puri, 1969; Rao and Rao, 1981; Salunke and Pimpalgaonkar, 1997) and 1.20% (Rao and Rao, 1981) with that of dried stem. Sharma et al. (2012) have reported that the thickness of *T. cordifolia* stem also has effect(s) on the yield of satwa with 1.6-2.0 cm thick stem giving maximum yield of satwa. These variations may be due to different ecotypes, size of the stem, collection time and levels of maturity of the plant. In the present study stem pieces of 1.6-2.0 cm were used for preparation of satwa and the yield of satwa ranged from 1.48%-1.60% in three different forms of *Tinospora*. Sharma et al. (2012) have reported that the yield of satwa from Neem-guduchi is highly variable and it depends upon multiple factors like size, environment,
nature of cellular activities etc. They have also reported that the medium sized stem (1.5-2.0cm) of Guduchi yields maximum satwa since there is maximum accumulation of starch at this stage during development. Sharma et al. (2015) have reported that the yield of Neem-guduchi satwa from male and female stem was 2.25 % and 3.18 % respectively since relatively higher starch and mucilage contents are found in female plants, suggesting that more yield of satwa can be obtained from them.

6.1.2 Nutritional Analysis of Three Tinospora forms

The nutritional composition of medicinal plants depends heavily on environmental and physiological conditions as well as the time of harvesting of the plant (Kutbay and Ok, 2003; Sharma et al., 2013b). Guduchyadi varga from Bhavprakash nighantu (1999) mentioned the time of collection of Guduchi as end of May. Accumulation of active components of a drug is also reported to be maximum during specific seasons in many medicinal plants (Kutbay and Ok, 2003; Nasreen et al., 2010; Patil and Gaikwad, 2011). The variability in the active components of an Ayurvedic drug due to these factors can be studied with the help of principles to determine different nutritional/pharmaceutical contents in the drug (Nasreen et al., 2010; Patil and Gaikwad, 2011; Geeta and Kumari, 2013; Mahima et al., 2013). In case of T. cordifolia, crude protein and fiber are reported to be the principal components of the powder from dried stem pieces (Hussain et al., 2009). There are reports about the proximate and elemental analysis of powder prepared from fresh or dried stem of T. cordifolia (Nile and Khobragade, 2009; Mahima et al., 2014).

Powder prepared from dried stem of T. cordifolia was found to be rich in phytochemicals like alkaloids, glycosides, sterols and carbohydrates (Nasreen et al., 2010; Tanwar et al., 2012). The presence of wide range of phytochemicals is also reported in different solvent extracts (methanol, petroleum ether, water, chloroform and ethyl acetate) of T. cordifolia stem (Sivakumar and Rajan, 2011; Pradhan et al., 2013). Nutritive value of the medicinal plants can be determined with the help of proximate and elemental analysis (Mahima et al., 2013). The crude protein content of T. cordifolia is of great importance to its nutritive value (Ajibade and Fagbohun, 2010). Satwa is a potential source of nutrition and has shown beneficial effects for boosting the immune system-response and body building (Ajibade and Fagbohun, 2010; Geeta and Kumari, 2013; Mahima et al., 2014).
The reports on the nutritional analysis of Guduchi satwa are available only for *T. cordifolia* satwa. There are no reports of nutritional and/or comparative analysis of *T. sinensis* and *Neem guduchi*. In view of this, the present analysis was undertaken to identify the nutritional richness of these *Tinospora* forms. The present study has lead to the first report of the nutritional analysis of satwa prepared from *T. cordifolia*, *T. sinensis* and *Neem-guduchi* (Chavan et al., 2014). In present study, the lipid, ash, and carbohydrate contents of *Neem-giloe* were greater than that of *T. cordifolia* and *T. sinensis*. *T. sinensis* showed higher amount of protein, starch, and crude fiber than *Neem-giloe* and *T. cordifolia*.

### 6.2 Drug Induced Liver Injury

The ability to prevent damage to the liver is called as hepatoprotection (Mishra et al., 2014). Hepatic damage may be caused due to viral hepatitis, bile duct obstruction, cholesterol overload, etc. and also due to some chemical factors such as overdose of several drugs, alcohol intake etc. With increase in the incidence of hepatotoxicity, there is a need to find effective ways for prevention or management of liver damage (Wang et al., 2009). The hepatoprotective efficacy of a drug is measured in terms of its ability to restore normal hepatic functions or to reduce damage to the liver (Yadav and Dixit, 2003). The drugs used for treating hepatic disorders in modern medicine have several undesirable side effects (Singh, 2013; Ananthi and Anuradha, 2015).

The two hepatotoxicants selected in this study are Acetaminophen (Paracetamol) and Alcohol (Ethanol) which are routinely used for medicinal or other purposes. The incidence of acetaminophen induced liver damage has been reported to be increased from 28% in 1998 to 52% in 2003, in USA (Larson et al., 2005). A recent Indian report has estimated that about 33% of the people consuming acetaminophen are subjected to liver damage (Marzilawati et al., 2012). Easy and over-the-counter availability of acetaminophen and self-medication (Kaufman et al., 2002; Larson et al., 2005; Abay and Amelo, 2010) is believed to be a reason behind intentional or unintentional overdose (Lee, 2004; Larson et al., 2005). Alcohol increases the hepatotoxicity of various xenobiotics and the interaction between alcohol and hepatotoxins is well recognized (Zimmerman, 1999). Alcoholic liver disease (ALD) is a common consequence of prolonged and heavy alcohol abuse. In
India, the incidence of alcohol induced hepatotoxicity is reported to be 31% (WHO, 2011).

In the present study, animals were dosed repeatedly with hepatotoxicant (acetaminophen and alcohol separately) and simultaneously treated with satwa from one of the three different forms of *Tinospora* or Omega-3 fatty acids (Flax oil and Fish oil) and combination of best *Tinospora* forms and best omega-3 fatty acids. Biochemical, histological and molecular analyses were performed to determine the beneficial effects of satwa from three *Tinospora* forms and omega-3 fatty acids and their combination.

### 6.3 Hepatoprotective Activity of Satwa against Acetaminophen Induced Hepatotoxicity

#### 6.3.1 Biochemical Parameters

Elevated levels of serum glutamic oxaloacetic transaminase (SGOT) indicate liver damage, which may be due to viral hepatitis, cardiac infarction and muscle injury while serum glutamic pyruvic transaminase (SGPT) is more specific to liver, and is thus a better parameter for detecting liver injury (Reitman and Frankel, 1957; Thapa and Walia, 2007). Increase in serum levels of alkaline phosphate (ALP) is due to increased synthesis of the enzyme in presence of increasing biliary pressure (Kind and King, 1954; Thapa and Walia, 2007). Serum bilirubin level is related to function of hepatic cells (Jendrassik and Grof, 1938; Thapa and Walia, 2007). Increased SGOT, SGPT and ALP are indicative of cellular leakage and reduced functional integrity of the liver cell membranes indicating hepatocellular damages (Gutierrez and Solis et al., 2009; Basu et al., 2012). In the current study, administration of acetaminophen caused a significant elevation of SGOT, SGPT, ALP, and total bilirubin when compared to healthy control, which is indication of hepatic damage. Several reports indicate elevated levels of liver function markers like SGOT, SGPT, ALP and bilirubin in rats subjected to acetaminophen induced liver injury (Duairaj et al., 2007; Ramachandran et al., 2010; Sundari et al., 2011; Basu et al., 2012; Galal et al., 2012). Our study reveals comparative hepatoprotective effect of *T. cordifolia*, *T. sinensis* and *Neem-giloe* satwa. The previous studies have reported the hepatoprotective effect of *T. cordifolia* alone against different hepatotoxicants
(Bishayi et al., 2002; Sharma and Pandey, 2010; Kavitha et al., 2011b; Venkatalakshmi and Ragadevi, 2012; Kumar et al., 2013b). In the present study, treatment of rats with Neem-giloe (200 mg/kg) decreased levels of SGOT and bilirubin while T. sinensis showed effects on improvements in serum SGPT and ALP against acetaminophen induced hepatotoxicity. Apart from liver protection, studies on T. sinensis indicate anti-inflammatory (Li et al., 2003) and anti-diabetic activities (Yonemitsu et al., 1993). Some reports also show the immunomodulatory activity of T. cordifolia (growing on neem tree) (Bhalerao et al., 2012; Narkhede et al., 2014).

Elevated levels of total cholesterol, phospholipids, triglycerides and free fatty acids in the plasma have been reported in acetaminophen treated rats which is an indication of reduced or impaired fat metabolism secondary to liver damage (Ramachandran et al., 2010; Haldar et al., 2011; Malarvizhi et al., 2012). Several reports indicate elevated levels of serum lipids (Total cholesterol, triglyceride, and LDL) in rats subjected to acetaminophen overdose (Duairaj et al., 2007; Sundari et al., 2011; Basu et al., 2012; Singh, 2013; Singh et al., 2015). In present study, animals treated with acetaminophen showed increase in the levels of cholesterol, triglyceride, very-low-density lipoprotein cholesterol (VLDL), low-density lipoprotein cholesterol (LDL), and decrease in high-density lipoprotein cholesterol (HDL) levels in serum and liver homogenates as compared to healthy animals. The earlier studies revealed that treatment with T. cordifolia root extract resulted in significant reduction in levels of serum and tissue cholesterol, phospholipids and free fatty acids in alloxan induced diabetic rats (Stanely et al., 1999). In present study, T. cordifolia satwa exhibited improvements in the serum levels of total cholesterol, HDL and LDL, T. sinensis satwa showed improvement in VLDL and triglycerides levels while Neem-giloe satwa showed significant improvements in total protein and lipid profile (HDL, LDL, VLDL, Triglyceride) in liver tissues. The alterations in the lipid profile due to chemically induced liver damage also leads to generation of several other chemical entities which may have direct or indirect effect on the liver functions (Basu et al., 2012).

Free radicals are generated during liver injury which attack many sub-cellular organelles and systems. Acetaminophen hepatotoxicity is mediated through oxidative stress due to activation of acetaminophen by cytochrome P450 (Zoubair et al., 2013). Excessive peroxidation causes increased reduced glutathione (GSH) consumption
(Nandy et al., 2012). GSH is a scavenger of toxic metabolites, including NAPQI, which is a metabolite of acetaminophen (Hsu et al., 2008). Depletion in the levels of reduced glutathione leads to compromised antioxidant capacity of the tissue. Acetaminophen overdose causes decrease in antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Lores et al., 1995; Duairaj et al., 2007; Basu et al., 2012) and increases lipid peroxidation levels in liver (Duairaj et al., 2007; Basu et al., 2012). In the present study, the activity of SOD and catalase as well as GSH contents in liver significantly decreased, indicating oxidative stress while lipid peroxidation (MDA) levels increased indicating increased lipid peroxidation. Interventions with Neem-giloe satwa showed significant increase in the levels of SOD, CAT, and GSH. Though statistically insignificant, animals treated with the satwa from T. sinensis showed decreased MDA levels in liver homogenates than that in acetaminophen treated group. The administration of T. cordifolia (50, 100, 200 gm/kg orally daily for 25 days) has been reported to exhibit protective effect by reducing the contents of thiobarbituric acid reactive substances (TBARS) and increasing GSH, ascorbic acid, protein, and the activities of antioxidant enzymes viz., SOD, CAT, glutathione peroxidase, glutathione S-transferase, (GST), and glutathione reductase (GR) in liver and kidney against aflatoxin B1 (AFB1) toxicity in mice (Gupta et al., 2011). Prince and Menon (2001) reported that administration of aqueous extract of the roots of T. cordifolia (5.0 g/kg for 6 weeks) resulted in significant reduction in TBARS and an increase in GSH, CAT and SOD in alloxan induced diabetic rats.

6.3.2 Histological Analysis

Histological study of liver sections of rats treated with acetaminophen displayed degenerative changes in hepatocytes and cells that line the blood sinusoids. The anatomical changes in liver due to injury or toxicity are known to be positively correlated with increase in transaminase activities (Galal et al., 2012). Acetaminophen induced liver injury frequently shows intense centrilobular necrosis and vasoconstriction (Manivannan et al., 2011), severe intense congestion, hydropic degeneration, pyknosis and occasional necrosis (Kanchana and Sadiq, 2011; Prabu et al., 2011), sinusoidal haemorrhages and dilatations with chronic inflammatory cell infiltrate in portal tracts (Sundari et al., 2011). In present study, sections of liver from
acetaminophen treated group showed mild congestion of central vein, hepatocytes exhibited apoptotic death with few intra and extracellular hyaline globules and ballooning and degeneration around central vein. Mild nucleomegaly was also visible under microscope. Significant recovery of hepatocellular lesions was observed in the animals treated with the satwa of *Neem-giloe* which restored the hepatic histology to near normal architecture. Such normal hepatic lobule architecture was found in the animals treated with 2 ml/100g of *T. cordifolia* for 30 days against CCL₄ induced hepatotoxicity (Kumar et al., 2013b). Normal liver histology, without any detectable necrosis and vacuolisation, was also observed in rats treated with *T. cordifolia* extract (200 mg/kg) once daily for 3 days against CCL₄ induced hepatotoxicity (Kavitha et al., 2011b).

### 6.3.3 Gene Expression

**Expression of Genes Involved in Lipid Metabolism**

PPAR\(\gamma\) (peroxisome proliferator-activated receptor-gamma) and SREBP1 (Sterol regulatory element-binding protein 1) are transcription factors and regulators of lipid homeostasis in hepatocytes and a target for fatty acids and hypolipidemic drugs (Eberle et al., 2004; Shan et al., 2008; Gong et al., 2014).

The proteins encoded by different PPAR genes have the ability to induce hepatic peroxisome proliferation in response to xenobiotic stimuli (Sahu, 2007). The three PPAR isoforms (PPAR\(\alpha\), PPAR\(\delta\) and PPAR\(\gamma\)) are believed to play a central role in regulation of carbohydrate and lipid metabolism, fatty acid metabolism and are also assumed to possess anti-inflammatory activity (Wang et al., 2014b). These PPAR isoforms inhibit the induction of pro-inflammatory cytokines and stimulate the production of anti-inflammatory molecules (Kostadinova et al., 2005). Dysregulation of some PPAR isoforms contribute to development of a wide range of liver diseases (Peyrou et al., 2012). The pathways for action of PPARs have been well studied. PPARs combine with PPRE (peroxisome proliferator response element) in the promoter region of their target genes involved in fatty acid transport and lipid catabolism (Sears et al., 2007). Differential effects could be explained by promoter and cell context and also by the availability of co-factors but at the same time there are site specific conformational changes in the receptors which are induced by PPAR\(\gamma\)
ligands that ultimately lead to chromatin modeling of target genes and differential promoter activation (Olefsky, 2000). Majority of studies deal with PPARγ in diabetic and obese mouse livers (Memon et al., 2000; Gavrilova et al., 2003; Bedoucha et al., 2001) but the mechanistic relationship of increase of PPARγ expression in hepatotoxicity, remains unclear till date.

Liver is a primary site of biotransformation and is critical in modulating metabolically and chemically induced toxicity and there are reports which suggest that PPARs modulate hepatotoxicity. Pioglitazone, a PPARγ agonist, inhibits CCl₄ (carbon tetrachloride) induced hepatic fibrosis through inhibition of inflammation and hepatic stellate cell proliferation indicating protective role of PPARγ in hepatotoxicity (Yuan et al., 2004). An isoform of PPAR, PPARβ, enhances chemically induced liver toxicity (Hellemans et al., 2003). Expression of PPARβ messenger RNA was increased in hepatic stellate cells, as they undergo spontaneous activation (Hellemans et al., 2003).

PPARγ has direct involvement in regulation of the functional expression of drug transporters, such as the ABCG2, ABCA1 etc. ABCG2, an ATP-binding cassette transporter is known to perform clearance of endogenous and exogenous toxic agents. Activation of PPARγ and consecutively increased amounts of the ABCG2 transporter protein were shown to significantly increase efflux of xenobiotics in human dendritic cells (Szatmari et al., 2006). Peroxisomal proliferators like clofibrate, aspirin, valproate, ethylhexanol, ciprofibrate and perfluorooctanoate might cause local toxicity due to inhibition of mitochondrial oxygen uptake (Keller et al., 1992). In chronic liver injury induced by carbon tetrachloride, for instance, a downregulation of PPARγ expression was observed in hepatocytes, while increased levels of these transcription factors were found in Kupffer cells associated with inverse correlation to levels of activated NFκβ (Orfila et al., 2005). PPARγ also plays an antitoxic role by inducing liver cells to deposit harmless lipids thereby preventing the accumulation of toxic lipids (Medina-Gomez et al., 2007). Miyahara et al. (2000), and Zhang et al. (2012) reported PPARγ deficiency in hepatic stellate cells associated with excessive formation of fibrotic tissue in the liver. The role of PPARγ in manifestation of inflammation is gaining momentum (Szeles, 2007) and expression in hepatoma cell line indicates its potential role in liver function (Koga et al., 2007). Down regulation of PPARγ mRNA expression has been reported in isoniazid induced hepatotoxicity.
(Mahmoud et al., 2014). In accordance with the above reports, the present study also reported down regulation of PPARγ expression in acetaminophen induced hepatotoxicity as compared with healthy control. Several medicinal plant extracts are reported to activate PPARγ (Local Food-Nutaceuticals Consortium, 2005; Christensen et al., 2009; Andaloussi et al., 2010; Vogl et al., 2013; Yang et al., 2013). Curcuminoids have also been reported to inhibit pro-inflammatory induction by enhancing PPARγ activation (Jacob et al., 2007). The protective effects of berberine against isoniazid-induced hepatotoxicity may be attributed to its ability to upregulate PPARγ and subsequently suppress NF-κβ, iNOS and release of proinflammatory cytokines (Mahmoud et al., 2014). The mechanism of action of hepatoprotection by several secondary metabolites from plants has been shown to be through reduction in oxidative stress which is achieved via activating PPARγ (Duval et al., 2014). Though statistically insignificant, the present study reported marginal improvement in PPARγ expression in the livers of rats treated with Neem-gilo satwa and T. sinensis satwa exhibiting near normal liver architecture.

The liver plays a central role in lipid metabolism through de novo lipid synthesis and fatty acid oxidation. SREBPs (Sterol regulatory element-binding proteins) are the important transcription factors which activate the expression of genes involved in the biosynthesis of TGs (Triglycerides), fatty acids and cholesterol. The transcription factors in SREBP family are master regulators of lipid metabolism, which control the expression of genes required for fatty acid and cholesterol biosynthesis. In mammals, there are three isoforms of SREBPs, SREBP- 1a, 1c and 2 (Horton et al., 2002). SREBP-1c is the predominant isoform of SREBP-1 in liver and SREBP-1c is mainly responsible for the biosynthesis of TGs. Studies have shown that transgenic mice overexpress SREBP-1c or SREBP-1a and produce massive fatty liver due to accumulation of TGs and cholesterylesters (Shimano et al., 1996; Shimomura et al., 1999). SREBP1 is involved in the activation of genes associated with fatty acid metabolism, and involved directly in cholesterol homeostasis (Horton et al., 1998; Pai et al., 1998). SREBP1 specifically activates several of the key genes involved in lipogenesis (Horton et al., 2002; Horton et al., 2003) like fatty acid synthase (FAS), and Acetyl-CoA carboxylase alpha (ACACA) (Ronnebaum et al., 2008). SREBPs are bound as precursors to the endoplasmic reticulum and nuclear envelope. When activated, they are released from the membrane and escorted to the Golgi complex by
SREBP cleavage-activating protein (SCAP) and then cleaved by specific proteases and translocated to the nucleus, where they bind to sterol regulatory element-1 (SRE1), which is a decameric sequence flanking the low density lipoprotein receptor gene and some genes involved in sterol biosynthesis (Sozio and Crabb, 2008). Sterols inhibit the cleavage of the precursor, and the mature nuclear form is rapidly catabolized, thereby reducing transcription. The genes which are activated by SREBP are in turn regulated by AMPK (AMP-Activated protein kinase) since AMPK directly phosphorylates and binds to SREBP-2 and SREBP-1c (Li et al., 2011) inhibiting the expression of FAS and ACC which are key lipogenic enzymes (Viollet et al., 2009).

SREBP-1 gene knockout mice show a very low basal expression of FAS hardly possess the ability to upregulate de novo lipogenesis (Juvet et al., 2003). SREBP1 gene expression was observed to be down-regulated in animals treated with single high dose of acetaminophen, carbon tetrachloride, tetracycline amiodarone (Fukushima et al., 2006). The present study however, involved daily dosing of acetaminophen for 15 days in rats. Thorough literature search indicated that the reports for effects of such treatment on SREBP expression are not yet available. In the present study, SREBP1 expression was reported to be higher in the animals which were repeatedly treated with high dose of acetaminophen for 15 days as compared to the healthy control.

Scanty references are available on the effect of herbal interventions on SREBP expression in animal models for hepatotoxicity. A study involving C57BL/6-Lep ob/ob mice reported the prevention of fatty liver by carbenoxolone intervention, an active component of Glycyrrhiza glabra. The hepatoprotective ability of the intervention was attributed to SREBP-1c inhibitory activity and anti-apoptotic action of the intervention (Rhee et al., 2012). The diosgenin fraction from fenugreek inhibits triglyceride accumulation in livers of diabetic obese KK-Ay mice as well as in HepG 2 cells with simultaneous decrease in the expressions of SREBP-1c, ACC and FAS (Uemura et al., 2011). In the present study, expression of SREBP-1 was significantly decreased in animals treated with the satwa of T. cordifolia, T. sinensis and Neem-giloe as compared to negative control.

Mammalian intracellular fatty acid-binding proteins (FABPs) comprise a superfamily of lipid-binding proteins which are involved in the fatty acid uptake,
intracellular transport and in regulating lipid metabolism, cellular signaling pathways and other lipid ligands (Wang et al., 2007; Storch and Thumser, 2010). FABP is highly expressed in adipocytes, liver, muscle, heart, brain and macrophages and the expression and activation of FABP1 has been reported to contribute to the pathogenesis of obesity, metabolic syndrome and associated inflammation (Makowski and Hotamisligil, 2004). Fatty acids transport, in and out of the cells, is a complicated process and is important for function and utilization of lipids (Kim et al., 2007). The effect of acetaminophen induced hepatotoxicity on FABP1 expression has been studied with relation to oxidative stress. A dose-dependent increase in oxidative stress induced by acetaminophen was associated with significantly low FABP1 expression (Gong et al., 2014). FABP1 also plays an early protective role in acetaminophen induced mitochondrial impairment through scavenging free radicals within the mitochondria itself as well as in the cytosol (Gong et al., 2014). The role of L-FABP in liver disease was further assessed by Wang et al. (2007). FABP1 has been reported to possess strong antioxidant properties (Yan et al., 2009). In accordance with this role of FABP, the present study observed decreased expression levels of FABP1 in acetaminophen treated group and significantly higher expression in animals treated with Neem-giloe satwa.

Expression of Genes Involved in Inflammation

NF-κβ (Nuclear factor-κβ) is one of the most important transcription factors and it is activated by inflammatory cytokines like TNF-α (Tumor necrosis factor-alpha) (Richard, 2001). Both are related to injury and inflammation, hepatitis, immunity, including various etiologies of muscle catabolism and osteoclastogenesis (Zwart et al., 2009). The NF-κβ pathway is complex and is activated by phosphorylation, ubiquitination, and proteolysis of the inhibitory protein IκB (I kappa B), which nominally binds NF-κβ in the cytosol in the inactive form (Zwart et al., 2009). Blazka et al. (1995) reported the up-regulation of TNF-α in liver of acetaminophen treated mice. Dambach et al. (2006) and Song et al. (2014) recently reported significantly up-regulated expression of TNF-α and NF-κβ in acetaminophen-induced hepatotoxicity in mice. Various inflammatory cytokines produced during drug induced liver injury have been reported to be involved in tissue damage (Ishida et al., 2002). Ishida et al. (2004) reported that, liver injury in mice
deficient in the 55KDa TNF receptor (TNF-Rp55), was attenuated after APAP challenge. In addition, APAP-induced mortality was reduced in TNF-Rp55 KO mice. Studies in mice deficient in CCR2, the primary receptor for the chemokine MCP-1 (Monocyte chemoattractant protein-1), showed that these mice had increased toxicity of TNF-α and IFN-α (Hogaboam et al., 2000). TNF-α is reported to promote tissue damage during acetaminophen toxicity (Boess et al., 1998). mRNA and protein expressions of TNF-α and NF-κβ were significantly upregulated in D-galactosamine–induced hepatotoxicity (Aristatile et al., 2013). Tu et al. (2012) observed significant increase in TNF-α in carbon tetrachloride intoxicated rats. The serum levels of pro-inflammatory cytokines, such as TNF-α and NF-κβ were significantly elevated in isoniazid induced hepatotoxicity in albino rats (Mahmoud et al., 2014). Roles of NF-κβ in suppression (Van Antwerp et al., 1996) as well as induction (Grilli et al., 1996) of apoptosis have been reported and NF-κβ is also thought to play a major role in liver regeneration (Ulloa et al., 2002; Tsung et al., 2005; Luedde and Schwabe, 2011). In the present study, NF-κβ and TNF-α gene expression were higher in acetaminophen induced hepatotoxicity as compared with healthy control group.

The diabetic rats, treated for 24 weeks with Tinospora cordifolia extract (250 mg/kg) exhibited significantly reduced amount of inflammatory markers such as TNF-α and IL-1β (Agrawal et al., 2012). Several chemical constituents like alkaloids, diterpenoid lactones, steroids, glycosides etc from different parts of T. cordifolia are known to inhibit the activity of NF-κβ and TNF-α (Mittal et al., 2014). G1-4A, an arabinogalatan, found in T. cordifolia, increases levels of TNF-α and IL-10 against endotoxic shock through modulation of cytokines and nitric oxide in mice (Desai et al., 2007). Treatment of albino rats with Berberine, an isoquinoline alkaloid, leads to decrease in the serum levels of TNF-α and NF-κβ in isoniazid and cyclophosphamide-induced hepatotoxicity (Germoush and Mahmoud, 2014; Mahmoud et al., 2014). Treatment of male Sprague-dawley rats with curcumin significantly reduced the levels of proinflammatory mediators (TNF- α, IL-6 and MCP-1) mRNA in carbon tetrachloride induced liver toxicity (Tu et al., 2012).

In present study, NF-κβ gene expression was found to be significantly decreased in the rats treated with the satwa of T. cordifolia, T. sinensis and Neem-giloe while there was statistically insignificant decrease in TNF-α gene expression in Neem-giloe satwa treated rats. Silymarin, a standard drug used in the present study,
has been reported to suppress NF-κβ gene expression in the hepatoma cell line HEPG2 (Saliou et al., 1998). Apart from the intervention groups in the present study, NF-κβ gene expression was also found to be significantly decreased in the rats treated with Silymarin (positive control).

Based on the results obtained from biochemical, histological and gene modulation studies in animals receiving interventions of satwa of *T. cordifolia*, *T. sinensis* and *Neem-giloe*, hepatoprotective activity of *Neem-giloe* was found to be better than that of *T. cordifolia* and *T. sinensis*, in acetaminophen induced hepatotoxicity.
Fig. 28. A Model for Probable Molecular Mechanism of Action of Satwa from Three different forms of *Tinospora*. A: Effect of *T. cordifolia*, B: Effect of *T. sinensis*, C: Effect of *Neem-giloe*.

APAP: Acetaminophen; PPARγ: Peroxisome proliferator-activated receptor γ; SREBP: Sterol regulatory element binding protein; NF-κβ: Nuclear factor kappa β; Acyl-CoA: Acetyl coenzyme A; FAS: Fatty acid synthase; TNF-α: Tumour necrosis factor α; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; CH: Total cholesterol.
6.4 Hepatoprotective Activity of Satwa against Ethanol Induced Hepatotoxicity

6.4.1 Biochemical Parameters

In the present study, administration of alcohol caused a significant elevation of SGOT, SGPT, ALP, and total bilirubin when compared to healthy control, which is indication of hepatic damage. Several reports indicate elevated levels of liver function markers like SGOT, SGPT, ALP and bilirubin in ethanol-induced hepatotoxicity in rats (Yue et al., 2006; Arulkumaran et al., 2009; Vidhya and Indira, 2009; Nigam and Paarakh, 2011; Singh and Gupta, 2011; Cui et al., 2013; Padmanabhan and Jangle 2014; Seif, 2014). In the present study, serum levels of SGOT, SGPT, ALP and bilirubin were significantly decreased after treatment with *T. sinensis* (200 mg/kg) as compared to ethanol treated group. The hepatoprotective activity of ethanolic extract of *T. sinensis* roots has previously been demonstrated in carbon tetrachloride induced hepatotoxicity in rats (Naik et al., 2013). Treatment of albino rats with acetone and aqueous extracts of *Adina cordifolia* showed significant decrease in the levels of serum markers (SGOT, SGPT, ALP and total bilirubin), indicating the protection of hepatic cells against ethanol induced hepatocellular injury (Sharma et al., 2012). Treatment with aqueous extract of *Andrographis paniculata* (50mg/kg, 100mg/kg, 200mg/kg of body weight) was found to protect the rats from hepatotoxic action of ethanol as evidenced by significant reduction in the elevated levels of SGOT, SGPT, ALP and bilirubin (Vetriselvan et al., 2011).

Alcohol has significant effect on lipid and lipoproteins metabolism. Alcohol induced liver injury is characterized by lipid accumulation in affected hepatocytes (Ontko, 1973). In the present study, animals treated with ethanol showed increase in the levels of serum and hepatic cholesterol, triglyceride, VLDL, LDL, along with decrease in HDL levels as compared to healthy control. Such abnormal level of lipids is an indication of disturbed lipid metabolism. Earlier studies also have reported increased levels of serum and tissue lipids (Triglyceride, total cholesterol, and phospholipids) in alcohol treated rats (Tomita et al., 2004; Kim et al., 2007; Arulkumaran et al., 2009; Cui et al., 2013; Samundeeswari et al., 2013). In the present study, *Neem-giloe* satwa exhibited normalization of serum lipid profile while *T. sinensis* satwa normalized the lipid profile in liver. Treatment of animals with *Neem-
giloe satwa also revealed significant increase in total protein content. An earlier study on polyherbal formulation, Vimliv, which contains *T. cordifolia*, showed significant improvement of lipid profile (Triglyceride, total cholesterol, phospholipid) in serum and liver tissue in ethanol-induced hepatic damage in female albino wistar rats (Samundeeswari et al., 2013). A recent report by Sharma and Dabur (2015) also indicated improvements in serum lipid profile after treatment of alcoholic volunteers with water extract of *T. cordifolia*. They also showed that the water extract helped in enhancing intestinal vitamin absorption and preventing multivitamin deficiency in liver due to alcohol intake.

Oxidative stress plays an important role in the development of ALD (Alcoholic liver disease) (Yurt and Celik, 2011). Recent literature of alcohol induced hepatotoxicity indicates decrease in the levels of SOD, GSH and catalase and increase in malondialdehyde, hydroperoxides in the liver homogenate of alcohol treated rats (Das and Vasudevan, 2006; Mallikarjuna et al., 2009; Shanmugam et al., 2010; Arun and Balasubramanian, 2011; Singh and Gupta, 2011; Rejitha et al., 2012; Seif, 2014). Administration of aqueous extract of stem and leaves (400 mg/kg body weight, orally) of *T. cordifolia* increased the activities of SOD and CAT and decreased the levels of SGOT, SGPT and ALP enzymes in lead nitrate induced hepatotoxicity in mice (Sharma and Pandey, 2010). In the present study, contents of hepatic SOD, GSH and catalase significantly decreased while hepatic MDA increased in the ethanol treated group. Treatment with *T. sinensis* satwa resulted in significant decrease in the levels of MDA and significant increase in the levels of SOD, CAT and GSH in the liver homogenate as compared with ethanol treated group.

### 6.4.2 Histological Analysis

The hepatic architecture in rats treated with alcohol frequently exhibits hepatocytic necrosis, inflammation in centrilobular region with portal triads (Arun and Balasubramanian, 2011), congestion, macrovesicular and microvesicular steatosis (Nigam and Paarakh, 2011), fatty changes in the hepatocytes with intense centrilobular necrosis and vacuolization (Singh and Gupta, 2011; Seif, 2014), tubular epithelial cell degeneration with mononuclear cell infiltration, oedema, necrosis (Samundeeswari et al., 2013), an enlargement of the hepatocytes, higher steatosis (fat accumulation) and inflammatory injury (Kupffer cell activation) (Cui et al., 2013). *T.
*cordifolia* has been reported to protect liver damage against carbon tetrachloride induced hepatotoxicity through preventing fibrosis, stimulating hepatic regeneration and through diminishing congestion, inflammation, vacuolation, fatty changes etc. (Bishayi et al., 2002; Sharma and Pandey, 2010). In the present study, sections of liver from ethanol treated group showed swollen hepatocytes with granular cytoplasm. Collections of few polymorphs were visible in hepatic parenchyma, suggesting foci of necrosis. Treatment with *T. sinensis* satwa demonstrated near normal liver histology as compared to ethanol treated group.

6.4.3 Gene Expression

Expression of Genes Involved in Lipid Metabolism

Excessive ethanol consumption leads to alcoholic liver disease (ALD) through multifactorial and complex mechanism. Several animal experiments have shown the effect of ethanol through regulation of hepatic expression of PPARγ and PPARγ agonists have been shown to prevent alcohol-induced liver injury (Enomoto et al., 2003; Ohata et al., 2004; Tomita et al., 2004). A recent study has shown that PPAR-α and PPARγ agonist treatments reduced severity of chronic alcohol induced liver injury including hepatic architectural disorder and steatosis (De la Monte et al., 2011). PPAR-α and PPARγ expressions at protein levels and mRNA concentrations were upregulated in the livers of Fischer rats with alcohol feeding (Luvizotto et al., 2010). Chronic alcohol administration significantly reduced the hepatic expression of PPARα, which is involved in lipid metabolism (Park et al., 2014). Alcohol intoxicated mice supplemented with *Aloe vera* polysaccharides exhibit marked increase in mRNA levels of PPAR-α which otherwise is down-regulated after alcohol treatment leading to liver damage (Cui et al., 2013). Treatment of albino rats with 8β-Glycyrrhetinic acid has been shown to exert hepatoprotective effects against cyclophosphamide-induced hepatotoxicity through up-regulation of PPARγ (Mahmoud and Al Dera, 2015). In present study also, PPARγ gene expression was seen to be significantly higher in *T. sinensis* treated group while the ethanol treated group showed lower PPARγ gene expression.

Accumulation of ethanol also affects expression of SREBP and SREBP target genes, thus further increasing lipid synthesis (Yin et al., 2007). You et al. (2002) have
reported that chronic ethanol feeding induces fatty acid synthesis pathway by activating SREBP-1, and this effect of ethanol may contribute to the development of alcoholic fatty liver. Acute ethanol (A single oral dose of 0.5 or 5g/kg of body weight) affects the expression levels of SREBP-1 and many other SREBP-1 target genes, thereby increasing fatty acid synthesis in male ICR mice (Yin et al., 2007) and male C57BL/6 mice (Ji and Kaplowitz, 2003). Cui et al. (2013) showed that alcohol consumption decreases AMPK-α2 expression and elevates SREBP-1c levels in mice. Huang et al. (2010) investigated the effects of Antrodia camphorata fruiting bodies against chronic alcohol consumption in rats and found that the expression level of SREBP-1c, Acetyl-CoA carboxylase, 3-hydroxy-3-methylglutaryl-CoA reductase, fatty acid synthase and malic enzyme was down-regulated. The studies on traditional Chinese medicines like Schisandra chinensis (Park et al., 2014) and Gentiana manshurica (Lu et al., 2012) have demonstrated prevention of alcohol induced liver damage through decreased expression of SREBP and decrease in SREBP-1 regulated fatty acid synthesis. The present study also reports higher expression of SREBP-1 in ethanol treated rats while its expression was significantly reduced in animals treated with Neem-giloe satwa.

FABP1 has been reported in many metabolic disease processes, such as cholestatic liver disease, cancer, diabetes, obesity, and atherosclerosis (Furuhashi and Hotamisligil, 2008). FABP1 prevents free fatty acid induced lipotoxicity and is known to be down regulated in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) in animal models as well as in NAFLD patients (Guzman et al., 2013). Administration of Radix Platycodi (PR), the roots of Platycodon grandiflorum (Traditional Oriental Medicine) significantly prevented alcohol-induced elevation of serum and liver lipids by normalizing the FABP expression in alcohol-treated rats (Kim et al., 2007). Nanji et al. (2004) also found reduced expression of FABP in alcohol-fed rats. Protein as well as mRNA expression of L-FABP showed significant decrease following ethanol consumption in mice (Smathers, 2011). In present study also, expression of FABP1 decreased in the ethanol treated group while significant increase in FABP1 expression was found in animals treated with T. sinensis and Neem-giloe satwa.
Acute ethanol administration causes prominent hepatic microvesicular steatosis with mild necrosis and increased levels of SGPT and TNF-α in mice (Song et al., 2006). Alcoholic hepatitis is characterized by hepatic inflammation with higher levels of TNF-α, IL-1, IL-8 in animal models as well as patients (McClain et al., 1999). Treatment of alcohol treated rats with *Sida cordifolia* is reported to decrease the hepatic expression of inflammatory markers like NF-κβ and TNF-α (Rejitha et al., 2012). Treatment of alcohol intoxicated mice with polysaccharides from *Aloe vera* is reported to decrease the expression of TNF-α (Cui et al., 2013). Roy et al. (1994) observed high TNF-α level in carbon tetrachloride treated rats while treatment of animals with Liv.52 (Polyherbal hepatoprotective formulation) decreased levels of the inflammatory marker. α-D glucan, a polysaccharide found in *T. cordifolia*, is reported to enhance generation of TNF-α and other cytokines from human peripheral blood mononuclear cells (Nair et al., 2004). In the present study also, expression of NF-κβ and TNF-α was increased in ethanol treated animals while significant decrease in the expression levels of the markers was found in the livers of the animals treated with *T. cordifolia satwa*, *T. sinensis satwa* and *Neem-giloe satwa*.

Based on the results obtained from biochemical, histological and gene modulation studies in animals receiving interventions of satwa of *T. cordifolia*, *T. sinensis* and *Neem-giloe*, hepatoprotective activity of *T. sinensis* was found to be better than that of *T. cordifolia* and *T. sinensis*, in ethanol induced hepatotoxicity.
Fig. 29. A Model for Probable Molecular Mechanism of Action of Satwa from Three different forms of *Tinospora*. A: Effect of *T. cordifolia*, B: Effect of *T. sinensis*, C: Effect of Neem-giloe

PPARγ: Peroxisome proliferator-activated receptor γ; SREBP: Sterol regulatory element binding protein; NF-κβ: Nuclear factor kappa β; Acyl-CoA: Acetyl coenzyme A; FAS: Fatty acid synthase; TNF-α: Tumour necrosis factor α; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; CH: Total cholesterol.
6.5 Hepatoprotective Activity of Flax Oil and Fish Oil against Acetaminophen Induced Hepatotoxicity

6.5.1 Biochemical Parameters

Omega-3 fatty acid supplementation is known to improve liver function markers in chronic diseases (Heller et al., 2004; Lee et al., 2007; Wu et al., 2012; Li et al., 2014). Omega-3 fatty acids are essential dietary nutrients for normal growth and development (Innis, 2004). Fish oil has been clinically and experimentally evaluated in earlier studies for its beneficial effects in cardiovascular diseases, cancer, rheumatoid arthritis, bone disease, psychiatric and immune disorders (Simopoulos, 1991; De Caterina et al., 1994; Liu et al., 2001). Khanchandani et al. (2014) revealed that the administration of omega-3 fatty acid (Fish oil) at a dose of 600 mg/kg b.w. to albino rabbits reduced the elevated serum liver enzymes like SGOT, SGPT, ALP, bilirubin when compared with CCl4 administered group which indicates heptoprotective nature of omega-3 fatty (Fish oil) acids. Treatment of albino rats with fish oil showed significant decrease in serum SGOT, SGPT and creatinine levels in ifosfamide induced toxicity (Asaad and Aziz, 2012). Fish oil at a dose of 300 mg/kg revealed significant lowering of enzymes like SGOT, SGPT but had a less marked effect on the levels of ALP and total bilirubin (Meganathan et al., 2011). Roy et al. (2007) reported that dietary supplementation of fish oil exhibited strong hepatoprotective activity against galactosamine induced liver damage in mice. In the present study also, treatment of rats with fish oil (500 mg/kg bw) showed significant reduction in the serum levels of SGOT, SGPT, ALP and total bilirubin against acetaminophen induced hepatotoxicity.

Fish oil predominantly contains EPA (Eicosapentaenoic acid) and DHA (Docosachexaenoic acid) (Egert et al., 2009; Skulas-Ray et al., 2011). Several studies have reported the triglyceride lowering effect of dietary fish oil (Rivellese et al., 1996; Montori et al., 2000; De Caterina et al., 2007; Devarshi et al., 2013; Lorente-Cebrian et al., 2013; Bremer et al., 2014). A clinical study has shown a significant increase in HDL and a decrease in cholesterol, triglyceride levels in hyperlipidemic individuals after receiving 1.3g fish oil in bread daily for 2 to 4 weeks (Liu et al., 2000). Flax oil and fish oil treatment reduced serum triglycerides and VLDL levels in STZ and STZ-NIC induced diabetic rats (Kaithwas and Majumdar, 2012; Devarshi et al., 2013).
Treatment with fish oil and flax oil showed reduction in the plasma levels of triglycerides and cholesterol in mice fed on high fat diet (Riediger et al., 2008). In the present study, flax oil treatment exhibited significant reduction in the serum levels of total cholesterol, LDL, triglycerides and VLDL as compared to acetaminophen treated group while fish oil treatment lead to significant increase in the HDL level. The hepatic lipid profile in the present study was also found to be normalized by flax and fish oil treatment.

Naqshbandi et al. (2011) reported antioxidant properties of fish oil through increase in the levels of SOD, CAT, GSH and decrease in the levels of MDA in liver homogenates of cisplatin-induced hepatotoxic rats. The antioxidant property of fish oil has also been demonstrated by decrease in the hepatic MDA level in acetaminophen induced hepatotoxicity by pre-treatment of rats with fish oil (Kalra et al., 2012). The animals fed on fish oil showed significant higher activities of catalase, glutathione peroxidase and superoxide dismutase in rat liver (Ruiz-Guiterrez et al., 1999). Besides improvement in antioxidant status, fish oil also decreases the hepatic hydroperoxide contents in atherosclerotic rabbits (Aguilera et al., 2003). Fish oil and flax oil are the richest sources of omega-3 fatty acids and the present study indicated that they are also potent antioxidants which significantly improve the antioxidant markers in acetaminophen treated rats (Chavan et al., 2013b). In the present study, fish oil exhibited higher antioxidant properties than flax oil, as indicated by significant increase in the levels of SOD, CAT and GSH and significant decrease in MDA levels in liver homogenates in acetaminophen induced hepatotoxicity.

6.5.2 Histological Analysis

Damage to the liver due to acetaminophen induced hepatotoxicity at histological level is discussed earlier in the thesis. In the present study, acetaminophen treated group showed swollen or occasionally apoptotic hepatocytes with coarse granular cytoplasm and compressed sinusoids. Treatment with both fish and flax oil exhibited strikingly normal liver histology without any anatomically detectable anomalies. The hepatoprotective activity of omega-3 fatty acids has been demonstrated by pre-treatment of albino mice with fish oil which reduces liver damage caused by galactosamine (Roy et al., 2007). Meganathan et al. (2011) observed healthy liver histology in rats treated with fish oil in acetaminophen induced
liver injury. Omega-3 fatty acid (Fish oil) treated groups exhibited protection of hepatic lobules with mild fatty changes and localized necrosis when compared with carbon tetrachloride treated group (Khanchandani et al., 2014). As described above, the present study also reports near normal liver histology in the animals treated with flax oil and fish oil.

6.5.3 Gene Expression

Expression of Genes Involved in Lipid Metabolism

Dietary omega-3 fatty acids play various physiological roles and serve as biological regulators. Polyunsaturated fatty acids are a fundamental part of cell membrane and they also act as signaling molecules triggering cascade of intra-cellular signaling (Hwang and Rhee, 1999; Merendino et al., 2013). In recent years, direct involvement of omega-3 fatty acids in regulation of gene expression has been established (Price et al., 2000; De Caterina and Massaro, 2005; Deckelbaum et al., 2006). Omega-3 fatty acids have been shown to decrease the transcriptional activation of many genes like adhesion molecules, chemoattractants, and inflammatory cytokines involved in endothelial activation in response to inflammatory and pro-atherogenic stimuli (De Caterina and Massaro, 2005). EPA and DHA, the predominant omega 3 fatty acids present in fish oil, have been reported to act as natural ligands for activation of PPARγ (Trombetta et al., 2007). Increase in the dietary polyunsaturated fatty acids upregulates PPARα and PPARγ expression in the spleen, liver and bursa of chickens (Selvaraj et al., 2010). In the present study, hepatic expression of PPARγ was significantly increased in animals treated with fish oil as compared to acetaminophen treated group which exhibited decreased levels of PPARγ expression.

Treatment of rats with flax oil and fish oil (Omega-3 fatty acid) showed down regulation of hepatic SREBP-1 expression along with decreased serum triglyceride levels (Davidson, 2006; Devarshi et al., 2013). There are numerous reports on omega-3 supplementation in animals indicating down regulation of SREBP-1c (Kim et al., 1999; Xu et al., 1999; Yahagi et al., 1999; Botolin et al., 2006; Kim et al., 2014). Fish oil supplementation up regulates FABP-1 mRNA, involved in DHA uptake and decreases the expression of SREBP-1 (Dutta-Roy, 2000; Gaca et al., 2012). In present study also, significant decrease in the expression of SREBP-1 was observed in rats.
treated with flax and fish oil and FABP1 was found to be up-regulated in flax oil as compared with acetaminophen treated animals.

**Expression of Genes Involved in Inflammation**

Omega-3 fatty acids such as EPA and DHA are bioactive dietary compounds. Omega-3 fatty acids intervention in rats prevented inflammation and severe hepatic steatosis when fed on methionine/choline deficient diets (Marsman et al., 2013). Omega-3 fatty acids decrease the production of inflammatory proteins which may be mediated by altered activation of key transcription factors regulating NF-κβ and TNF-α (Babcock et al., 2000; Novak et al., 2003; Kang and Weylandt, 2008; Scorletti and Byrne, 2013). TNF-α has been linked to hepatotoxicity, sepsis, and the inflammatory response (Wielockx et al., 2001; Lee, 2007). In a mouse model, TNF-α induction was observed in apoptosis and necrosis of hepatocytes leading to liver failure (Wielockx et al., 2001). In the present study, expression of TNF-α and NF-κβ was higher in acetaminophen treated group, indicating inflammatory response in the hepatocytes. The TNF-α expression was significantly reduced in animals treated with flax and fish oil while reduction in NF-κβ was observed in rats treated with flax oil.

Based on the results obtained from biochemical, histological and gene modulation studies in animals receiving interventions of polyunsaturated fatty acids (flax oil and fish oil), hepatoprotective activity of fish oil was found to be better than that of flax oil, in acetaminophen induced hepatotoxicity.
Fig. 30. A Model for Probable Molecular Mechanism of Action of Flax Oil and Fish Oil Interventions. A: Effect of Flax Oil, B: Effect of Fish Oil

APAP: Acetaminophen; PPARγ: Peroxisome proliferator-activated receptor γ; SREBP: Sterol regulatory element binding protein; NF-κβ: Nuclear factor kappa β; Acyl-CoA: Acetyl coenzyme A; FAS: Fatty acid synthase; TNF-α: Tumour necrosis factor α; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; CH: Total cholesterol.
6.6 Hepatoprotective Activity of Flax Oil and Fish Oil against Ethanol Induced Hepatotoxicity

6.6.1 Biochemical Parameters

In the present study, flax oil intervention in ethanol treated rats produced significant normalization of liver function. Phenolic components in flaxseed have been shown to be effective in restoration of increased activities of liver function enzymes (Kasote et al., 2012). Ismail et al. (2009) have reported normalization of serum SGPT and SGOT levels after flax oil treatment in carbon tetrachloride intoxicated rats. Shakir and Madhusudhan (2007) reported that rats treated with flaxseed chutney showed decrease in the levels of liver markers such as SGOT, SGPT, ALP and bilirubin in serum and liver homogenate against carbon tetrachloride induced hepatotoxicity. In present study also, treatment of rats with flax oil (500 mg/kg) significantly reduced levels of SGOT, SGPT, ALP and bilirubin as compared to ethanol treated group.

Raw flaxseeds as well as dietary flaxseeds as baked products, are known to have hypolipidemic, hypoglycemic and hypocholesterolemic effects (Cunnane et al., 1995; Shakir and Madhusudhan, 2007). Several animal and human intervention studies indicate that flaxseed oil has beneficial effects on serum lipid profile (Mahmud et al., 2004; Vijaimohan et al., 2006; Riediger et al., 2008; Bassett et al., 2009; Newairy and Abdou, 2009; Kaithwas and Mujumdar, 2012). Administration of 15% flaxseed chutney or flaxseed oil to rats showed improvements of the lipid profile (Increased level of HDL, decreased level of total cholesterol and LDL) in serum and liver homogenates (Shakir and Madhusudhan, 2007). Ismail et al. (2009) demonstrated that supplementation of flaxseed oil resulted in significant reduction in serum total cholesterol, LDL and VLDL in rats treated with carbon tetrachloride. High cholesterol diet-induced hypercholesterolemic rats exhibited lower serum total cholesterol, triacylglycerols, LDL, VLDL, phospholipids, and increase in HDL after flaxseed oil intervention (Hussein et al., 2014). In the present study also, flax oil showed normolipidemic effects with decreased levels of total cholesterol, LDL, triglycerides, VLDL and increased HDL in serum as well as liver homogenates.
Hepatoprotective and anti-oxidant activity of flaxseed has been demonstrated through restoration of antioxidant enzymes in the liver of flaxseed pre-treated animals challenged with carbon tetrachloride (Rajesha et al., 2006). Treatment of the hull fraction of flaxseed also resulted in a significant increase in hepatic anti-oxidant enzymes such as SOD, CAT, peroxidase as compared to carbon tetrachloride treated group which is attributed to secoisolariciresinol diglucoside (SDG) content of the flaxseed hull (Rajesha et al., 2010). Treatment of rats with flax lignans showed decrease in TBARS and increase in the activities of glutathione S-transferase, SOD, glutathione reductase and CAT as compared to lead acetate treated rats (Newairy and Abdou, 2009). Several studies reported reduced levels of lipid peroxides (MDA) in liver tissue in flaxseed oil treated animals (Lee and Prasad, 2003; Abdel-Moneim et al., 2011). The present study also reports significant increase in activity of antioxidant enzymes (SOD, CAT), increase in reduced glutathione and reduced levels of lipid peroxides in flax oil treated animals as compared to ethanol treated group.

6.6.2 Histological Analysis

Several studies on liver damage due to ethanol induced hepatotoxicity have already been discussed. In the present study, animals treated with flax oil showed near normal hepatic architecture. There are several reports which indicate almost normal liver architecture with minor histological anomalies after treatment with omega-3 fatty acids like flax oil or fish oil (Ismail et al., 2009; Meganathan et al., 2011; Kasote et al., 2012; Khanchandani et al., 2014).

6.6.3 Gene Expression

Expression of Genes Involved in Lipid Metabolism

In the present study, PPARγ level was significantly increased in fish oil treated animals and SREBP-1 was significantly decreased in flax oil and fish oil as compared to ethanol treated animals. Omega-3 fatty acids are known to have their effects on modulation of gene expression in cultured hepatocytes as well as in the animal livers. The mRNA and protein expression of SREBP-1c was found to be suppressed after treatment of the cells or animals with PUFAs (Sekiya et al., 2000; Hannah et al., 2001; Yoshikawa et al., 2002; Nakatani et al., 2003). Fish oil has been reported to increase the expression of PPARγ mRNA in mice liver (Yamazaki et al.,
2007). Fish oil also has effects on reduction of SREBP-1c and increase in the PPARα expression in ethanol induced fatty liver (Wada et al., 2008).

Nanji et al. (2004) have reported that the expression of FABP decreases with increase in the extent of fatty liver in ethanol intoxicated rats. They detected lowest expression of L-FABP in the animals which developed severe fatty liver. Intestinal expression of L-FABP was found to be upregulated in mice treated with sunflower oil which was shown to be due to linoleic acid in sunflower oil (Poirier et al., 1997). In the present study also, near normal liver function and histology was associated with flax oil treatment which showed increase in the level of FABP1 expression as compared to ethanol treated animals.

**Expression of Genes Involved in Inflammation**

Fish oil supplementation in healthy human volunteers lead to decreased production of inflammatory cytokines (like TNF-α, IL-1 and IL-6) by mononuclear cells (Caughey et al., 1996; Baumann et al., 1999; Trebble et al., 2003). Baumann et al. (1999) have exclusively demonstrated that only omega-3 and not omega-6 and omega-9 fatty acids, can significantly alter the expression levels of the genes involved in inflammation and atherosclerosis. Treatment of mice with omega-3 fatty acids reduced serum alanine aminotransferase (SGPT) levels and decreased inflammatory response with decreased plasma TNF-α levels and this treatment also led to reduced hepatic expression of TNF-α, IL-1β, IFN-γ and IL-6 as compared to lipopolysaccharide/D-galactosamine-induced hepatitis model (Schmocker et al., 2007). In the present study, expression of NF-κB and TNF-α was significantly decreased in flax oil treated animals as compared to alcohol treated animals.

Based on the results obtained from biochemical, histological and gene modulation studies in animals receiving interventions of polyunsaturated fatty acids (flax oil and fish oil), hepatoprotective activity of flax oil was found to be better than that of fish oil, in ethanol induced hepatotoxicity.
Fig. 31. A Model for Probable Molecular Mechanism of Action of Flax Oil and Fish Oil Interventions. A: Effect of Flax Oil, B: Effect of Fish Oil

PPARγ: Peroxisome proliferator-activated receptor γ; SREBP: Sterol regulatory element binding protein; NF-κβ: Nuclear factor kappa β; Acyl-CoA: Acetyl coenzyme A; FAS: Fatty acid synthase; TNF-α: Tumour necrosis factor α; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; CH: Total cholesterol.
6.7 Protective and Corrective Effects of Combination of Neem-giloe Satwa and Fish Oil against Acetaminophen Induced Hepatotoxicity

6.7.1 Biochemical Parameters

Hepatoprotective effects of Neem-giloe satwa and fish oil were separately evaluated against acetaminophen induced hepatotoxicity. The studies revealed that the comparative hepatoprotective activity of Neem-giloe satwa and fish oil was higher than other two satwa (T. cordifolia and T. sinensis) and flax oil, respectively. The present study was carried out to evaluate protective and corrective effects of combination of Neem-giloe satwa and fish oil against acetaminophen induced hepatotoxicity.

Herbal medicines and nutraceutical supplements have been shown to have preventive role in several diseases. Several medicinal herbs with hepatoprotective properties have been reported in literature, e.g. Allium sativum (Garlic) (Ajayi et al., 2009), Silybum marianum (Milk Thistle) (Freitag et al., 2015), Glycyrrhiza glabra (Licorice Root) (Sharma and Agrawal, 2014), Curcuma longa (Turmeric root) (Bae et al., 2006) and Neem-guduchi (Chavan et al., 2013a; Nagarkar et al., 2013), Tinospora cordifolia (Bishayi et al., 2002). In Asian countries, several studies revealed that many dietary food items and supplements possess hepatoprotective activity (Shukla and Kumar, 2013). There are a number of different food items and supplements e.g. hepatoprotective spices like Turmeric (Luper, 1999), Coriander (Samojlik et al., 2010), Garlic (Ajai et al., 2009), Red chili (Kim et al., 2005); hepatoprotective fruits like Grapes (Carbo et al., 1999), Custard apple (Chavan et al., 2011), Apple (Miura et al., 2007), Pomegranate (Toklu et al., 2007); hepatoprotective vegetables and grains like Carrot (Bishayee et al., 1995), Sweet corn (Guo et al., 2009), Soy (Hu et al., 2004), hepatoprotective drinks like Green tea (Luper, 1999), Coffee (Wang et al., 2009); hepatoprotective omega-3 fatty acids like flax oil (Shakir and Madhusudhan, 2007; Ismail et al., 2009; Kasote et al., 2012) and fish oil (Meganathan et al., 2011; Asaad and Aziz, 2012; Khanchandani et al., 2014). Food ingredients contain several phytochemicals beneficial in liver injuries and possess potential to prevent or reverse different kinds of liver injuries. There are several studies reported on either herbal or nutritional supplements (Omega-3 fatty acid) against different hepatotoxicants.
A recent report shows that treatment of rats with a combination of Glycyrrhizin and omega-3 fatty acid (fish oil) leads to significant decrease in SGOT and SGPT activities as compared to thioacetamide treated rats (Abo El-Magd et al., 2015). In the current study, protective and corrective treatment of rats with a combination of *Neem-giloë* satwa and fish oil showed significant decrease in the levels of SGOT, SGPT, ALP and bilirubin as compared to acetaminophen treated groups. In a single-blind, placebo-controlled, crossover study of combinatorial treatment of fish oil and garlic showed decrease in cholesterol, triglyceride, and LDL levels, as well as increase in HDL as compared to placebo treated group (Morcos, 1997). In present study also, protective and corrective treatment of combination of *Neem-giloë* satwa and fish oil showed improvement in the lipid profile (decrease in cholesterol, LDL, VLDL and triglyceride and increase in HDL) in serum and liver homogenates. Total protein of liver homogenate was significantly improved in corrective effect of the combination as compared to acetaminophen treated group.

A combination of Glycyrrhizin and fish oil to thioacetamide intoxicated rats exhibited strong antioxidant activity with significant increase in hepatic MDA levels (Abo El-Magd et al., 2015). In the present study, combination of *Neem-giloë* satwa and fish oil was also found to have antioxidant effects and it decreased the extent of lipid peroxidation and improved the levels of SOD, reduced glutathione and CAT as compared to acetaminophen treated group.

### 6.7.2 Histological Analysis

In the present study, sections of liver from the animals treated with a single dose of acetaminophen on 8th day showed swelling and degenerative changes in hepatocytes with compressed sinusoids. The protective treatment of combination of *Neem-giloë* satwa and fish oil showed improvement in hepatic architecture which still showed some hepatocytes with degenerative changes. The corrective treatment of combination of *Neem-giloë* satwa and fish oil showed near normal liver histology as compared to that in the animals treated with acetaminophen which exhibited congestion of central vein and swollen hepatocytes with ballooning degeneration.
6.7.3 Gene Expression

Expression of Genes Involved in Lipid Metabolism

Thorough literature search indicated lack of references on expression studies of genes from lipid metabolism, in acetaminophen induced hepatotoxicity and combination of herbal and nutraceutical interventions.

In the present study, the animals with the intervention of combination of satwa and fish oil, exhibited significant up-regulation of PPARγ and FABP1 while expression of SREBP-1 was down-regulated.

Expression of Genes Involved in Inflammation

A recent report by Abo El-Magd et al. (2015) has reported increase in the expression of NF-κβ in hepatic tissues of thioacetamide treated rats which was reduced after treatment with a combination of glycyrrhizin and fish oil (Abo El-Magd et al., 2015). In the present study also, protective and corrective treatment of combination of interventions showed significant down-regulation of NF-κβ and TNF-α.
Fig. 32. A Model for Probable Molecular Mechanism of Action of Neem-giloe and Fish oil. A: Protective Effect of Combination of Neem-giloe Satwa, B: Corrective Effect of Combination of Neem-giloe Satwa

APAP: Acetaminophen; PPARγ: Peroxisome proliferator-activated receptor γ; SREBP: Sterol regulatory element binding protein; NF-κB: Nuclear factor kappa β; Acyl-CoA: Acetyl coenzyme A; FAS: Fatty acid synthase; TNFα: Tumour necrosis factor α; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; CH: Total cholesterol.
6.8 Prophylactic Effect of Combination of *Tinospora sinensis* Satwa and Flax Oil against Ethanol Induced Hepatotoxicity

6.8.1 Biochemical Parameters

Hepatoprotective effects of *T. sinensis* satwa and flax oil were separately evaluated against ethanol induced hepatotoxicity. The studies revealed that the comparative hepatoprotective activity of *T. sinensis* satwa and flax oil was higher than other two satwa (*T. cordifolia* and *Neem-giloe*) and fish oil, respectively. The present study was carried out to study the prophylactic effects of combination of *T. sinensis* satwa and flax oil against ethanol induced hepatotoxicity. In the present study, combination of *T. sinensis* satwa and flax oil showed significant reduction of SGOT, SGPT, ALP and bilirubin as compared to ethanol treated group. The treatment also normalized serum and hepatic lipid profile and improved hepatic antioxidant status. A report by Wahba and Ibrahim (2013) showed improvement in the serum levels of liver function markers, lipid profile as well as increase in tissue antioxidant enzymes after treatment with flax oil in combination with vitamin E in potassium bromate treated rats.

6.8.2 Histological Analysis

Effects of ethanol treatment and interventions on the hepatic architecture are described in the results section. There are no previous reports describing hepatic architecture in ethanol intoxicated rats treated with combination of herbal and nutraceutical interventions.

6.8.3 Gene Expression

Expression of Genes Involved in Lipid Metabolism

PPARγ level was significantly up regulated and SREBP-1 level was found normalized in treatment with combination of *T. sinensis* satwa and flax oil. In prophylactic effect, FABP1 was down regulated in ethanol treated group while it was up regulated in treatment with combination of *T. sinensis* satwa and flax oil.

There are no previous reports on the gene expression studies of combination of interventions in ethanol induced hepatotoxicity. In the present studies, PPARγ was
significantly up-regulated while NF-κβ was shown to be significantly down-regulated in intervention group.

**Expression of Genes Involved in Inflammation**

In the present study, NF-κβ and TNF-α expressions were up regulated in ethanol treated animals. NF-κβ gene expression was found significantly decreased in the animals treated with a combination of *T. sinensis* satwa and flax oil. TNF-α expression was significantly decreased in healthy control and no significant change was observed due to treatment of combination of *T. sinensis* satwa and flax oil.

Treatment of animals with combinations of *Neem-giloe* and fish oil and *T. sinensis* and flax oil in acetaminophen and ethanol induced hepatotoxicity respectively, showed comparatively better results than intervention of any one of them. So combination of herbal and nutritional intervention has shown beneficial effects on liver disease.
**Fig. 33.** A Model for Probable Mechanism of Action of *T. sinensis* Satwa and Flax oil

PPARγ: Peroxisome proliferator-activated receptor γ; SREBP: Sterol regulatory element binding protein; NF-κβ: Nuclear factor kappa β; Acyl-CoA: Acetyl coenzyme A; FAS: Fatty acid synthase; TNF-α: Tumour necrosis factor α; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; CH: Total cholesterol.