4.1. CHANGES IN THE WEIGHT OF BODY, LIVER AND KIDNEY

Fig. 5a-c represents body, liver and kidney weight changes in both control and experimental animals. The body weight of the rats which declined due to the aflatoxin, recovered after treatment with each one of the drugs [E. caryophyllata (T4), its alcoholic extract (T6) and eugenol (T8)]. The maximum recovery was found in sylimarin treated group while the minimum recovery was found in clove. The alcoholic extract, eugenol were equally more efficient than the entire clove. The control animals did not show any significant variation in the body and organ weights when compare to the drug treated animals [E. caryophyllata (T3), alcoholic extract (T5), eugenol (T7) and sylimarin (T9)]. But among the animals treated only with the drug, alcoholic extract (T5) induces maximum increase in the body weight and eugenol the minimum weight (T7).

Tersitore et al., (1987) have shown that tumour growth elicited marked loss of body weight in growing asitic hepatoma bearing rats. This may be due to decreased food intake and/or absorption, which contribute to muscle waste in tumour. Tumour may act as nitrogen trap and the cells are more efficient in utilizing aminoacids for gluconeogenesis than growth (Shelman et al., 1950).

Moreover the host responds to increased tumour load by decreased muscle protein synthesis and muscle break down. Amino acids resulting from breakdown are subsequently used by liver, further increasing the host metabolic burden (Waterhouse et al., 1979). This could probably be the reason for the loss of weight due to aflatoxicosis.

In aflatoxicosis condition, the weight of liver and kidney (not significant) decrease largely due to protein degradation during tumour growth. Protein metabolic perturbations in host, although causing tissue waste may themselves favour the growth of tumour itself (Tersitore et al., 1987).

The recovery in the body weight of clove, eugenol and alcoholic extract treated rats may be due to antiaflatoxicosis potency of E. caryophyllata. All the drugs causes disappearance of troublesome symptoms by subjective improvement. Absence of significant variation
among the various experimental animals reveals the non-toxic nature of
these drugs. A similar observation has been noted in the body and organ
weight gain at the optimum dose of *Tridax procumbens* (Susila
Appadurai, 2001) against aflatoxicosis.

4.2. HEMATOLOGICAL PARAMETERS

Table 7 shows the levels of haemoglobin, RBC, WBC, PT and PCV
in the control and experimental groups of rats. Increased RBC and WBC
counts and increased levels of haemoglobin and PCV, in the *E.
caryophyllata*, its extract and eugenol treated groups, indicated the
protective effects of eugenol, clove and its alcoholic extract on the
haemopoietic system. Alcoholic extract induces the maximum recovery in
many of the haematological parameters (Haemoglobin, RBC and WBC).
But Eugenol brought about the maximum recovery in PCV and sylimarin
in PT. The proven hepatoprotective agent (sylimarin) was next in its
recovery efficiency in all the haematological parameters studied.

The blood cells are the mobile units of the body’s protective system
(Guyton and Hall, 1991). Decreased white blood count (p<0.001) in the
aflatoxin fed animals indicate decreased resistance of the body to toxicity
induced by AFB$_1$. Decreased RBC count (p<0.001), haemoglobin
(p<0.001) and PCV (p<0.001) also indicate the severity of hepatic damage
induced by AFB$_1$. Decrease in the haemoglobin levels might be due to
increased catabolism and degradation of haemoglobin to bilirubin.
Reduction in Hb content can be related to decrease in RBC number
which in turn indicates anaemic induction (Sarathchandra *et al.*, 1996).
Induction of anaemic status in toxicity and cancer are well established. A
mild degree of anaemia is one of the complications observed in HCC
patients (Murray-Lyon, 1983). Increased RBC and WBC contents and
increase levels of haemoglobin and PCV in the drug treated groups along
with the toxin indicate the protective effect of the plant and its extract on
the haemopoietic system. The morphology of the blood cells also supports
the above observations (*Plate 2 & 3*).
The PT was significantly (p<0.001) prolonged in the aflatoxin fed animals as compared to control animals. This reflects the defects in the intrinsic and extrinsic pathways of coagulation system due to AFB<sub>1</sub> administration. PT measures the rate at which prothrombin is converted to thrombin in the presence of thromboplastin, calcium, fibrinogen and other coagulation factors. The prolongation of PT in the aflatoxic rats might be due to the fact that the liver may be damaged and that it cannot adequately synthesize the clotting factors (Johnson, 1995). Previous reports have showed prolonged partial thromboplastin time in liver diseases according to the degree of hepatic failure (Denninger, 1999).

The PT was significantly (p<0.001) decreased in the *E. caryophyllata*, its alcoholic extract and eugenol treated group of rats as compared to aflatoxin fed group of rats. This indicates the ability of clove and its extract to correct the defects in the intrinsic and extrinsic pathways of the coagulation system.

### 4.3. BLOOD GLUCOSE, HEPATIC GLYCOGEN, LACTATE AND PYRUVATE

**Fig. 6a-d** shows the levels of blood glucose hepatic glycogen, lactate and pyruvate in the liver of the control and experimental groups of rats. Hypoglycemia during liver injury is a consequence of impaired gluconeogenesis and inability to mobilize glycogen stores (Ellis and Wedon, 1996). This correlates well with the decreased levels of liver glycogen and blood glucose observed in the aflatoxin fed rats as compared to control rats in this study. The level was restored to normal in experimental groups which were fed with clove along with aflatoxin treatment. No significant alteration of glucose content was observed in their respective control groups.

Of the various drugs used against aflatoxicosis, treatment with sylimarin proves to be the most effective in blood glucose and pyruvate and eugenol in liver glycogen and lactate. Next to sylimarin and eugenol, alcoholic fraction was effective in alleviation. In all the carbohydrate
fractions studied (except blood glucose) treatment with clove seems to be least effective.

Cancer cells have increased rates of glucose catabolism, compared to healthy cells and the malignant tumours in experimental group acts as a glucose trap (Shat pot, 1979). Decreased blood glucose level in tumour condition suggested that the glucose availability is not sufficient to provide both the host and the tumour. Glucose is diverted away from host tissues not only for direct utilization by the tumour but also as an important precursor for the synthesis of substances that may be required for tumour growth. Thus high metabolic demand for glucose is experienced by tumour bearing animals (Mulligan and Tisaldale, 1991).

The blood glucose content might have been restored to near normal level in drug treated animals, due to perhaps the antitumour potency of *E. caryophyllata*. Because of the decreased tumour burden in this group of animals, the excessive utilization of glucose would have retarded.

In the transition to hepatic malignancy, there occurred abruptly complete change in the type of glycogen metabolism. In liver, the glycogen content was significantly reduced (P<0.001) in the aflatoxin fed group. In the drug treated groups, the level was restored to near normal levels.

One feature common to HCC (Hepato Cellular Carcinoma) induced by aflatoxin is the depletion of liver glycogen. The glycogen reduction indicates transformation of the preneoplastic to neoplastic cells. This may be due to excess demand of glucose by the tumour and hence the glycogen reserves in the liver is exhausted or becomes non existent (Mc Fadzean and Young, 1969). The decrease in activities of glycogen synthetase and glycogen phosphorylase have also been observed in rats affected with hepatoma (Hammond and Balinsky, 1978). Thus in neoplastic liver, the pathway leading to glycogen synthesis is diverted to glycolysis (Mc Fadzean and Young, 1969) since both the pathways utilize glucose as the substrate.

The glycogen content was recovered to near normal level in the experimental groups. (AFB₁ and the drugs) in comparison to the aflatoxin alone fed groups. Administration of drugs might have stimulate liver cells
to convert more glucose to glycogen and thus glycogen content restored to normal.

Based upon the above experimental results, it may be suggested that, *E. caryophyllata* and its alcoholic extract have definitely a protective role on carbohydrate metabolism in aflatoxicosis. This action is probably due to the synergistic effect of the various compound in the clove and the alcoholic extract.

It is well known that clinical and experimental liver diseases are accompanied by the alteration of carbohydrate and amino acid metabolisms for energy production and that hepatic gluconeogenesis from amino acid is markedly depressed (Felig *et al.*, 1970 and Monier and Wagle, 1971). Impaired gluconeogenesis has been reported to result in the accumulation of lactate and pyruvate in the liver (Record and Albenti, 1972). This could probably account for the increased levels of lactate and pyruvate in the liver of aflatoxin fed animals as compared to normal rats.

Friedrich *et al.*, (1969) and Sato *et al.*, (1978) have demonstrated the induced glycogenesis, and reduced liver specific marker enzymes of gluconeogenesis (Glucose-6-phosphatase, fructose-1-6, bisphosphatase) and glycogen phosphorylase in DENA treatment. These enzymes gradually were replaced with the non-hepatic fetal enzymes on DENA treatment.

The two stages of hepatocarcinogenesis can be distinguished by demonstration of accumulation of glycogen. In the first stage (i.e. after 3 months), glycogen storage increases, which probably characterizes a proneoplastic cell population which is associated with the increased activity of glycogen synthase. The second stage is marked by a gradual reduction of the glycogen of the proneoplastic cells into neoplastic cells (Bannasch *et al.*, 1980). The increased accumulation of glycogen may be related to decreased glycogen phosphorylase activity. The eugenol co-treatment with the toxin according to Sato *et al.*, (1978), probably reduced the glycogen deposition and induced the decreased glycogen phosphorylase activity (a result of DENA treatment), resulting in the decreased accumulation of glycogen.
The glycogen content decreased with the progression of hepatocarcinogenesis. The glycolytic and gluconeogenic enzymes increased remarkably just before birth and the reverse occurs as the primary tumours progress and is specific to neoplastic transformation (Middleton and Walker 1972).

The enhanced activity of G6PD observed reference the proliferate activity of tumour cells. A signification part of the increased glucose utilization denotes the increased activity of G6PD of the pentose phosphate shunt for increased DNA synthesis (Ikezaki et al., 1992).

Increase in hexokinase activity was also observed in tumour cells. Because hexokinase is the first enzyme in the glycolytic pathway, its activity reflects the maximal rate of glucose utilization (Ikezaki et al., 1992). This was supported in a study with PB+DENA treatment. Eugenol co-treatment showed a change in enzyme activity, suggesting that eugenol can inhibit the proliferate activity of tumour cells and that it could induce a reversal of glycolysis by scavenging the free radicals.

A significant increase in the liver lactate and pyruvate levels during aflatoxicosis has been found in our study. Treatment with Eugenol, clove and its alcoholic extract to aflatoxicosis induction showed a tendency for the restoration of altered liver lactate and pyruvate levels towards near normality thereby giving an induction of the protective effects of the plant and its alcoholic extract against aflatoxicosis in rats.

4.4. PROTEIN BOUND CARBOHYDRATE COMPONENTS

The constant attention to the study of glycoprotein components is explained by the fact that they play a very important role in different functions of cells, especially in transporting different substances through biomolecules inside the cells and in the regulation of cellular adhesion, recognition of signals from outside, intercellular interactions etc., (Umiyal et al., 1998).

The level of glycoprotein components viz., hexose, hexosamine and sialic acid in liver are depicted in Fig. 7a-c. All the three glycoprotein components were significantly increased (hexose P<0.001, hexosamine
P<0.001, Sialic acid P<0.05) in AFB₁ induced toxic conditions. Sialic acid, a sensitive and specific marker of malignancy was dramatically raised than the other two glycoprotein components. The glycoprotein contents were almost normalized in Eugenol, clove and its alcoholic extract treated groups of animals. Their respective control group of animals did not show any significant variations in these parameters except sialic acid. Either eugenol or Sylimarin is highly active in the alleviation process.

Abnormal increase in the level of glycoprotein components has been related to the changes in hepatic cells during neoplastic transformation. Huang et al., 2001 postulated that, the presence of tumour in hepatocytes induces the synthesis of glycoproteins, which subsequently appear in circulation. Large amounts of hexose, hexosamine and sialic acid were reported in hepatoma conditions (Schimizu and Funaskoshi, 1970).

Sialic acid is the acylated derivative of neuraminic acid and exists as terminal component of the non-reducing end of carbohydrate chains of glycoprotein. Elevated level of sialic acid can be useful for early detection of cancer, stating the prognosis of disease, degree of metastasis and recurrence (Shanmugam and Natarajan, 1985). Increased activity of sialyl transferase leads to increased expression of sialic acid in hepatoma condition (Caughman and Breen, 1995) which may be one of the necessities of neoplastic cells helping malignancy. The influence of sialic acid on the oncogenecity of tumour cells has been studied by many investigators as the main determinant of cell surface negative charge, electrophoretic mobility and the loss of contact inhibition. It also acts as an antigen marking agents and as a component of cell surface involved in the adherence of tumour cells to mesothelial membrane to form metastasis (Prasad, 1986).

The reversal of the content of glycoprotein components to near normal level in eugenol, clove and its alcoholic extract treated groups of animals may be due to its potent antitumour activity. Eugenol, clove and its extract may alter all membrane glycoprotein synthesis and structure.
4.5. LIVER PROTEIN AND SERUM PROTEIN

**Fig. 8a** shows the levels of liver protein, serum protein (**Fig. 8b**), albumin, globulin and A/G ratio in the control and experimental groups of rats. The aflatoxin fed group showed decreased levels of liver protein, serum protein, albumin, A/G ratio and increase in the globulin levels as compared to the control rats. This finding is in accordance with the earlier findings (Pier, 1973; Huff *et al*., 1983; Jassar *et al*., 1993; Devegowda *et al*., 1994; Sodhi *et al*., 1996 and Kececi, 1998). Huff *et al*. (1983) stated that the most sensitive indicators of aflatoxicosis is reduction in serum protein and albumin. This is due to the binding of aflatoxin to DNA and thereby impairing messenger RNA synthesis and selective inhibition of the enzyme activity of RNA polymerase, resulting in blockage of protein synthesis (Leeson *et al*., 1995).

Albumin is the most abundant circulatory protein and its synthesis is a typical function of normal liver cells. The serum albumin level has been used as a test of liver function because it is affected by hepatic protein synthesis (Mc Intyre and Rosalki, 1992). The significant decrease in albumin levels in hepatitis – induced rats could be attributed to suppressed protein synthesis in liver and subsequent impaired hepatic function following D-galactosamine/ lipopolysaccharide administration (Tiegs *et al*., 1989). Several studies report hypoalbuminemia during hepatic dysfunction which may also be due to increased catabolism (Roturmund *et al*., 1970; Landel *et al*., 1985) and also in other gastrointestinal malignancies (Landel *et al*., 1985).

The liver is an important site of protein synthesis and it has the highest rate of synthesis of tissue. Major protein mass of the organism is severely affected by cancer. Protein waste implies underlying metabolic imbalance which is being expressed by an elevation in the apparent protein degradation rate with no changes in the apparent synthetic rate (Tersitore *et al*., 1987). Reduced liver protein in Morris hepatoma bearing rats and walker 256 carcinoma in other reports (Landel *et al*., 1985) have also suggested the increased protein degradation. Recycling of amino acid has been decreased in tumour condition resulting in enhanced efflux of
these amino acids from the tissues. Thus the host responds to increased tumour load by increasing tissue protein breakdown (Tersitore et al., 1987).

The elevation of globulin content in aflatoxicosis induced animals appears to be compensatory, as the ratio of albumin to globulin showed a significant fall in this group of animals. A decrease in liver protein, serum total protein and albumin and an increase in globulin fraction was also observed during hepatic dysfunction in previous studies (Premalatha and Sachidanandam, 1998). Similar conditions were also observed in birds fed with aflatoxin (Shukla and Pachauri, 1995; Susila Appadurai, 2001).

In rats administered with eugenol, clove and its alcoholic extract and sylimarin along with the aflatoxin, the levels of protein, albumin, globulin and A/G ratio were resumed to near normal levels in most of the conditions. Among these, eugenol shows the maximum effect on the toxin next to the proven hepatoprotective agent, sylimarin. This observation infers a protective effect by Eugenol and the alcoholic fraction on impaired hepatic function caused by aflatoxin.

4.6. LIVER MARKER ENZYMES

Of all the macromolecules that leak from the damaged tissues, enzymes, because of their tissue specificity and catalytic activity are the best markers of tissue damage (Hearse, 1979). Determination of the activity of hepatic enzymes released into the blood by the damaged liver is one of the most useful tools in the study of hepatotoxicity (Plaa and Hewitt, 1989). The specific and non specific biochemical parameters which were known to be altered by hepatotoxins were measured as markers for evaluating the hepatoprotective activity of many drugs (Zimmermann, 1978).

The clinical and diagnostic values associated with changes in activities of blood enzymes such as lactate dehydrogenase (LDH), aspartate transaminase (SGOT) and alanine transaminase (SGPT) has long been recognized. Since liver is the organ primarily affected by toxic agents, the study of serum enzyme activity has been found to be of great
importance in the assessment of liver damage (Plaa and Hearitt, 1982). Hence in the present study, an attempt was made to study the activities of certain marker enzymes of hepatic function like SGOT, LDH, γ-glutamyl transaminase (γ-GT) and alkalaine phophotase (ALP) in the serum.

**Fig. 9a-e** depicts the activities of marker enzymes *viz.*, SGOT, SGPT, LDH, ALP and γ-GT in serum of control and experimental animals. All the enzymes were significantly increased (P<0.001) in aflatoxin treated rats as compared to the control rats. Recoupment of these enzyme levels to near normal level was observed in all the treatment groups. In all the marker enzymes studied Sylimarin followed by alcoholic extract brings about the best recoupment, the least being in eugenol or clove. Thus, a synergistic effect of compounds than a single pure compound seems to be more favorable for the liver marker enzymes.

In toxic liver injuries, variable changes in the activities of these enzymes can be found in the serum based on the kind of toxin and extent of cellular damage (Plaa and Zimmermann, 1997; Devi and Devaki, 1998). Increased activities of the enzymes in plasma may be due to leakage of enzymes from the neoplastic cells into blood or may be due to the release of enzymes from normal tissue invaded by tumour or may be due to the possible effect of tumour on remote tissue leading to the loss of its enzyme and release into the blood (Schwartz *et al.*, 1962).

Hepatotoxic compounds are known to cause marked elevation in the activities of transaminase, LDH and ALP in the serum (Wang and Teigs *et al.*, 1986; Teigs *et al.*, 1989; Basnet *et al.*, 1996 and Hase *et al.*, 1997). In agreement with the results obtained in the previous investigations the present study elicited a significant increase in the activities of these enzymes in the serum of the rats exposed to aflatoxin, indicating the security of hepato cellular injury. The increased activity of these enzymes reflect a alteration in the plasma membrane integrity and/or permeability as a response to the hepatotoxin (Sakaguchi *et al.*, 1981).

The alcoholic extract of *E. caryophyllata* used in the present study seems to offer more significant protection and maintenance to the
structural integrity of the hepatocellular membrane. This is evident from the fact that the treatment of the rats with clove extract significantly prevented the toxicity of aflatoxin on the liver as indicated by the decreased activities of the marker enzymes of hepatic function studies. Treatment with alcoholic extract attenuated the aflatoxin induced increase activities of these enzymes. A subsequent recovery towards normal level in the activities of these enzymes strongly suggest the possibility of clove and its alcoholic extract as a conditioner of the hepatocytes. Accelerating the regeneration of parenchymal cells, this may be thus achieved probably through protecting the membrane fragility and decreasing the leakage of marker enzymes into the circulation. In the clove, alcoholic extract and eugenol alone treated rats, there was no significant change in the activities of these enzymes as compared to the levels reached in the control rats (Fig. 9a-e). This proves the absence of any toxic effect of clove on the mammalian system.

The aflatoxicosis, induced altered enzyme activities in rats were brought back to near normal level in all the treated groups. Prasad, (1987) has noted that an ayurvedic drug which contains extracts of the Semicarpus anacardium nuts has the ability to decrease aminotransferase levels in tumour bearing mice. This action might be attributed to the presence of flavanoids that decreased the ALT activity in bromobenzene intoxicated animals (Sanz et al., 1994). Recoupment of ALP γ-GT. AST, ALT and LDH suggest that clove and its alcoholic extract stimulates a protective mechanism against abnormal liver functions. This function could probably be due to the synergistic effect of the antihepatotoxic chemicals (ascorbic acid, casuarictin, gallic acid, hyperoside, oleanolic acid, pedunculagin, quercetin, stigmosterol, tellimagrandin-I, etc.) and hepatoprotective chemicals (β-sitosterol, ellagic acid, Eugenol, kaempferol, oleanolic acid, quercetin, rhamnetin, etc.) reported in clove (Duke, 1999).

Eugenol was found to cause a decrease in serum LDH and ALP levels (Sen et al., 1992; Parasakthy et al., 1993). They also reported that
eugenol decreased the serum marker enzymes, AST, ALT, LDH in CCL4 intoxicated rats.

Such an increase in LDH activity and its isoenzyme could be due to specific involvement of M4 organs (i.e) muscle, liver and lung. Damage to these tissues leads to an increase in serum LDH5 isoenzyme. These results were also consistent with results obtained by Roman, (1969). This increase could also be indicative of an anaerobic trend in metabolism which is a defensive adaptation to hypoxic conditions (Chvapil and Peng1975) in cancer cells.

The reduction of this isoenzyme in eugenol treatment shows that eugenol plays a role in reducing DENA+PB induced liver damage.

AST, ALT & ALP serve as reliable markers of liver function and are frequently elevated in the serum as an indication of hepatocellular disruption (Marshall and Bangert, 1995). AST is elevated more often to a greater extend that ALT in HCC, AST is more related to tumour growth than ALT, which reflects parenchymal damage (Moss, 1994). Antioxidants succeed in restricting the effective of transaminase from liver and blood (Rana and Kumar, 1988). Eugenol co-administration restored these enzyme activities at normal levels. These observations could confirm the improved liver function in eugenol treated rats. Changes in ALP activity may be a consequence of changes in the permeability of plasma membrane in addition to changes in the balance between synthesis and degradation of enzymes ALP is transported into the serum in the form of complexes with lipid and undergoes further complexing in serum, contributing to the rise in total activity. (Hardonk and Koudstall, 1976; Moss, 1994). Eugenol maintained these levels and reduced the tumour burden in the liver.

In the present study also presence of flavanoids and phenolic compounds in the alcoholic extract of E. caryophyllata may be responsible for the antiaflatoxicosis activity. Plant flavanoids were shown to possess anticarcinogenic activity against certain experimental tumours (Glusker and Rolisi, 1986). Flavanoids exert cytoprotective effects through the inhibition of several enzymes (Kandaswami et al., 1991). Flavanoids
also express protection against genetic damage by AFB$_1$ and the formation of precancerous lesion (Francis et al., 1989).

4.7. MEMBRANE BOUND ENZYMES (ATPases)

Total ATPase (Na$^+$ K$^+$ ATPase, Mg$^{2+}$ ATPase and Ca$^{2+}$ ATPase) are intimately associated with the plasma membrane and participates in the energy requiring translocation of sodium, potassium, calcium and magnesium (Muriel, 1995). ATPase also maintains cellular electrolyte concentration and transmembrane electrolyte concentration and transmembrane electrochemical gradients.

Fig. 10a-d shows the activities of the membrane bound phosphates such as Ca$^{2+}$ ATPase, Na$^+$ K$^+$ ATPase, Mg$^{2+}$ ATPase and total ATPase in liver of the control and experimental group of rats. Results indicate that the activities of all the membrane bound phosphatases studied were decreased in the liver of the aflatoxin fed group. AFB$_1$ induced hepatotoxicity is characterized by severe derangement of subcellular metabolism and structural alterations of cell membrane (Premalatha and Sachidanandam, 1998). Decreased activities of ATPases in the aflatoxin fed group indicates alterations in the energy metabolism and disturbances of liver function.

Among the ATPases the plasma membrane Na$^+$/K$^+$ ATPase is concerned with the maintenance of low intracellular concentrations of Na$^+$ and subsequently cellular water content (Chandramohan et al., 1996). The findings in aflatoxin fed animals similar to those, reported by Berstein et al., (1981) who stated that the decrease in Na$^+$/K$^+$ ATPase activity occurs during tumour growth with a close correlation with the degree of malignance of the tumour. Generally in hepatoma, membrane forming tertiary capillaries are reduced and dilated or constructed with no microvilli. Such a typical capillaries contain no ATPase. Inhibition of Na$^+$/K$^+$ ATPase has fascinating implication for cellular metabolism, since 20-50% of the total cellular ATPase is used by plasma membrane Na$^+$/K$^+$ ATPase. Na$^+$/K$^+$ ATPase is also found to be inhibited by high cholesterol which affects flexibility of membrane. The increased serum cholesterol in
cancerous conditions leads to increased membrane cholesterol in liver cells which could cause a systemic decrease in plasma membrane Na\(^+\) K\(^+\) ATPase activity (Yeagle, 1983).

Ca\(^{2+}\) ATPase regulates the calcium pump activity and intracellular calcium function as a messenger in the regulation and control of cellular processes that play a central role in mediating muscle contraction, neurosecretion and other Ca\(^{2+}\) mediated cell functions (Alkon, 1988). Decreased Ca\(^{2+}\) ATPase activity has been reported during oxidant stress due to hydroperoxides and drugs in hepatocyte (Orrenius et al., 1984; Muriel, 1995). AFB\(_1\) could maximally increase hepatic Ca\(^{2+}\) influx in whole homogenate (Tosulkao and Glinsukon, 1992). Since, plasma membrane Ca\(^{2+}\) ATPase extrude Ca\(^{2+}\) from the cytoplasm of all the cells (Horward et al., 1994). Blocking of intracellular Ca\(^{2+}\) ATPase in cancer conditions resulting in fast Ca\(^{2+}\) accumulation (Rizzato et al., 1994). This could be the reason for membrane damage in AFB\(_1\) associated toxicity, because excessive Ca\(^{2+}\) influx has been suggested as a final phenomenon leading to the cellular death (Tosulkao and Glinsukon, 1992).

The inhibition of activity of Mg\(^{2+}\) ATPase was observed in aflatoxicosis conditions. Foster et al., (1974) have also reported the decreased activity of enzyme in Zajdela ascetic hepatoma. The activity of Mg\(^{2+}\) ATPase in normal liver cells is 140% higher than those in hepatoma cells (Ohinishi et al., 1982).

The decreased activity of ATPase in AFB\(_1\) induced aflatoxic animals may be due to increased lipid peroxidation (LPO) which occurs in toxic conditions. AFB\(_1\) is a membrane active compound capable of inducing chromosomal damage through the release of reactive oxygen species (ROS) (Amstad et al., 1984). Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity and carcinogenicity of aflatoxins (Shen et al., 1994). Peroxidation of membrane lipids initiated the loss of membrane integrity and membrane bound enzyme activities which in turn brought about a disturbance of cellular homeostatis (Tosulkao and Glinsukon, 1992).
ATPases are very sensitive to peroxidation reaction and abnormal lipoperoxides affects ATPase activities. Na⁺/K⁺ ATPase and Mg²⁺ ATPase activities were decreased due to the excessive production of TBARS (Rauchve et al., 1995). The decrement in Ca²⁺ ATPase activity is due to increased LPO or marked MDA formation which occurs in cancer condition. The inhibition may also be due to thiol oxidation. This was accompanied by parallel decrease in cytoplasmic GSH level and increased generation of lipid peroxidation by producing MDA (Habbel et al., 1986).

The restoration activities of all the three deranged ATPase to near normal level in the treatment groups (except AFB₁ treated) can be attributed to the potent membrane stabilizing effect of E. caryophyllata and its alcoholic fractions. Parasakthy et al., (1996) have recorded the alleviation of CCl₄ induced erythrocyte membrane damage by eugenol in rats. Similar result was also observed by Premalatha et al., (1997) in AFB₁ induced hepatocellular carcinoma with Semicarpus anacardium nut extract.

The anesthetic property of clove and eugenol has been well documented by Kreydiyyeh et al., (2000) attribute this property to the inhibitory effect of eugenol on the Na⁺/K⁺ ATPase.

4.8. BILIRUBIN

Bilirubin is an endogenous organic anion that binds reversibly to albumin and is transported to the liver, where it is conjugated to glucuronic acid and excreted in bile (Friedman et al., 1996). Determination of serum bilirubin serves as an index for the assessment of hepatic function and any abnormal increase in the levels of bilirubin in the serum indicate hepatobiliary disease and serve disturbance of hepatocellular function (Martin and Friedman, 1998).

Bilirubin normally present in the serum represents a balance between input from production and hepatic removal of the pigment Hyperbilirubinemia therefore may result from (i) over production of bilirubin (ii) impaired uptake conjugation or excretion of bilirubin from
the serum. (iii) regulation of unconjugated or conjugated bilirubin from damaged hepatocytes or bile ducts.

**Fig. 11a-c** shows the levels of total conjugated and unconjugated bilirubin in the serum of the control and experimental group of rats. Aflatoxicosis is characterized by increased levels of bilirubin in the serum (Premalatha and Sacchithanandan, 1998). This correlates well with the increased levels of serum bilirubin (total, conjugated and unconjugated) in the aflatoxin fed group as compared to the control group in the present study. This is also showing the severity of hepatic dysfunction caused by the hepatotoxin. The increased levels of unconjugated and conjugated bilirubin in the present study could result from an impairment of uptake or conjugation, coupled with decreased excretion of the pigment (McIntyre and Rosalki 1992; Pratt and Kapalan, 1999). In hepatic tumours, haemolysis plus deranged liver function leads to hyperbilirubinaemia (Issalbecher, 1991).

Stabilization in the levels of serum bilirubin in the *E. caryophyllata*, its extract and eugenol treated groups as compared to aflatoxin alone fed group clearly indicate the improvement in the functional status of the liver. Ravikumar et al, (2005) have noted that the alcoholic extract of *T. procumbens* could bring down the Bilirubin level in D-galactosamine / Lipopolysaccharide induced hepatitis in rats.

### 4.9. UREA AND CREATININE

**Fig. 12a&amp;b** depicts the levels of blood urea and serum creatinine in control and experiment animals. Blood urea was significantly (P<0.001) decreased whereas serum creatinine was slightly increased in aflatoxin fed group of animals. These levels were reversed to near normal in clove, its alcoholic extract and eugenol. Their respective control animals [T3, T5, T7] did not show any significant alterations in these parameters.

The mode of disposal of nitrogen is by the formation of urea, creatinine and creatinine. The amino nitrogen resulting from the utilization of amino acids for energy requirements is excreted largely as urea (Schmike, 1962). Urea production is intimately related to the
metabolic pathway for disposal of ammonia, the toxic end product of nitrogen metabolism. The lowered urea level in aflatoxicosis conditions may be due to reduced hepatic urea synthesis which leads to reduction in blood urea (nitrogen) and this is an index of hepatocellular function defect. Diminished urea synthesis with a resultant decrease in the removal of ammonia, enhances the metabolic disturbances in cancer condition (Mc Intyre and Rosalki, 1992).

The slightly increased creatinine level during aflatoxicosis conditions may be due to impaired renal function which occurs as a secondary event to reduce hepatocellular function (Mc Intyre and Rosalki, 1992).

The reversal of these altered urea (maximum by clove) and creatinine (maximum by alcoholic extract) levels to near normal state in the treated animals could be attributed to strong inhibitory effect of *E. caryophyllata* on aflatoxicosis conditions. Eugenol is also efficient in the alleviation processes both in urea and creatinine.

4.10. NUCLEIC ACIDS

**Fig. 13 a,b** shows the levels of nucleic acids in liver of control and experimental animals. Increased level of DNA (P<0.001) with subsequent increase in RNA (P<0.05) were observed in aflatoxin fed animals. These levels were decreased to near normality in the group of rats fed with aflatoxin along with clove, its alcoholic extract and eugenol. No significant alterations of nucleic acids were observed in their respective control groups.

Neoplasms are associated with abnormalities in their DNA content which increased with the degree of malignancy. The determination of DNA content was more meaningful with regard to biologic and functional aspects of the tumour because it is indicative of proliferative activity in tumour conditions. DNA content is found to be an independent indicator of prognosis, since the size of tumour often correlates well with DNA content in tumour (Ellis *et al.*, 1991). The increased DNA content in the liver of aflatoxin fed rats may be due to increased expression of enzymes
necessary for DNA synthesis in tumour cells with regression of many enzymes related to differentiated cell functions. Reports have also suggested an abnormal amount of DNA in many cancers, including breast carcinoma, lung carcinoma and endometrial adeno carcinoma. The RNA level in liver of aflatoxin fed animals was also increased as the DNA content (Fig. 13a). The increased content may lead to an increased transcription which might have resulted in the elevated RNA content.

Low levels of antioxidant enzymes will cause the accumulation of free radicals, which result in damage to DNA, lipids and proteins and perhaps, finally lead to cancer. DNA is a potential biological target for many initiators during carcinogenesis. Oxygen radical affects lead to DNA damage. They also directly affect the protein components of the DNA repair apparatus itself. DNA alternations are inherited as mutations (Burden, 1993). The DNA damage caused by free radicals is seen in the form of base damage. One major product of base damage in DNA is thymine glycol. Any chemical carcinogenesis that can generate free radicals, in principle, induces the formation of thymine glycols (Floyd, 1982).

DNA synthesis is minimum in hepatocytes in the absence of DNA-damaging agents; these cells have a very slow rate of cell division (Bridges, 1990). The development of HCC is associated with increased DNA synthesis (Pierre et al., 1996). The period of maximum sensitivity of DNA to chemical damage is approximately 18-36 hr after stimulation of cell proliferation, which corresponds to the period of maximum rate of DNA synthesis, namely the S phase (Kaufmann et al., 1991; Rabes et al., 1972) have shown as increased incorporation of [³H] thymidine into the altered foci of carcinogen treated rats.

The increased incorporation of [³H] thymidine into DNA in the present study suggests increased replication and the subsequent uncontrolled proliferation of malignant cells. The incorporation of [³H] thymidine into DNA was reduced by eugenol co-treatment, probably by inhibiting the DNA synthesis and due to its cytotoxic action on cancer
cells. Methotrexate, a well known anticancer drug was also found to inhibit DNA synthesis as seen by decreased thymidine incorporation into DNA (Rang, et al., 1995).

In the groups fed with clove, its alcoholic extract and eugenol no significant alterations of nucleic acids revealed that they have selective action attacking only the toxin induced cells without harming the normal cells. Phillips, (1990) also supports this view stating that Eugenol is devoid of biologically significant DNA binding activity in vivo.

4.11. ANTIOXIDANT ENZYMES

There is a strong association between oxygen free radicals (OFR) and cancer development (Fischer et al., 1988). Strong evidence indicates that oxygen free radicals play an important role in the initiation as well as the promotion phase of carcinogenesis (Slaga et al., 1981).

Even though oxygen is necessary for aerobic life it can also participate in potentially toxic reactions involving OFR that damage membranes (proteins and nucleic acids). OFR reactions and oxidative damage are in most cases held in check by antioxidant defense mechanisms. However, where an excessive amount of OFRs are produced, defense mechanisms are impaired. Oxidative damage may occur and this appears to be important in contributing to several pathological conditions (Floyd, 1990).

Prime targets of free radical attack are the unsaturated bonds in membrane lipids, consequence peroxidation results in a loss in membrane fluidity and receptor alignment and potentially in cellular lysis. Free radical damage to sulfur containing enzymes and other protein culminates inactivation, cross linking and denaturation of these enzymes. Oxidative damage to carbohydrates alters many of the cellular receptor function (Sies, 1985: Taylor et al., 1986). OFR damage DNA causing mutation that are carcinogenic, free radicals such as sulphur oxide anion, peroxy radicals and the hydroxy radicals cause DNA strand breaks, which is the most frequently observed single lesion (Brawn and Fridovich, 1981; Floyd and Schneiden, 1990). Scholty et al., (1990)
reported increased levels of ROS in neoplastic liver nodules in rats initiated with DENA.

**Table 8** shows the activities of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and glucose 6-phosphate dehydrogenase in the control and experimental groups of rats. Results indicate that in the rats fed with aflatoxin the activities of all the antioxidant enzymes studied were significantly lower (p<0.001) as compared to the control group. The recoupment of these enzymes to near normal level were found in the aflatoxin fed rats treated with each one of the drugs chosen, when compared to the rats fed with aflatoxin alone. No significant alterations of the enzyme levels were observed in the groups treated with drug alone when compared to control animals.

The clove seems to be least rejuvenating drug while sylimarin and alcoholic extract are very active in the rejuvenation process.

The decreased activities of the enzyme of glutathione antioxidant defence system (glutathione peroxidase, glutathione-S-transferase, glutathione reductase), catalase and glucose-6-phosphate dehydrogenase indicate defective antioxidant defenses and decreased free radical scavenging action in the aflatoxin fed rats.

Catalase catalyses the disproportionation of H$_2$O$_2$ forming neutral products O$_2$ and H$_2$O. Although both catalase and GPX combine to metabolize H$_2$O$_2$, catalase contributes more to H$_2$O$_2$ metabolism at higher concentration (Freeman and Crapo, 1982). The decreased activity of the enzyme in aflatoxin fed group may be due to the induction of H$_2$O$_2$ by AFB$_1$. Liver catalase activity is depressed in all tumour bearing animals in correlation with the degree of malignancy of the tumour, suggesting the impairment of free radical scavenger system in hepato carcinoma (Bellisola *et al.*, 1987).

GPX (Glutathione Peroxidase), another enzyme thought to be first line of defence against oxidative damage reduces the level of H$_2$O$_2$ and lipid hydroperoxides. The decreased activity in cancer condition may be due to excessive production of lipid hydroperoxides. GPX level was also
relatively low in hepatoma 27 (Perkin et al., 1997). Reduction in SOD and GPX in hepatocarcinoma conditions would be expected to have direct consequences, but reduction in GPX is found to be more deleterious than SOD. This greater relative importance of GPX over SOD can be attributed to the ability of GPX to detoxify H$_2$O$_2$ formed by SOD (Simmons and Jamall, 1988).

May be due to the antiperoxidative property of the clove, the enzyme activities were recouped back to normal in clove and alcoholic extract treated animals (T4 & T6) and thus they act as a possible element to protect cells from free radical damage. The flavanoids (especially quercetin) present in the plant (Duke, 1999) and extract have the protective effect against lipoperoxidative damage which depend on its hydrogen donating capacity of hydroxyl group in each molecule (Saija et al., 1995).

Eugenol co-treatment inhibited lipid peroxidation at the level of initiation, promotion or both by the removal of DNA metabolites by acting as an anti oxidant. The inhibition by the effect of eugenol on lipid peroxidation by scavenging radical intermediates when incubated with rat liver mitochondria was reported by Nagababu and Lakshmaih, (1992). Eugenol did not undergo any percitable chemical change during the course of peroxidation (Nagababu & Lakshmaih, 1992).

Glutathione-S-transferase is believed to play a key role in the protection of tissues from toxic reactions and it is known to possess GPX activity towards lipid peroxides. GST catalyses the peroxidation reaction using GSH but unlike GPX, H$_2$O$_2$ cannot be used as a substrate, i.e., it catalyses only lipid peroxides (Nakawoza et al., 1996). GST has a high conjugation activity toward 4-hydroxynoneal, which is one of the most potent aldehyde products of LPO (Jenson et al., 1986). Due to the excessive production of lipid peroxides GST activity is found to be reduced in aflatoxicosis conditions. Cancers originating from liver and kidney, both of which are organs normally processing high GST activity show less activity than in respective control tissues (Tisicid and Sato,
GST expression was found to be reduced particularly in hepato
cellular carcinoma (Malders et al., 1995).

GSH conjugates formed via GSH-transferase activity are also
excreted from the cell, resulting in intra cellular GSH depletion and
increased cellular susceptibility to continued oxidative stress. The intra
 cellular concentrations of GSH play a vital role in protecting the cell from
oxidative stress (Rosser and Gores, 1995).

GSH redox cycle is balanced by the formation of NADPH by G-6-P-
D and its utilization by Glutathione Reductase (GR). GR has a key role in
the regulation of GSH. The activity of GR is decreased in aflatoxin fed
group of rats. Due to increased oxidative stress in this group of animals,
the GR is inactivated. AFB_1 epoxide conjugation with GSH results with
accumulation of oxidized glutathione (GSSG). The toxic manifestation of
cellular oxidative stress is increased when the concentration of
intracellular GSSG also increases (Freeman and Crapo, 1982). Continuous endogenous production of ROS including H_2O_2 and lipid
peroxides also create a constant production of GSSG (Reed and Fariss,
1984). The activation of GR prevents the reduction of GSSG. This leads to
the depletion of GSH level, which enhances the susceptibility of the host
to toxicity. Thus inactivation of GR during oxidative stress is the key
aspect of GSH redox cycle (Reed, 1986).

The resumption in GR activity in drug treated (clove, its alcoholic
extract) animals may be due to reduced oxidative stress experienced by
these animals. These drugs may prevent hydroperoxide formation which
lead to the increase in activity of GR.

G-6-P-D, a key enzyme of the hexose monophosphate (HMP) shunt
pathway was found to be increased in aflatoxin fed rats. G6PD plays an
important role in the carcinogenic process and will be a good marker for
the early diagnosis of tumours. The production of free radicals and
superoxide radicals during cancer condition leads to secondary formation
of H_2O_2 that is capable of stimulating HMP pathway (Varners et al., 1984).
Increased level of G6PD have been observed in the liver of azodye induced
hepatocarcinoma and in hepatoma bearing rats (Poivier and Pitot, 1970).
HMP shunt pathway is increased in hepatic cancer, since this pathway provides precursors for the synthesis of nucleic acids and phospholipids. The enhanced rate of synthesis of these compounds may be essential to keep with rapid cell division and membrane biosynthesis during tumor growth (Arora and Pedron, 1988).

The decreased G6PD activity in drugs (clove its alcoholic extract & eugenol) treated animals may be due to the potent anticancer (anti-aflatoxicosis) activity of clove. The drugs probably by inhibiting G6PD activity prevents uncontrolled synthesis of nucleic acids (Table 8).

SOD may play a central role in protecting cancer cells against ROS (Yamaguchi et al., 1994). SOD activity and superoxide generation may be different from normal in vitro tumour cells. The liver, richest in SOD (Perkin et al., 1977) followed by the kidney in normal animals, were found to have decreased activity in hepatocarcinoma bearing animals. The increased superoxide radical levels in tumour cells (Oberley and Buettner, 1979) may explain the decreased activity of the enzyme in malignant tissues. The above authors have also reported that in hepatoma 27, Zajdela hepatoma and Lewis lung carcinoma, total specific SOD activity was found to be 3-5 fold lower than normal homologous tissue. Also Erlich ascites tumour cells had about one half of the total SOD activity of normal liver cells (Sahu et al., 1977).

Due to the antioxidant potency of the clove and its alcoholic extract, the activity of SOD was found to be normalized in these groups.

By an unknown mechanism, cells with lower SOD, CAT and GPX, higher GST and G6 PD or changed GR were selected over the normal cells and finally developed into tumours with abnormalities in anti oxidant enzymes (Sun, 1990). Eugenol co treatment altered the anti oxidant system. Sun, (1990) reported that eugenol inhibited superoxide formation and lipid peroxidation, and had radical scavenging activity. Eugenol was also reported to act as an in vivo antioxidant (Nagababu and Lakshmaiah, 1992 and Parasakthhy et al., 1995). These properties of eugenol may be responsible for its chemo preventive action.
4.12. NON-ENZYMATIC ANTIOXIDANTS

**Fig. 14a-e** depicts the levels of non-enzymatic antioxidants α-tocopherol, ascorbic acid, vitamin A, uric acid, total reduced glutathione and ceruloplasmin in the control and experimental group of rats. The results shows that the levels of all the non-enzymatic antioxidants studies were significantly reduced (P<0.001) in the aflatoxin fed rats. The antioxidant levels were brought back to near normal in various drug treated animals (P<0.001) when compared with aflatoxin fed group. No significant variation of all these parameters were found in the remaining groups.

Among the non enzymatic antioxidants total reduced glutathione (GSH) plays a critical role in important cellular functions, which includes maintenance of thiol status of proteins, the destruction of H₂O₂, lipid peroxides and free radicals, translocation of amino acids across cell membranes, the detoxification of foreign compounds and the biotransformation of drugs (James and Hrabison, 1982). Apart from its direct free radical scavenging properties and ability to conjugate with several electrophilic intermediates that are capable of initiating lipid-peroxidation, GSH is the physiological co-substrate of the conjugating enzyme system. Further more glutathione dependent cystolic and microsomal factors were reported which protect against lipid peroxidation (Nicotera and Orrenius, 1986). The decreased level of GSH in aflatoxin fed group animals may be due to its utilization by the amount of free radicals. Increased lipid peroxides also correlates with depletion of GSH and this effect is expected at a step, prior of poly unsaturated fatty acids (PUFA) (Burk, 1983). (Table 8)

Many authors have reported the lower GSH content in Morris and Yoshide hepatoma (Fiala et al., 1973) and the synthesis of GSH was 14 times greater in normal liver than Novikoff hepatoma (Wirth and Theorgeirsson, 1978). GSH depletion may sensitize tumour cells to the cytostatic effect without promoting toxicity towards normal host cells (Romine and Kessal, 1986). The administration of AFB₁ also depletes GSH and this depletion which not only compromise cellular defence
against attack by reactive molecules but it also imparts profound effects on normal hepatocellular function.

The GSH levels were increased to near normal in all the treatment (except AFB$_1$ alone) groups, which may be due to the antioxidant activity of clove. Duke 1999 listed the following chemicals as antioxidant in clove:- Acetyl Eugenol, ascorbic acid, β – carotene, β – sitosterol, campesterol, ellagic acid, ellagittannin, eugenol, gallic acid, hyperoside, isoeugenol, isoquercitin, kaempferol, methyl eugenol, myricetin, quercetin, stigmasterol, vanillin, etc.,

Vitamin E (α-tocophenol) is one of the most significant antioxidants in animal cells and is thought to act as a chain breaking antioxidant, by donating its labile hydrogen atom from phenolic hydroxyl groups to propagating lipid peroxyl and alkoxy-radical intermediates of lipid peroxidation thus terminating the chain reaction (Wiseman, 1996). Vitamin E has been found to have potent antioxidant activity due to its ability to penetrate to a precise site into the membrane which may be the important feature of protection against highly reactive radicals (Premalatha and Sachidanandam, 1999). Significantly decreased Vitamin E levels in aflatoxin fed group might be due to the excessive utilization of this antioxidants for quenching enormous free radicals produced in these conditions. The level of vitamin E has increased in clove, its alcoholic extract and eugenol treated rats which by its antioxidative nature results in the rejuvenation.

Vitamin C (ascorbic acid) is an excellent hydrophilic antioxidant (Frei et al., 1986). The availability of vitamin C is a determined factor in controlling and potentiating many aspects of host resistance. It can protect cell membranes and lipoprotein particles from oxidative damage by regenerating the antioxidant from vitamin E (Buettner, 1993). Thus vitamin C and Vitamin E act synergistically in scavenging a wide variety of reactive oxygen species (ROS) (Beyer, 1994). The decreased level of vitamin C was found in aflatoxin fed group animals. The ascorbic molecule must be involved in the feed back inhibition of lysosoma L
glycosidases responsible for malignant invasiveness (Cameron et al., 1979).

The recoupment of vitamin C to near normal level in drugs treated rats was recorded. Clove by inducing the regulation of ascorbic acid has acted as a potent antiaflatoxic drug. Flavanoids also have the ability to regenerate the reducing agent, ascorbate (Acker et al., 1996) and Tridax contains various flavanoids (WOI, 1976).

Uric acid the end product of purine metabolism, has been proven to be a selective antioxidant, capable especially of reacting with free radicals and hypochlorous acid (Hasugawa and Kuroda, 1989). Urate protects ascorbate against oxidation by cupric ions and also against iron induced oxidation. Urate possesses preventive antioxidant activity in addition to its chain-breaking action (Wayner et al., 1987). Decreased levels of uric acid in the aflatoxin fed rats might be due to increased production of free radicals and subsequent lipid peroxidation. The marked increase in uric acid levels in the clove, its alcoholic extract and eugenol administered groups as compared to aflatoxin fed group would have resulted in the free radical scavenging activity of the plant and its extract on lipid peroxidation chain reaction. Nagababu and Lakshmaiah, (1992) reported the antioxidant efficiency of eugenol. It may inhibit lipid peroxidation at the level of initiation, propagation or both. Formation of phenoxy radical and reconversion to eugenol appears to be the major reactions involved in quenching the radical intermediates.

4.13. LIPIDS

Mammalian liver normally contains about five percent lipid. This is found in the hepatic cells and in the kuppffer cells in the form of small droplets. Under the influence of various pathological and physiological disturbances, the lipid content may rise to 25 to 30 percent. Since the liver is active in synthesis, breakdown and modification of the plasma lipids and since there is a large flow of blood through the liver, the lipid content of this organ can rather quickly increase or decrease (West et al., 1992).
Fig. 15a-d shows the levels of total lipids in liver and the cholesterol, triglycerides and phospholipids in the liver and serum of control and experimental groups of rats. The results show that the levels of all the parameters mentioned above, manifested a significant increase (P<0.001) in the aflatoxin fed rats except the levels of phospholipids which showed a significant decrease (P<0.001) as compared to control rats. All these manifestations were reversed back to near control values in groups treated with E. caryophyllata, alcoholic extract, eugenol and sylimarin along with aflatoxin. Although the lipid levels were slightly altered in the respective control groups when compared to the normal, the changes were significant.

A number of agents that produce liver injury also cause the accumulation of abnormal amounts of fat, predominantly triglycerides in the hepatic parenchymal cells. In general, accumulation of triglycerides can be thought of as resulting from an imbalance between the rate of synthesis and the rate of release of triglycerides by the parenchymal cells into the systematic circulation (Plaa and Hewith, 1989). Non esterified fatty acids (NEFAs) removed from the circulation or synthesized endogenously are processed through two main pathways in liver, (a) mitochondrial β–oxidation for production of metabolic energy and (b) incorporation into complex lipids, especially triglycerides, phospholipids, cholesterol esters and glycolipids (Dianzani, 1978). Once synthesized, the complex lipids may be used for production of cellular membranes (structural lipids) or be continuously secreted from the liver into the blood. The latter pathway appear to be of greatest interest in the triglyceride accumulation observed in the fatty liver caused by the administration of the hepatotoxin (Koff et al., 1971).

The liver plays a major role in cholesterol metabolism in mammals. During tumour growth, the animals progressively developed marked changes in the liver and distribution of total cholesterol (Dessi et al., 1982). High rates of cholesterol synthesis was observed in aflatoxin fed rats. Similar elevation in cholesterol levels was reported in hepatoma cells (Diatlovitskia and Bergelson, 1982) and in N-nitrosodiethylamine
induced hepato carcinoma (Tang et al., 1992). Deregulated cholestrogenesis observed in tumours, implicated an over production, which could result in the enrichment of tumour cell membranes with cholesterol. This may be capable of inducing cell population into enormously enhanced proliferative activity (Coleman, 1986). Cholesterol metabolism in the body is regulated by continuous exchange of cholesterol between tissues and blood. The increased total cholesterol level in serum of aflatoxin fed rats may be due to decreased uptake of cholesterol from blood. Blood supply to hepatomas is decreased and hence 80% decrease in uptake of blood born substances occurred in hepatoma conditions (Ericksun et al., 1978). Similar mechanism is possible in aflatoxicosis also.

The decreased cholesterol content to near normal in the treated groups (E. caryophyllata, its extract and eugenol) can be due to strong hypocholesteromic activity of E. caryophyllata. This may inhibit cholesterol synthesis and accumulation. Ethanol extract of Semicarpus anacardium nut helped in the mobilization of lipids especially cholesterol from liver and prevents its deposition (Sharma et al., 1995). Lowering the elevated level of cholesterol not only retard progression but can even cause faster regression of the toxicity of aflatoxin (Ravikumar et al., 2005).

Triglycerides are probably metabolized by lipoprotein lipase (LPL) and the reaction products, free fatty acid (FFA) and glycerol may then be translocated into the liver readily crossing the liver cell membrane (Felt and Benny, 1971). The hyper triglyceridemia observed in AFB1 induced toxicity may be due to the clearance defects associated with deficient LPL activity. Increased fatty acid mobilization from peripheral adipose tissues and decreased triglycerides clearance from blood circulation are considered as causes for the hepatoma induced hypertriglyceridemia (Redgrave et al., 1984). Hyper triglyceridemia which is frequently observed in various degrees in tumour bearing animals in combination with increased VLDL and decreased HDL, which is very suggestive of a
defective catabolism rather than elevated hepatic synthesis of triglycerols rich lipoproteins (Damen et al., 1984).

The decreased content of triglycerides in clove, its extract and eugenol administered animals may be due to its hypolipidemic activity. Administration of alcoholic extract of *E. caryophyllata* has proved to reduce the triglyceride content in galactosamine / lipopolysaccharide induced hepatitis in rats (Ravikumar et al., 2005). The hypolipidemic effect of *E. caryophyllata* and its extract can be attributed to the presence of oleanolic acid, in it (Duke, 1999).

Phospholipids are known to play a significant role in the molecular organization and the activity of membrane bound enzymes. They also play an important role in maintaining the structural integrity of the hepatocellular membrane (Bachmann et al., 1977). The decreased level of phospholipids (p<0.001) in the aflatoxin fed animals implicates the alteration and disturbance in the phospholipids metabolism after the administration of AFB₁. It is similar to the diethyl nitrosamine induced hepatocarcinoma (Tang et al., 1992). The Ca²⁺ dependent enzyme, phospholipase A₂ is a key enzyme in the arachidonic cascade, and this enzyme may play an important role in the increased lipid peroxide formation in the animals fed with aflatoxin. The increased concentration of intracellular calcium caused by the administration of toxin, increases the activity of phospholipase A₂ which in turn causes the hydrolysis of lower membrane phospholipids to release arachidonic acid (Kramer, 1993). This might account for the decreased levels of phospholipids observed in the liver of AFB₁ challenged rats.

The considerable increase in the levels of phospholipids in the *E. caryophyllata*, its extract and eugenol treated rats suggest that *E. caryophyllata* might decrease the activity of the enzyme phospholipase A₂ probably by maintaining the levels of intracellular calcium within the live in normal levels. This intern decreases the degree of hydrolysis of membrane phospholipids to release arachidonic acid. This might also be one of the probable mechanisms which is responsible for the antiinflammatory activity of the plant extract.
**Fig. 16a-c** shows the level of HDL, LDL and VLDL in the serum of control and experimental groups of rats. In the total lipids and the various components of the lipid profile of the serum and liver tissue, tannin seems to be the least active drug in bringing back the toxic impact to near normal contain. The most active drug seems to be the alcoholic fraction in the case of phospholipids and the triglycerides of the liver and HDL, VLDL and triglycerides of serum. Sylimarin was the best drug for total lipids of the liver. In the case of cholesterol and LDL of serum, clove seems to act as the best drug.

The formation of serum lipoproteins is dependent upon the metabolism of precursor (nascent) lipoproteins secreted from the liver and intestine (Wu and Windmueller, 1979). The conversion of nascent to mature lipoproteins involves enzymatic modification of lipids by lipoprotein lipase (LPL), hepatic lipase (HL), and lecithin cholesterol acyl transferase (LCAT) and the exchange of apoproteins and lipids with other lipoproteins and cells (Cryer, 1981). Sirowej and Katterman, (1978) and Cartwright *et al.*, 1982, showed that twenty four hours after a single injection of D-galactosamine, serum LCAT activity is depressed, cholesterol esters are reduced and discoidal LDL and HDL accumulate in plasma.

In accordance with these reports, the present study elicited a significant increase (p< 0.001) in serum VLDL and LDL in the aflatoxin fed rats as compared to control rats. The abnormalities in the lipid and lipoprotein composition produced by AFB$_1$ have been attributed to the decreased LCAT activity and concomitant defects in lipoprotein metabolism. The decreased levels of LDL and VLDL in the *E. caryophyllata*, its extract and eugenol treated groups as compared to aflatoxin fed rats may be due to the optimal activity of serum lipoprotein lipase and due to the antioxidant effect of the plant extract (Duke, 1999).

HDL is considered to be a beneficial lipoprotein (Gordon *et al.*, 1977). It helps in the scavenging of cholesterol from the extra hepatic tissues in the presence of lecithin cholesterol acyl transferase and brings it to the liver (Kavitha and Nalini, 2000). Decreased levels (p< 0.001) of
serum HDL was seen in the aflatoxin fed rats as compared to the control rats. The lowered HDL levels can be attributed to the decreased serum lipoprotein lipase and lecithin cholesteryl acyl transferase activity. The considerable increase in the levels of HDL in the *E. caryophyllata*, its extract and eugenol treated rats as compared to aflatoxin fed group (p<0.001) may be due to the delayed clearance and increased synthesis of HDL constituents. In this context Nikkila *et al.*, (1978) have shown that elevated activity of plasma lipoprotein lipase leads to an increase in HDL production and reduction in LDL constituents. Therefore the increased HDL levels might be due to the increased activities of lipoprotein lipase and lecithin cholesterol acyl transferase.

4.14. SODIUM, POTASSIUM AND CALCIUM

Minerals are essential elements in many biological functions such as electron transport chain and biological oxidation, in mitochondria and in antioxidant enzyme activity. Alteration in the cell mineral metabolism has been reported to be an important pathogenic step in toxin induced abnormalities, and is related to its biological features (Miyahara *et al.*, 1982).

Table 9 shows the levels of calcium, sodium and potassium in the serum of normal and experimental groups of rats. Results indicate that the levels of calcium and potassium were significantly increased (P<0.001) whereas the levels of sodium significantly decreased (P<0.001) in the serum of the aflatoxin fed animals. This could be due to the failure of the sodium pump that resulted in the decrease in serum sodium and rise in serum potassium concentrations.

In the mammalian cells, the intracellular concentration of Na\(^+\) and K\(^+\) is regulated by a transport system in the cell membrane, which converts chemical energy by the hydrolysis of ATP into a rectorial movement of sodium and of the cell and potassium into the cell against electrochemical gradient (Skou, 1971). Active calcium transport and resultant low calcium concentration are the necessary conditions for active Na\(^+\)/K\(^+\) transport. Since sodium and calcium are thought to be the
competitive at a number of sites, it seems likely that a high concentration of total calcium in toxin induced conditions would compete with sodium at specific sites at the inner surface of the membrane. This may lead to the decrease in sodium content (Shanne et al., 1980). Also failure of sodium pump results in a depletion of plasma Na\(^+\) and a rise in plasma K\(^+\) concentration. This leads to hyponatremia and hyperkalemia which are the most common electrolyte abnormalities in aflatoxicosis condition.

Calcium is a messenger of great importance to cells. It conveys information to a large number of processes, among them are the various aspects of mobility, division of cells, export of products of cellular activity by fusion of intracellular vesicles within the plasma membrane, production of metabolites at various steps of biosynthetic and breakdown pathways (Carafoli, 1995). Primary liver tumour may be present as hypercalcemic crisis and the measurement of serum calcium level is indicative of liver failure. Reitman and Frankel (1964), have also reported higher calcium content in the Morris hepatoma of the rat than in normal liver tissue. Direct proportionality was found between concentration of calcium in plasma and mitotic activity in the tissue (Perris, 1971). The influx of Ca\(^{2+}\) into the cell is uncontrolled in cancerous condition, leading to the disturbances in equilibrium between intracellular and extracellular calcium concentration (Cittiadini et al., 1981). Such calcium influx across cell membranes and consequent rise of intracellular, free calcium lead to the increased DNA synthesis (Gefland et al., 1984). The strong glycolytic, enhancement in cancer cell is directly depend on cell calcium increase and thus energy and membrane permeability play a different role in control of net intracellular calcium content of neoplastic cells (Cittiadini et al., 1981).

A significant decrease in the levels of calcium, potassium and increase in the levels of sodium in the group of Eugenol, clove and its extract treatment with aflatoxin fed rats as compared to aflatoxin alone fed group was recorded. This gives a supportive evidence for the protection offered by the plant and its alchoholic extract against aflatoxicosis in rats.
4.15. LIPID PEROXIDES, IRON AND FERRITIN

Table 10 shows the levels of lipid peroxides, iron and ferritin in the liver of control and experimental groups of rats. Results indicate that the levels of lipid peroxides and iron was increased (p<0.001) whereas the levels of liver ferritin was significantly (p<0.001) decreased in the rats fed with aflatoxin as compared to control rats, thereby showing the severity of oxidative stress induced as a result of the administration of the hepatotoxin.

The direction and magnitude of lipid peroxidation is the evidence most frequently cited to support the involvement of free radical reactions in toxicology and cancer. Lipid peroxide merits investigation since it is a very damaging process in biological system.

Increased level of lipid peroxides in aflatoxicosis condition may be due to excessive generation of superoxide radicals and H$_2$O$_2$. During the course of carcinogenesis, a considerable amount of ROS are generated and these radicals are capable of abstracting hydrogen atom from PUFA and thereby initiate lipid peroxides. Increased level of lipid peroxides are seen after severely impaired liver diseases condition (Olinesu et al., 1994).

The increment in the level of lipid peroxides in aflatoxicosis bearing animals may be due to the uncompromised production of free radicals by AFB$_1$. Shen et al (1994) have shown that there was a significant, persistent and dose dependent increase in malonaldehyde (MDA) and conjugated dienes level (both are end products of lipid peroxidation) after AFB$_1$ administration. Tosulkao and Glinsukon (1992) have also demonstrated the AFB$_1$ mediated effect on the formation of lipid peroxides. MDA production has been reported to cause intra-strand cross linkages with protein resulting in lipid peroxides-protein interaction. This leads to free radical formation in proteins resulting in polymerization which is considered to be more damaging to biomembranes (Longani and Davies, 1980).

The decreased level of lipid peroxides in clove, its extract and eugenol treated animals leads to the inference that *E. caryophyllata* and
its extract counteracts the abnormal increase in lipid peroxides induced by AFB<sub>1</sub>. This might be due to the antioxidative nature of the <i>E. caryophyllata</i> (Duke, 1999). Thus clove and its alcoholic extract have the ability to counteract adverse biological effect of AFB<sub>1</sub>.

Organisms take great care in the handling of iron (Weinberg, 1990) using both transport (such as transferrin) and storage (such as ferritin and hemosiderin) proteins so as to minimize the amount of free iron within cells and in extracellular fluid. However oxidative stress can itself provide iron for iron from ferritin and H<sub>2</sub>O<sub>2</sub> can degrade hemoprotein to release iron (Halliwell <i>et al.</i>, 1995).

The modification induced in the apolar residues of the membrane phosphoglycerides by active oxygen generation are considered to bring about structural alterations in the membrane. Therefore biomembranes and subcellular organelles are the major sites of lipid peroxide damage, during toxic conditions in rats. Earlier studies by Sakaguchi <i>et al.</i> (1989) suggested that Ca<sup>2+</sup> may participate as one of the mechanism of the free radical formation in the liver of mice during endotoxaemia. In accordance with these reports, we also observed a significant increase in the levels of lipid peroxides in rats fed with aflatoxin. The increased level of iron and decreased level of ferritin in aflatoxicosis was probably due to increased lipid peroxidation and excessive production of free radicals.

The restoration of altered levels of iron and ferritin in the rats treated with clove and its extract with aflatoxin as compared to aflatoxin alone treated animals implicates the inhibitory effect of the plant and its extract on the above said parameters.

4.16. ELECTROPHORESIS

The electrophoresis profiles of a liver tissue of rat reveals different types of protein bands in an experimental tissue. The treated and control liver tissue samples, electrophorogram (Plate 4) of ten samples from L1 to L10 exhibited different kinds of polypeptides in coomassive brilliant blue stained gel; molecular weights ranged from 200 KDa to 450 KDa.
The staining intensity of the some of major polypeptides were found to be lower in lane 2 (L2), lane 3 (L3), lane 5 (L5), lane 7 (L7) and lane 9 (L9). However, in lane 2 (L2) which was treated with aflatoxin was found to be more altered in the higher molecular weight polypeptide fractions.

The staining intensity of polypeptides of molecular weight 118 and 116 has predominantly disappeared in lane 2 (L2) which was treated with aflatoxin.

From the changes in the total polypeptide fractions in the molecular weight 118 and 116, it is also found that some of low molecular weight proteins ranged from 56-45 KDa have disappeared in L2, L5, L7 and L9.

The protein profiles in lane 4 (L4), lane 6 (L6), lane (L8) and lane (L10) were found to be similar to that of control and has no major differences in any of the particular polypeptide fractions in the stained gel.

From these observations it can be clearly inferred that when the toxin or drug was given alone, the banding was faint with lesser number of bands but when they were given together, there were darker and more number of bands.

**4.17. HISTOPATHOLOGICAL STUDIES**

**Liver**

Assessment of the hepatoprotective potential of a drug is incomplete without histological description of changes in the architecture of the liver. The liver cells of the control animals revealed a normal architecture (Plates 5 & 6).

In aflatoxin fed group of animals, the hepatocyte showed marked congestion of central vein along with intense cytoplasmic granularity. There was a slight increase in Kupffer cell activity. The severity of the toxicity is evidenced by the observation of pathological changes in the architecture of the liver viz., infiltration of inflammatory cells, Kupffer cell hyperplasia, neutrophil accumulation focal necrosis and degenerative changes in the hepatocytes. This shows histologic features of
development of pure, well differentiated liver cell abnormalities. Biliary proliferation is not seen in any of the rat livers. These lesions observed after AFB\textsubscript{1} treatment are in agreement with previous report on the administration of AFB\textsubscript{1} (Angsubhakorn et al., 1990).

The basic nature of this toxin is perhaps to disturb the internal milieu of the sensitive cells in such a manner that their biosynthetic pathways get disturbed. As a consequence, many cells fail to complete their division cycle and may even get damaged and destroyed. Many of the cells that escape destruction (resistant cells) start dividing more rapidly to compensate the loss. This compensatory division probably becomes unbridled of all the possible control mechanism, resulting in neoplasia (Bilgrami et al., 1986).

The toxicity of aflatoxin with the accompanying regenerative hyperplasia of parenchymal cells, in AFB\textsubscript{1} toxin fed animals contribute to the development of hepatocellular carcinoma in atleast two ways. First, cell replication is essential for carcinogenesis. Second, the carcinogen altered hepatocytes appear better, able to survive and grow in the face of a general hepatotoxin (Roebuck et al., 1991). There have been reports which have disturbed the presence of Kupffer cells within these areas of hyperplasia and concluded that these cells represent earliest evidence of malignancy. Hyperplastic areas progress into larger neoplastic nodules and finally into hepatocellular carcinoma. Hepatocytes undergo a sequence of alterations recognized morphologically by a progression from altered foci to nodules and from nodules to cancer (Sell and Leffert, 1982).

The nodules appearing in aflatoxin fed group of animals represent transitional stages from normal liver parenchyma to hepatocellular carcinoma and may be considered as the direct forerunners of the latter. Kalengavi and Desmet (1975) have also reported the development of liver cell hyperplastic foci and nodules in AFB\textsubscript{1} induced carcinogenesis.

Rats given with \textit{E. caryophyllata}, its alcoholic extract, eugenol and sylimarin along with aflatoxin showed considerable recovery from hepatotoxicants as observed by the absence of the adverse toxic changes.
in the liver. Thus the results of the liver histology confirmed the protective effects of *E. caryophyllata* against the toxicity of aflatoxin.

When administered without the toxin there was no severe cell damage proving that the drugs are not hepatotoxic. The observation is in accordance with that of Zhao and Brien, (1996) who have studied the prevention of CCl₄ – induced liver necrosis in mice by naturally occurring methelynedioxybenzenes.

**Kidney**

Being an organ of excretion, kidney filters and removes the toxins of the body. Obvious any toxin makes its impact on the histology of kidney. Hence histopathological study on kidney, is undertaken to evaluate the efficacy of clove in ameliorating the harmful effects of aflatoxin on kidney.

Photomicrographs (Plates 7&8) compares the histology of the cross section of the kidney of the experimental groups. The cross sections of the kidney of the control group of rats reveal number of normal glomeruli, distal and proximal convoluted tubules. Cross section of the aflatoxin fed kidney shows the lesions in these three important structures of a nephron due to the impact of aflatoxin on the renal cell membrane. The prominent changes are noticed in glomeruli. They are less organized with a collapsing of the outer layer. Distal tubule is less visible. Proximal tubules show degenerative changes, such as necrosis and vacuolation of the epithelium. Reports have suggested the presence of epithelial cell proliferation, anaplastic cells in corticomedullary region and nuclear enlargement of tubular epithelium in kidney in AFB₁- induced hepatocellular carcinoma (Rati et al., 1991). the rats fed with *E. caryophyllata*, its alcoholic extract, eugenol and sylimarin did not show any abnormal changes in the cross section of kidney as compared to control rats. This infers the absence of adverse effects of the drugs. The amelioration of adverse histologic patterns observed in aflatoxin treated groups may be due to the administration of the drugs along with toxin which augments the nodular formation due to toxin.
4.18. HPLC ANALYSIS

The HPLC analysis of the alcoholic fraction at the specific conditions (as given in the chapter Materials and Methods) revealed four peaks (Fig. 17) with the following retention time and peak area.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention Time</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.230</td>
<td>18.2</td>
</tr>
<tr>
<td>2.</td>
<td>2.640</td>
<td>4.9</td>
</tr>
<tr>
<td>3.</td>
<td>3.017</td>
<td>42.6</td>
</tr>
<tr>
<td>4.</td>
<td>3.350</td>
<td>33.5</td>
</tr>
<tr>
<td>5.</td>
<td>6.347</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The HPLC finger print profile at 254 nm in the methanol mobile system generally reveals steroids and terpenoids. Farnesol, sitosterol, stigmasterol and campesterol are probably be the fractions of E. caryophyllata (Duke, 1999).

β-sitosterol has been reported to occur along with eugenol in E. caryophyllata (Duke, 1999).

Isoquercetin, quercetin and luteolin are the different flavanoids reported in clove. Duke, (1999) has listed a number of compounds in clove (Table 1). From the literatures surveyed and the HPLC analysis, it can be concluded that, E. caryophyllata contains various groups of secondary metabolites such as sterols, eugenol and flavanoids all of which have been reported to be antioxidants.

4.19. RELATIVE ANTIAGLATOXIC EFFICIENCY OF THE EXPERIMENTAL DRUGS

The metabolic derangement brought about by aflatoxin, especially in the hepatic function of the rat was alleviated to various degrees by the different drugs in the experimental concentrations. Sylimarin, the standard hepatoprotective drug seems to be the best. The alleviation has
been to the maximum level in the body weight, prothrombin time, blood glucose, indirect bilirubin, pyruvate, sialic acid, DNA, liver protein, serum total protein, albumin, globulin, A/G ratio, SGOT, SGPT, ALP, LDH, GGT, GST, ATPases, total lipids, total cholesterol, G-6-P-D, glutathione reductase, catalase, total reduced glutathione, cerealoplasmin, uric acid, ascorbic acid, α-tocopherol, Na⁺ and K⁺. This could be because the concentration selected for the present study is the appropriate one for its antihepatotoxic potential.

Out of the three forms in which *E. caryophyllata* has been observed for its antiaflatoxic potential, the alcoholic fraction is the best form (Fig. 18). More alleviation than even Sylimarin has been noted in kidney weight, haemoglobin, RBC, WBC, total cholesterol (serum), triglycerides, LDL, total bilirubin, direct bilirubin, SOD, total ATPase, Mg²⁺ ATPase, lipid peroxide, iron, ferritin and creatinine. This could be because of the relatively purified state of the various alcohol soluble components. *E. caryophyllata* has been reported to contain kaempheol, quercetin and rhamnetin as Antiaflatoxic agents. Apart from this number compounds with antihepatotoxic potential and hepatoprotective abilities have been reported in clove (Duke, 1999). Farnesol, sitosterol, stigmasterol and campesterol have also been identified to be present in the alcoholic fraction through HPLC analysis in this study. The highest hepatoprotective potential could be because of the synergistic effect of all these secondary metabolites together.

In most of the hepatoprotective herbal medicines, a combination of various herbs are administered for their performance. Supporting this view, Cai et al., (2004) states that, in the Chinese drug formulations, bioactive constituent of one plant is activated by the components of the other plant.

The standard drug sylimarin used in this study is also a multiple compound found as flavolignan. It has been more or less established beyond any reasonable doubt that sylimarin is produced in the plant by means of a radical coupling of a flavonoid and a coniferyl alcohol (Wagner et al., 1968 as cited by Kar, 2003).
The *E. caryophyllata* has also been antiaflatoxic, though not as efficient as the alcoholic fraction and eugenol. In the case of only urea the alleviation by *E. caryophyllata* was the maximum. This could be because concentration of the hepatoprotective principles were probably lesser in clove than the alcoholic fraction. Moreover the non alcoholic fraction would have probably had some antagonistic principles which have been eliminated due to the extraction with the alcohol.

Comparatively the lesser efficiency of eugenol could support the view that the higher efficiency of the alcoholic extract is a synergistic effect of various fractions. The alcoholic fraction contains number of proven hepatoprotective drugs apart from eugenol. Eugenol by itself brought about maximum alleviation in the parameters such as RNA, calcium, PCV, phospholipids, Ca$^{2+}$ ATPase, hexoseamine, hexose, glycogen, lactate, liver weight, liver protein, serum triglycerides, HDL and VLDL. Thus the presence of number of secondary metabolites in the alcoholic extract of *E. caryophyllata* have been acting as a multiple drug in alleviating aflatoxicosis.

The outcome of the histopathological and electrophoretic studies are supporting evidences for the hepatoprotective and antiaflatoxic efficacy of *E. caryophyllata*. 