Chapter 2
INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and it was reported to spread further with continuance and increasing incidence in 21st century. It was identified as the leading cause of death in the United States, it accounts for 25% of all the deaths in humans presently. Dietary factors continue to play a complex and multi role in the etiology of cancer. Nutrition accounts for up to one third of the total cause of cancer, apart from cigarette smoking and chronic inflammation and infection (Sugimura, 2002). Cancers most commonly associated with diet include esophageal, stomach, colon, liver and the prostate.

The liver is one of the largest organs in the body. It plays a vital role as a neutralizer of exo and endotoxins. Liver has significantly marked role in regulation of physiological processes. Several vital functions such as metabolism, secretion and storage, detoxification of a variety of drugs and xenobiotics, occur in liver. The bile secreted by the liver plays an important role in digestion. Liver diseases are among the most serious ailment (Hari Kumar et al., 2011). Various exogenous and endogenous factors are known to affect the normal pattern of cell growth, by which cell becomes a cancerous.

Hepatocellular carcinoma (HCC) is one of the most common cancers with poor prognosis. The limited treatment options and poor treatment success make hepatocellular carcinoma (HCC) one of the leading causes of death in developing countries. Recent studies during the last few years have shown new hopes in the treatment of this dreadful diseases by measures aimed at the close relationship between free radical production and hepatic cancer.
Indeed, curative treatment such as tumour resection and orthotopic liver transplantation are not feasible in advanced stages of Hepatocellular carcinoma (HCC).

Hepatocellular carcinoma (HCC) is an aggressive cancer with a worst outcome largely due to metastasis and postsurgical recurrence. Thus, the inhibition of invasion and metastasis is of great importance in its therapies (Zhi-Dong Wang et al., 2010). Therefore, the search for effective chemotherapeutic agents is most important to improve the survival rate of patients with advanced or recurrent HCC. Apart from this, HCC is well known for multi-drug resistance and poor response to current chemotherapeutic agents.

Many of herbal medicines (HM) and its active ingredients have been identified as potential modifiers of cancer (Sawadogo et al., 2012). Herbal medicines yield important breakthroughs in cancer prevention and treatment. Herbal medicine is presently used as first line aids in numerous cultures across the world (Kraft K., 2009). Recent research have proved to indicate several possible mechanisms of action for herbal medicines, or their bioactive components, may act alone or in concert to reduce cancer risk through their anti-oxidant (Shahin Sharif Ali et al., 2008), and anti-tumorigenic properties, as well as their direct suppressive effect on carcinogen bioactivities. Hence, there is an increasingly convincing rationale for employing HMs against cancer.

Medicinal plants have been used for healing and preventative health for about thousands of years all around the world. The use of Herbal medicines in cancer prevention has not been thoroughly evaluated despite growing evidence that the public is utilizing a wide range of HMs (Brown et al., 2001). Herbal medicine is still the main source for agent that can be used for
cancer treatment and prevention. Herbal medicine is considered the second method to fight cancer utilized by cancer patient in developed countries (Kennedy J, 2005).

Zhi-Dong Wang et al., 2010 stated that Medicinal plants or ethnopharmacology which were used in folklore medicine continue to be an important source of discovery and development of novel or potential therapeutic agents for treatment of cancer. Since the increase in the use of synthetic chemicals in cancer therapy has led to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources which are therapeutically effective, culturally acceptable and economically within the research of the poor people (Fauziah et al., 2005).

Hollman P and Katan M., 1997 and Liu RH., 2003 stated that most of the protective effect of fruits and vegetables exist only due to the presence of phytochemicals, which are the non-nutrient plant compounds such as the carotenoids, flavonoids, isoflavonoids, and phenolic acids. Different phytochemicals have been found to possess a range of activities, which may help in protecting against chronic disease. For example, phytochemicals may inhibit cancer cell proliferation regulate inflammatory and immune response, and protect against lipid oxidation. The case study done by Subbaramaiah K and Dannenberg AJ in 2003 delivered the most valuable information that the plants which were used for long time were well known for anticancer property and such as *Nigella sativa* (black seed), *Cinnamomum cassia* (Cinnamon), *Panax ginseng* (Ginseng), *Camellia sinensis* (Green tea), *Allium sativum* (garlic) and *Zingiber officinale Rosc.* (Ginger).
Importance of Rat as animal model

Recent PubMed data searches have revealed the fact that over 1 million publications were made using rat as experimental model. Aitman in 2008 stated that the use of rat has become a standardized physiological and toxicological model in pharmaceutical industry. It has recently reported that rat models were highly suited to study human disease, than mouse (Aitman et al., 2008). The physiology is easier to monitor in rat and it is likely corresponding to human condition. The rat is more intelligent than the mouse and is capable of learning a wider variety of tasks that are important to cognitive research. The size of the animal enhances its use as a disease model, not just because of the ability to perform surgical procedures, but also because of the proportional size of important substructures in organs that affects both how much of the organ is involved in an experimental lesion and the distance effects of drug administration to specific anatomical areas. The rat is the primary model for mechanistic studies of human reproduction. In models of diabetes, the rat model behaves more like the human disease in important ways, including the ability of environmental agents (e.g. toxins, stress, diet and vaccination) to modify the disease. For drug studies, the size of the rat enables serial blood draws. This is by no means a comprehensive list, but gives a sense of the depth and range of use for this animal model (Philip M. Iannaccone and Howard J. Jacob, 2009).

Induction of HCC by Diethyl nitrosamine (DEN)

Diethyl nitrosamine is an N-nitroso alkyl compound, categorized as a potent hepatotoxin and used as hepatocarcinogen in experimental animals, producing reproducible tumors after repeated administration (Jose et al., 1998, Gayathri et al., 2009, Yamada et al., 2006). Liao et al., in 2001 identified the presence of diethyl nitrosamine in a wide variety of foods like cheese,
soybean, smoked, salted and dried fish, cured meat and alcoholic beverages. Diethylnitrosamine (DEN), was also identified in tobacco smoke, cured and fried meals, cosmetics and pharmaceutical agents, has been established to be a powerful hepatocarcinogen in rats (El-Shahat et al., 2012). It is also found in minute concentrations in baby bottle nipples (Brown, 1999). Metabolism of certain therapeutic drugs is also reported to produce NDEA (Akintonwa, 1985). Many biochemical and molecular changes caused by chemical carcinogens acting through free radical metabolites induces oxidative stress leading to tumor promotion (Trush et al., 1991). Nitrosamines compounds induce oxidative stress and retrogradation of the antioxidant defense mechanism due to overproduction of reactive oxygen species (ROS) and lipid peroxidation which result in a wide range of disorders in a variety of rodent organs, especially liver (Thirunavukkarasu and Sakthisekaran., 2003).

The hepatocarcinogenesis induced by DEN was proposed to induce the mechanisms of the alteration of DNA structure, the formation of alkyl DNA adducts and the induction of chromosomal aberrations and micronuclei in the liver (Al-Rejaie et al., 2009 and Verna et al., 1996). In addition to a single injection of DEN followed by partial hepatectomy and coupled with 2-acetyl-aminofluorene (2-AAF) (Solt et al., 1983). Chuang et al., 2000 and Shiota et al., 1999, stated that the sequential administration of DEN for several weeks could also induce HCC in rodents.
Physical properties of Diethyl nitrosamine (DEN)

CAS no : 55-18-5

Synonyms : diethylamine, N- nitroso; N,N- diethylnitrosamine; DEN; DENA; DANA

Structure :

\[
\text{N-} \quad \text{N-} \quad \text{O}
\]

Mol wt : 102.1

Boiling point : 177 °C (Lit)

Refractive index : n20/D 1.437

Density : 0.95 g/mL (lit.)

Storage temperature : 2-8 °C

Form : Liquid

Colour : Yellow

Silymarin

Silymarin is a polyphenolic mixture of flavonoligands derived from the seeds of the milk thistle plant (*Silybum marianum* L. Gaertn). Silymarin is composed primarily of silibinin (≈90%) together with small amounts of other silibinin stereoisomers, such as isosilybin, dihydrosilybin, silydianin and silychristin (Kulms *et al.*, 1999). Silymarin has been clinically applied for the treatment of liver diseases as an encapsulated, standardized extract, since it is not water-soluble (Luper, 1998). Silymarin has been indicated to possess hepatoprotective (Freedman *et al.*, 2011 and Polyak *et al.*, 2010) and antihepatocarcinogenic (Bousserouel *et al.*, 2012, Féher *et al.*, 2012 and Varghese *et al.*, 2005) properties.

Silymarin structure

As silymarin has promisingly observed to have anti-inflammatory, anti-oxidative and anti-carcinogenic effects (Stege *et al.*, 2000 and Aragane *et al.*, 1998), it has been tested in various *in vitro* and *in vivo* models for its efficacy in prevention of skin carcinogenesis (Aragane *et al.*, 1998).
The mechanisms of the antihepatocarcinogenic effects induced by silymarin include the inhibition of cell proliferation and the stimulation of apoptosis (Gopalakrishnan et al., 2013).

Preclinical cell culture and animal efficacy testing models are currently used to identify, assess, to prioritize chemical agents and natural products with the goal of preventing different types of human cancer. If little is known about a potential agent, the first step is a sequential series of short-term in vitro prescreens of mechanistic, biochemical assays. These assays would provide quantitative data to establish an early indication of chemopreventive efficacy and to assist in prioritizing agents for further evaluation in longer-term in vitro transformation bioassays and whole animal models. Promising chemical agents or combinations of agents that work through different inhibitory mechanisms are subsequently tested in well established chemically induced or spontaneous animal tumor/cancer models (Vernon E. Steele et al., 2005).

Alpha-fetoprotein – AFP

Alpha-fetoprotein (alpha-fetoprotein, AFP) is a Glycoprotein, belonging to the intriguing class of onco-development protein. It is generally designated as tumour marker; AFP is recognized as an important blood component, possessing specific diagnostic significance. Total AFP was divided into three different glycoforms, AFP-L1, AFP-L2, and AFP-L3-based on their binding capability to lectin Lens culinaris agglutinin (LCA). High percentage of AFP-L3 has been shown to be associated with poor differentiation and biologically malignant characteristics, poor liver function, and larger tumor mass (Khien et al., 2001). AFP is a glycoprotein produced by the fetal liver and yolk sac during pregnancy. Serum AFP levels are often elevated in HCC, but this is not always the case. Chen et al., in 1984 observed the elevated AFP levels initially in the early stages of HCC and the drop or even normalized level before rising again as disease
progressed. Elevated levels of AFP up to pathological range in adults were correlated with the appearance of several malignant and chronic conditions, such as hepatocellular carcinoma (HCC) and chronic liver disease, respectively (Baig JA et al., 2009). Alpha-fetoprotein (AFP) was considered as a current gold standard and most commonly used biomarkers (Farinati et al., 2006) for patients at risk for HCC, along with ultrasound every 6 to 12 months. Serum AFP levels of more than 400 ng/mL are considered diagnostic; however, such high values are observed only in a small percentage of patients with HCC. Ultrasound surveillance even performed at every three monthly intervals cannot improve detection of small HCC because of limitations in recall procedures (Farinati et al., 2006).

The first serologic assay for detection and clinical follow up of patients with hepatocellular carcinoma was alpha-fetoprotein (AFP) which has been the standard tumor biomarker for HCC for many years. It is a glycoprotein produced by the fetal liver and yolk sac during pregnancy. Serum AFP levels are often elevated in HCC, but this is not always the case. AFP levels may be elevated initially in the early stages of HCC and then drop or even normalize before rising again as disease progression occurs (Chen et al., 1984). Additionally, AFP elevation has also been recognized in the presence of acute and chronic viral hepatitis as well as in patients with cirrhosis caused by hepatitis C. Given the multiple indications that present with elevated AFP levels, it is necessary to evaluate the significance of serum concentrations. In general, consistently elevated serum AFP levels greater than 500 ng/ml are indicative of HCC. Lower serum concentrations which are only transient in nature are more often present in benign liver disease (Wu, 1990). If a patient has known risk factors for HCC, such as the presence of cirrhosis, increasing levels of AFP have been shown to correlate with the development of HCC.
Unfortunately, AFP serum concentrations do not correlate well with the prognostic values of HCC such as tumor size, stage, or disease progression and ethnic variability may also exist. Furthermore, in some cases of HCC, AFP elevations are not apparent at all (Chen et al., 1984).

**Liver marker enzymes**

It has been reported that the analysis of liver enzymes in serum reflects cellular damage. Liver function enzymes such as Serum Glutamate Pyruvate Transaminase [SGPT], Serum Glutamate Oxaloacetate Transaminase [SGOT], Gamma glutamyl transpeptidase [γGT] and Alkaline phosphatase [ALP] and other biochemical parameters such as total protein, albumin, globulin are generally used in humans and animals as indicators of liver injury as well as liver response to medicine (Kim and Park., 1994).

**Oxidative stress**

Clinical, epidemiological and experimental studies provide evidence implicating the role of free radicals on the etiology of cancer (Cerutti, 1998).

Oxidative stress is non - other than a disturbance in the oxidant-antioxidant balance leading to potential cellular damage. Normally cells could tolerate a mild degree of oxidative stress, as they have sufficient antioxidant defense capacity and repair systems, which facilitate to recognize and remove molecules damaged by oxidation. The imbalance can result from an overabundance of reactive oxygen species (ROS) from other factors or due to lack of antioxidant capacity caused by disturbances in production and distribution. (Dröge in 2003) described ROS as potential carcinogens as they play vital roles in mutagenesis, tumor promotion, and progression. Perry et al., in (2000) observed that the excess ROS can damage lipids, protein or DNA, inhibiting normal function if it is not regulated properly. ROS alterations in different
signaling pathways may modulate gene expression, cell adhesion, cell metabolism, cell cycle and cell death. Choi et al., in (2006), stated that these events may induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation.

Oxidative stress increases when reactive forms of oxygen are produced faster than they can be safely neutralized by antioxidant mechanisms and/or from a decrease in antioxidant defense, which might lead the way to damage of biological macromolecules and disruption of normal metabolism and physiology. This condition can contribute and/or lead to the onset of health disorders and play a damaging role in a number of liver disorders (Favier, A. 2006).

**Enzymatic and Non-Enzymatic Antioxidants**

Cells have different antioxidant systems including low molecular weight antioxidant molecules like glutathione [GSH] and various antioxidant enzymes to defend themselves against free radicals attacks. Glutathione peroxidase [GPx] is a selenoprotein, which reduces lipidic or non – lipidic hydroperoxides, as well as H₂O₂ while oxidizing GSH. Alterations in the enzymatic systems allow cells to undergo normal differentiation. Natural antioxidants have a wide range of free radical scavenging activity and alter intracellular redox potential.

In human Lipid peroxidation is implicated in the pathogenesis of several hepatic disorders, (Favier A. 2006; Farinati et al., 1995; Barbaro et al., 1999; Madan et al., 2006). In inflammation related to viral infection results in increased lipid peroxidation with decreased antioxidant levels. Chrobot et al., 2000; Favier A, 2006, observed that the lipid peroxides formed may be chemotactic for the neutrophils causing increased inflammation, which further drives oxidant-mediated injury in the liver. They also have demonstrated an increase in
malondialdehyde (MDA) levels and decrease of the antioxidant capacity in acute and chronic hepatitis.

Superoxide dismutase activity is one of the major enzymatic antioxidant mechanisms against superoxide radical, in preventing liver toxicity induced by oxidative stress (Yagmurca et al., 2007). Catalase and GSHPx was observed to catalyze dismutation of the superoxide anion (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) which then convert H$_2$O$_2$ to water thus providing protection against ROS (Sayed-Ahmed et al., 2010). Superoxide dismutase (SOD) and catalase (CAT) was found to show counteractive deleterious action of reactive oxygen metabolites and protect from cellular and molecular damage (Abei in 1984). They were also determined to act as anticarcinogens, and inhibitors at initiation and promotion/transformation stage in carcinogenesis. Reduced glutathione (GSH), glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST) have been assumed as significant markers of chemoprevention owing to their antioxidant and detoxification properties (Saydam et al., 1997).

The superoxide dismutases (SODs) are ubiquitous components of cellular antioxidant systems. As described by McCord and Fridovich over 36 years ago, these proteins protect redox sensitive cellular machinery from damage by catalyzing the disproportionation of superoxide anion to oxygen and hydrogen peroxide (McCord et al., 1969). Several diverse families of SODs have been widely studied and disruptions or accentuations of their function are associated to varying degrees with several diseases in man such as arteriosclerosis Diabetes mellitus (Haskins et al., 2004 and Ookawara et al., 1992) and Down syndrome (Engidawork and Lubec., 2001).

The activity of each of the superoxide dismutase families relies upon a specific redox active metal ion, and depending on the SOD molecules, this could either be a manganese, iron,
copper or nickel ion. All known SODs require a redox active transition metal in the active site in order to accomplish the catalytic breakdown of superoxide anion. The metal cofactors catalyze both a one-electron oxidation and a one-electron reduction of separate superoxide anions to give the overall disproportionation reaction. These reactions typically require no external source of redox equivalents and are thus self-contained components of the antioxidant machinery. This allows SODs to function in a variety of intracellular and extracellular environments. Other antioxidant enzymes are known to consume superoxide, such as the prokaryotic superoxide reductases (SORs). In contrast to the SODs, these enzymes require an external source of electrons which are typically supplied by accessory redox proteins, such as a ferrodoxin (Kurtz, 2004). Under some biochemical conditions the SOD proteins can bypass the dismutation cycle and use external redox equivalents. For instance, catalytic SOR or superoxide oxidase (SOO) activities have been reported for some SOD proteins; however, a physiological role for such reaction pathways is not known (Liochev and Fridobich, 2000).

Superoxide dismutases are typically soluble secreted or cytosolic proteins but are also found in a number of subcellular compartments such as the cell envelope of gram-negative bacteria or the mitochondria of eukaryotic cells, as well as the extracellular milieu. While there is no similarity in sequence or structure between Cu/ZnSOD and Mn/FeSOD families, these metalloproteins do share some intriguing properties. For instance, both are quite stable relative to most proteins found in mesophilic organisms. Members of these families of enzymes are found across the continuum of life: examples are known in prokaryotes, archea and eukaryotes.
The Cu/ZnSODs and MnSOD, are the only forms found in yeast and mammals. Excellent overviews of the prokaryotic nickel and iron SODs can be found elsewhere (Barondeau et al., 2004 and Wintjens et al., 2004).

Catalase (EC 1.11.1.6) is an enzyme which is present mainly in the peroxisomes of mammalian cells. It is a tetrameric enzyme consisting of four identical, tetrahedrally arranged subunits of 60 kDa, each containing heme group and NADPH in its active center. Depending on the concentration of of $\text{H}_2\text{O}_2$, catalase has two enzymatic activities. If the concentration of $\text{H}_2\text{O}_2$ is high, catalase acts catalytically, i.e. removes $\text{H}_2\text{O}_2$ by forming $\text{H}_2\text{O}$ and $\text{O}_2$ (catalatic reaction). If the concentration of $\text{H}_2\text{O}_2$ is low and in the presence of a suitable hydrogen donor, e.g. ethanol, Methanol, phenol, and others, catalase acts peroxidically, removing $\text{H}_2\text{O}_2$, but oxidizing its substrate (peroxidatic reaction). (Scibior and Czeczot, 2006).

Vitamin C is essential to a healthy diet as well as a highly effective antioxidant. It is a substrate for ascorbate peroxidase. Vitamin E is a fat-soluble antioxidant that is the major antioxidant found in lipid-phase membranes. It blocks the production of ROS formed when fat undergoes oxidation. Several studies have clearly shown that serum levels of vitamin E are significantly reduced in patients with alcoholic liver disease (Bjørneboe et al., 1998, Leo et al., 1993). Vitamin E levels negatively correlate with production of oxidative stress products and directly correlate with the extent of liver damage (Masalkar and Abhang, 2005). Therefore, maintenance of normal concentrations of vitamin E seems to be essential to prevent lipid peroxidation induced by alcohol consumption. Works from several laboratories have indicated that mitochondrial damage may present a common early event in cell injury (Fulda et al., 1998). Mitochondrial damage was prevented by vitamin E (Sakurai and Cederbaum., 1998). Vitamin E
or C alone or in combination can facilitate scavenging free radicals generated in liver tissue (Zaidi et al., 2005).

As both the tuber extracts of *Ipomoea batatas* and *Solanum tuberosum* revealed that it posses many phytochemicals and antioxidant scavenging activity as described in Chapter 1, it provoked our interest to identify its effect on cancer induced rats, so we planned to carry our work with male Wistar albino rats as experimental model. Liver cancer was induced with (DEN) Diethyl nitrosamine.

**MATERIALS AND METHODS**

**Chemicals**

Chemicals N-nitrosodiethylamine and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and chemicals used in the study were of analytical reagent grade and of highest quality available, and were purchased from reliable firms and institutes [SRL, MERCK, RANBAXY, HIMEDIA, SIGMA and SUYOG]. Standard kits for LPO, SOD and CAT were obtained from Cayman Chemicals, USA.

**Selection of Animals and Ethics**

Healthy male Wistar albino rats weighing 150-200g were obtained from BRULAC, Saveetha University, Chennai-77 for this study. Animals were housed in cages under proper environmental conditions at room temperature 22-24°C and 12 h light/dark cycle and fed with a commercial pellet diet and water *ad-libitum*. The animals had free access to water. The animals were acclimatized to the laboratory conditions for two weeks before beginning the experiment. The experiment continued for 13 weeks on which constant weight of diet was given for animals. All the experiments were designed and conducted according to the Control and Supervision of

**Preparation of Plant Material**

The fresh tubers of *Ipomoea batatas* and *Solanum tuberosum* was collected from Plant cultivator during the month of August–October. They were dried in shade and powdered mechanically and prepared as mentioned in chapter 1.

**Induction of Experimental Animal**

The diethylnitrosamine was administered at a standard dose of 200 mg/kg body weight, as an intraperitoneal injection (Solt D, 1983). The aqueous extract of *Ipomoea batatas* and ethylacetate extract of *Solanum tuberosum* was given 250 mg/kg b.wt (Aderonke O Olowu *et al.*, 2011, Akeem *et al.*, 2011). Silymarin (2.5 mg/kg b.w) was used as a standard drug (Parthasarathy R *et al.*, 2007). Animals were divided into five groups as follows.

- **Group I:** Normal rats (n = 6, the animals were given normal diet)
- **Group II:** Hepatocarcinoma induced rats (n = 6, the animals were given DEN)
- **Group III:** Post-treated rats (n = 6, the animals were given DEN + *Ipomoea batatas*)
- **Group IV:** Post-treated rats (n = 6, the animals were given DEN + *Solanum tuberosum*)
- **Group V:** Drug control rats (n = 6, the animals were given DEN + Silymarin).
Treatment Protocol (Table 1)

Group I – Control animals received normal saline only. In Group II, III and IV animals liver cancer was induced with DEN by a single intraperitoneally injection (200 mg/kg body weight). After two weeks carcinogenic effect was promoted in all the groups by Phenobarbital (0.05%, PB). Promoter was supplemented to the animals through drinking water up to 14 successive weeks (Yoshiji et al., 1991). At the beginng of 15th week Groups III animals received *Ipomoea batatas* (250 mg/kg body weight/day) (Aderonke O Olowu et al., 2011) dissolved in saline and fed orally for 30 consecutive days. Groups IV animals received *Solanum tuberosum* (250 mg/kg body weight/day) (Akeem et al., 2011) for 30 days. Group V animals received Silymarin (2.5 mg/kg body weight) (Parthasarathy et al., 2007) after cancer induction and this group served as positive control.

Collection of Serum and Tissue Samples

After the experimental period the animals were sacrificed by cervical decapitation. Rats were treated as per the treatment protocol. Body weights of these rats were monitored sequentially in control and experimental animals. The blood was collected by retro orbital artery bleeding. Blood sample was centrifuged for 10 minutes at 3000 rpm to separate the serum, which was kept at −20 °C until further analysis. Liver was excised immediately and kept in physiological saline. Ten percentage homogenate was prepared with fresh tissue in 0.01 M Tris-HCl buffer (pH 7.4) Tissue homogenates were used for further analysis.
Histopathological Examination

Tissue specimens were fixed in 10% buffered formalin for at least 4 hrs immediately after sacrifice. Specimens were dehydrated by passing through ascending grades of alcohol, cleared in xylene, impregnated and embedded in paraffin. These sections were cut [3–5 µm] and were stained using haematoxylin – eosin and mounted in DPX and visualized under microscope for histological changes.

Statistical Analysis

Statistical analysis was done by using SPSS package. Values are mean ± SEM for six animals in each group and the significance of difference between mean values were determined by one – way analysis of variance [ANOVA].

Estimation of Bilirubin

Bilirubin in serum was estimated by Van den bergh reaction (Jendrassik and Jrof., 1938). Two forms of bilirubin are present in serum. Conjugated bilirubin yields purple coloured azo bilirubin upon treatment with diazotized sulphanilic acid but un-conjugated form reacts with diazotized sulphanilic acid only upon addition of methanol. The intensity of colour developed was read at 540 nm which is directly proportional to amount to bilirubin present.

Reagents

1. Diazot reaction: This reagent is prepared freshly by mixing 0.3ml of solution A with 10 ml of solution B.
Solution A: 1 g of sulphanilic acid was dissolved in 15ml of concentrated hydrochloric acid and made up to 1 liter with water. This acts as Van den bergh A solution.

Solution B: Dissolve 0.5 g of sodium nitrite in water and made up to 100 ml. This is called Van den bergh B solution.

2. Diazo blank 1.5% (v/v) Hydrochloric acid.

3. Bilirubin standard: 10mg of bilirubin dissolved in 10 ml of above was diluted to 100 ml with methanol which acts as working standard.

4. Absolute methanol.

Procedure

To 0.2 ml of serum 0.5 ml of dizao reagent was added followed by 1.8 ml of distilled water. To this complex add 2.5 ml of methanol. Series of standards were taken and made up to 2 ml with distilled water and 3 ml of methanol was added. To the standard solution of 0.5 ml of diazo reagent was added. Blank contains diazo solution and distilled water. All the test tubes were mixed well and allowed to stand for 30 minutes. The purple colour developed was read at 540 nm. The intensity of purple colour is directly proportional to concentration of bilirubin present in serum. The values are expressed as mg/dl of serum.
Estimation of Total Protein

The total proteins were determined using Folin – Ciocalteau reagent (Lowry et al., 1951) The phenolic group of tyrosine and tryptophan residues in protein produce a blue purple colour complex, with maximum absorption in the region of 660 nm wavelength, with Folin – Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of colour developed depends on the amount of these aromatic amino acids present and thus vary for different proteins.

Reagents

1. BSA stock solution (1mg/ml)

2. Analytical Reagents:

   a. 50 ml of 2% sodium carbonate was mixed with 50 ml of 0.1 N NaOH solutions (0.4 gm in 100 ml distilled water.)

   b. 10 ml of 1.56% copper sulphate solution was mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepare analytical reagents by making 2 ml of (b) with 100 ml of (a)

3. Folin – Ciocalteau reagent solution – Dilute commercial reagent with 1:2 volume of distilled water on the day of use.
**Procedure**

0.2 ml of serum was taken in the test tube and 2 ml of alkaline copper reagent were added and mixed well. Bovine serum albumin solution and water acts as standard and blank respectively to which 2 ml of alkaline copper reagent was added. All the tubes were incubated at room temperature for 10 minutes. 0.2 ml of Folin–Ciocalteau reagent solution was added to all the test tubes and incubated for 30 minutes. The colour developed is read at 660 nm using spectrophotometer. The values are expressed as g/dl of serum.

**Estimation of serum Albumin**

Serum albumin is estimated by dye binding method (Doumas et al., 1971). Albumin binds specifically to bromocresol green (BCG). The intensity of green colour formed by albumin BCG complex is directly proportional to albumin concentration in serum which is read at 630 nm.

**Standard albumin solution**

1. 200 mg of bovine serum albumin was dissolved in 100 ml of distilled water and 2 ml of standard was diluted with 100 ml of distilled water which acts as working standard. (40 mg/ml)

**Procedure**

0.1 ml of serum is mixed with 3 ml of albumin colour reagents (BCG) 0.1 ml of sodium chloride was treated as the blank. 3 ml of albumin colour reagent was added to test tubes containing blank
and standard solution. All the tubes were mixed well and allowed to stand for 10 minutes. The colour formed was read at 630 nm. The values are expressed as g/dl of serum.

**Estimation of Creatinine**

Creatinine was estimated by the alkaline picrate method of Owen *et al.*, 1954. Creatinine forms a coloured complex with picrate in alkaline method.

**Reagents**

1. Picric acid, 0.04 M: 916 mg of picric acid was dissolved in 100 ml of distilled water.

2. Sodium hydroxide: 0.75 N was prepared in distilled water.

3. Standard Creatinine: 100 mg of creatinine was dissolved in 0.1 N hydrochloride and made up to 100 ml. One ml of this solution was diluted to 10 ml with distilled water to give a working standard containing 100 µg/ml.

**Procedure**

0.1 ml of serum sample was made up to 2 ml with distilled water. Standards in the range of 10–40 µg were also made up to 2 ml. Blank contained only distilled water. To all the tubes, 1 ml of picric acid followed by 1 ml of sodium hydroxide was added. The formation of coloured complex is directly proportional to creatinine concentration in the sample which was read at 540 nm in a spectrophotometer after 15 minutes. The values are expressed as mg/dl of serum.
Estimation of Urea

Blood Urea nitrogen was estimated by the method of Natelson et al., (1983) by measuring the coloured complex formed with diacetyl monoxime in acidic medium.

Reagents

1. 10% Sodium tungstate: 10 grams of sodium tungstate was weighed and dissolved in 100 ml of distilled water.

2. 2/3 N Sulphuric acid

3. Diacetyl monoxime in 2% acetic acid

4. Sulphuric acid – phosphoric acid reagent: 140 ml of water was mixed with 150 ml of 75% of phosphoric acid and then 50 ml of concentrated sulphuric acid was added slowly.

Standard: 250 mg of Urea was dissolved in 100 ml of distilled water.

This solution was diluted 1 to 100 to give a solution containing 25 µg/ml which was used as the working standard.

Procedure

0.1 ml of serum sample was mixed with 3.3 ml of distilled water. 0.3 ml of sodium tungstate and sulphuric acid added mixed well and centrifuged. 1.0 ml of supernatant was taken and 0.4 ml of diacetyl monoxime, 1.6 ml of Sulphuric acid-phosphoric acid reagent was added. The tubes were placed in a boiling water bath for 30 min and cooled. The colour developed was
read at 480 nm in a spectrophotometer against water as blank. A series of standard were treated similarly. Blood urea level is expressed as mg/dl.

**Estimation of Blood Glucose**

Blood glucose estimated by the method of Sasaki *et al.*, 1972

**Reagents**

1. 10% TCA

2. O-Toluidene reagent:

   12.5 g of thiourea and 12.0 g of boric acid were dissolved in 50 ml of distilled water by heating over mild flame. 75 ml of redistilled O-Toluidene and 375 ml of acetic acid were mixed with to urea – boric acid mixture and the total volume was made up to 500 ml with distilled water. The reagent was left in a refrigerator.

**Standard Glucose Solution**

10 mg of glucose was dissolved in 100 ml of 0.2% benzoic acid in water.

**Procedure**

0.1 ml of blood was mixed with 1.9 ml of TCA solution to precipitate proteins and then centrifuged. 1 ml of the supernatant was mixed with 4 ml of O-Toluidene reagent and kept in a boiling water bath for 15 minutes. The greenish blue colour developed was read at 620 nm in a spectrophotometer.
A set of standard glucose were also treated similarly. Blood glucose levels were arrived using the standard curves. The values were expressed as mg/dl of blood.

**Estimation Cholesterol**

Serum cholesterol was estimated by Zak’s method (Zlatkis et al, 1953), Ferric chloride reagent precipitates the serum and extracts the cholesterol. Cholesterol reacts with concentrated sulphuric acid to form pink colour complex which is read at 540 nm. The intensity of pink coloured complex is directly proportional to concentration of cholesterol in serum.

**Reagents**

1. Ferric chloride reagent: 0.05% ferric chloride in glacial acetic acid (aldehyde free).
2. Concentrated sulphuric acid.
3. Cholesterol stock standard: 200 mg of cholesterol dissolved in 100ml acetic acid and 4 ml of stock is diluted with 100 ml of ferric chloride acetic acid reagent, (80 µg/ml)

**Procedure**

0.1ml of serum was added to 9.9 of ferric chloride reagent in a glass stoppered centrifuge tube for the preparation of protein free filtrate. The content were mixed and allowed to stand for 20–30 minutes. To 5 ml of protein free filtrate, 3ml of concentrated sulphuric acid was added and mixed by swirling. Standards were also treated similarly. Blank contains ferric chloride acetic acid reagent with concentrated sulphuric acid. All the test tubes were allowed to stand for 20
minutes and intensity of colour developed was read at 540 nm. The values are expressed as mg/dl of serum.

**Estimation of Serum Triglyceride**

Triglycerides in serum were estimated by the method of Rice (1970)

**Reagents**

1. Isopropanol

2. Activated alumina: 10g of aluminium oxide was taken in a beaker and 30 ml of distilled water was added. The suspension was stirred well and the supernatant was discarded. The suspension was washed with distilled water and the process was repeated several times until the supernatant becomes clear. The water was discarded and aluminium oxide was activated in an oven at 90°C overnight. The dry powder was used.

3. Alkaline potassium hydroxide: 10 g of potassium hydroxide was dissolved in 75ml of distilled water and 25 ml of isopropanol was added to the solution.

4. Sodium metaperiodate reagent: 7.7 g of anhydrous ammonium acetate was dissolved in 70ml of distilled water. To this 6 ml of acetic acid was added followed by 65mg of sodium metaperiodate. The solution was mixed well and made up to 100 ml with distilled water.

5. Acetyl acetone reagent: 0.4ml of redistilled acetyl acetone was added to 100 ml of isopropanol and mixed well. It was stored in the refrigerator.
6. Standard triglyceride solution: Stock Solution: 1 ml of glycerol trioleate was dissolved and made up to 100 ml with isopropanol.

**Procedure**

To 0.1ml of serum, 3.9ml of isopropanol was added followed by 50 mg of activated alumina. It was mixed well and left for 15 minutes. It was then centrifuged and 2 ml of the supernatant was taken for analysis. 6ml of alkaline potassium hydroxide was added to all the tubes. The tubes were incubated at 60°C for 10 minutes. The tubes were cooled and 1ml of sodium metaperiodate reagent was added to the tubes followed by the addition of 0.5ml of acetyl acetone reagent. The tubes were cooled and the colour developed was read at 405nm in a UV spectrophotometer against the blank. The values are expressed as mg/dl.

**Assay of Serum Glutamate Oxaloacetate Transaminase [E.C.2.6.1.1]**

The activity of SGOT was analysed by the method of King J 1965a

**Reagents**

1. Phosphate buffer: 100mM, pH:7.4

2. Substrate: 2.66 gms of DL- Aspartate and 32 mg of L – α ketoglutarate were dissolved in 20.5 ml of 1N Sodium hydroxide by gentle heating. This is made up to 100ml with buffer.

3. Dinitrophenyl hydrazine : 1mM of Dinitrophenyl hydrazine in 2N Hydrochloric acid.

4. Sodium hydroxide: 0.4N solution.
5. Standard pyruvate: 11 mg of Sodium pyruvate was dissolved in 100ml of phosphate buffer.

**Procedure**

In different tubes, 1ml of buffered substrate was added. 0.1ml of serum/ homogenate was added and incubated at 37°C for one hour. Then 1ml of Dinitrophenyl hydrazine reagent was added to arrest the reaction. To the blank, 0.1 ml of serum/homogenate was added only after the addition of Dinitrophenyl hydrazine reagent. The tubes were kept aside for 15 minutes, and then 10ml of sodium hydroxide was added and read at 520nm in a UV spectrophotometer. The enzyme activity was expressed as IU/L.

**Assay of Serum Glutamate Pyruvate Transaminase [EC 2.6.1.2]**

The activity of SGPT was assayed by the method of KingJ 1965 [b]

**Reagents**

1. Phosphate buffer: 100mM, pH 7.4

2. Substrate: 1.78 gm D L- Alanine and 38 mg of α ketoglutarate were dissolved in buffer. 0.5ml of 1N Sodium hydroxide was added by gentle heating. This is made upto 100 ml with buffer.

3. Dinitrophenyl hydrazine: 1mM dinitrophenyl hydrazine in 2N hydrochloric acid

4. Sodium hydroxide: 0.4N Solution
5. Standard pyruvate: 11mg of sodium pyruvate was dissolved in 100ml of phosphate buffer.

Procedure

In different tubes, 1ml of buffered substrate was added. 0.1ml of serum was added and incubated at 37°C for 30 minutes. Then 1ml of dinitrophenyl hydrazine reagent was added to arrest the reaction. To the blank, 0.1ml of serum was added after the addition of Dinitrophenyl hydrazine reagent. The tubes were kept aside for 15 minutes and then 10ml of Sodium hydroxide was added and read at 520nm in UV Spectrophotometer. The enzyme activity was expressed as IU/L.

Assay of Alkaline Phosphatase (EC.3.1.3.1)

ALP was assayed by the method of King J, 1965a.

Reagents

- Sodium carbonate – sodium bicarbonate buffer, pH10.
- Disodium phenyl phosphate
- 0.5N Sodium Hydroxide
- 20% Sodium carbonate
- Folin phenol’s Reagent
Procedure

In different tubes, 1ml of buffered substrate was added and preincubated for 37\(^\circ\)C for 5 minutes followed by 0.1ml of serum/ homogenate and incubated at 37\(^\circ\)C for 15 minutes. Then 0.8ml of NaOH was added to arrest the reaction. To the blank, 0.1ml of serum was added only after the addition of NaOH. The contents were centrifuged for 2 minutes. 1ml of supernatant was taken and made up to 4ml with distilled water. Blank comprises of 4ml of distilled water. 0.5ml of Folin’s Phenol reagent and 1.5 ml of Na\(_2\)CO\(_3\) were added to all the tubes including the blank and read at 640nm in UV spectrophotometer. The enzyme activity was expressed as IU/L.

Assay of Gamma Glutamyl Transpeptidase (EC 2.3.2.2)

The activity of gamma glutamyl transpeptidase was estimated according to the method of Orlowski, M. and Meister A., 1965.

Reagents

- 0.1M Tris – HCl buffer, pH 8.5
- Substrate (30.37 mg of L \(\gamma\) glutamyl p-nitroanilide was dissolved in 10ml of water by heating at 50–60\(^\circ\)C)
- Glycyl glycine (13.2 mg/10ml)
- p-Nitroaniline
Procedure

The incubation mixture contained 0.5ml of substrate, 1ml of Tris- HCl buffer 2.2ml of glycyl glycine, 0.2ml of serum and the total volume was made up to 4ml with distilled water. After incubation for 30min at 37°C, the samples were heated at 100°C for 5min and centrifuged. The amount of p–nitroaniline in the supernatant was measured at 410nm. The activity of gamma glutamyl transpeptidase was expressed as µ mole of p–nitroaniline formed/min/mg protein or IU/L.

Assay of Lipid peroxidation (LPO)

The level of lipid peroxides was assayed by the method of Ohkawa et al., 1979.

Reagents

- 8.1% Of Sodium dodecyl sulphate (SDS)
- 20% Acetic acid, pH 3.5 adjusted with NaOH
- 0.8% Thiobarbituric acid (TBA)
- n- Butanol/ pyridine mixture (15:1 v/v)
- 1,1,3,3- tetraethoxypropane

Procedure

To 0.2ml of homogenate, 0.2ml of SDS, 1.5ML of acetic acid and 1.5ml of TBA were added. The mixture was made up to 4ml with water and then heated in an oil bath at 90°C for 60 min. After cooling, 1 ml of water and 5ml of n-butanol/ pyridine mixture were added. After centrifugation at 4000 rpm for 10 min, the absorbance of organic layer was measured at 532 nm. The level of lipid peroxides was expressed as n moles of MDA formed/mg protein.
ENZYMATIC ANTIOXIDANTS

Assay of Superoxide Dismutase (SOD) (EC 1.15.1.1)

The activity of superoxide dismutase was determined by the method of Marklund and Marklund (1974).

Reagents

1. 0.1M Tris-HCl buffer, pH 8.2 containing 2 mM of diethylene triamine pentia acetic acid.
2. 0.05 M Tris – HCl buffer, pH 7.4.
3. Pyrogallol solution: 25.2 mg was dissolved in 1 ml of 0.05 M Tris-HCl buffer (pH 7.4) in an aluminium foil wrapped and stoppered test tube.
4. Pyrogallol working solution: At the time of assay, 0.5 ml was diluted to 50 ml with the 0.05 M Tris-HCl buffer, pH 7.4.
5. Absolute ethanol.
6. Chloroform

Procedure

To a known amount of tissue aliquot or serum, 0.25 ml of ethanol and 0.15 ml of chloroform were added. After 15 min of shaking in a mechanical shaker, the suspension was centrifuged and the supernatant obtained constituted the enzyme extract. The reaction mixture for auto – oxidation consisted of 2 ml of Tris-HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol and 1.5 ml of water. Initially the rate of auto – oxidation of pyrogallol was noted at an interval of 1 min to 3 min. The assay mixture for the enzyme contained 2 ml of the buffer, 0.5 ml of
pyrogallol, aliquots of the enzyme preparation and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted.

The enzyme activity was defined as the enzyme required for 50% inhibition of pyrogallol auto-oxidation (units/mg protein)

**Assay of catalase (EC 1.11.1.6)**

The activity of catalase was assayed by the method of Sinha (1972).

**Reagents**

1. 0.01 M phosphate buffer, pH 7.0
2. 0.2M H₂O₂
3. Stock dichromate / acetic acid solution. This reagent was prepared by mixing 5% solution of potassium dichromate with glacial acetic acid (1:3 by volumes)
4. Working dichromatic/acetic acid solution: The stock was diluted to 1:5 with water to prepare the working dichromatic/acetic acid solution.

**Procedure**

The assay mixture contained 0.5 ml of Hydrogen peroxide (H₂O₂), 1 ml buffer and 0.4 ml water. 0.2 ml of the diluted enzyme was added to initiate the reaction. 2 ml of the dichromate/acetic acid reagent was added after 15, 30, 45 and 60 sec of incubation. To the control tube, the enzyme was added after the addition of the acid reagent. The tubes were then heated for 10 min and colour developed was read at 610 nm. The activity of catalase is expressed as µmoles of H₂O₂ consumed/min/mg protein.
Assay of glutathione peroxidase (GPx) (EC 1.11.1.9)

The activity of GPx was assayed by the method of Rotruck et al., 1973)

Reagents

1. 0.32 M Phoshate buffer, pH 7.0
2. 0.8mM EDTA
3. 10 mM Sodium azide
4. 3 mM reduced glutathione
5. 2.5mM H₂O₂
6. 10% TCA
7. 0.3 M Disodium hydrogen phosphate
8. DTNB solution(40 mg of DTNB in 100 ml of 1% sodium citrate)
9. Reduced glutathione

Procedure

The reaction mixture consisted of 0.2 ml each of EDTA, sodium azide, H₂O₂, 0.4 ml of phosphate buffer, 0.1 ml homogenate serum and was incubated at 37°C and reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml DTNB were added and the colour developed was read at 420 nm immediately.

The activity of GPx was expressed as µ moles of glutathione oxidized/min/mg protein.
NON–ENZYMATIC ANTIOXIDANTS

Estimation of Reduced Glutathione (GSH)

The concentration of reduced glutathione was measured by the method of Moron et al. (1979)

**Reagents**

1. 10% TCA
2. 0.6 mM 5, 5’-dithiobis-2-nitrobenzonic acid (DTNB) in 0.2 M sodium phosphate
3. 0.2 M Phosphate buffer, pH 8.0

**Procedure**

1 ml of homogenate/serum was precipitated with 1 ml of TCA and the precipitate was removed by centrifugation. To 0.5 ml of supernatant, 2 ml of DTNB was added and the total volume was made up to 3 ml with phosphate buffer. The absorbance was read at 412 nm.

The level of glutathione was expressed as mg/mg protein.

Estimation of Ascorbic Acid

The concentration of ascorbic acid was estimated by the method of Omaye et al., 1979

**Reagents**

1. 5% TCA
2. 65% H$_2$SO$_4$
3. DNPH-Thiourea-CuSO₄ (DTC) reagent: 3 g DNPH, 0.4 g Thiourea and 0.05 g CuSO₄ was dissolved in 9N H₂SO₄ and made up to 100 ml with the same.

4. Standard: Ascorbic acid was made in 5% TCA in the range of 0 to 20 mg/ml.

**Procedure**

Aliquots of homogenate or serum precipitated with 5% ice cold TCA and centrifuged for 20 min at 3500 x g, 1 ml of the supernatant was mixed with 0.2ml of DTC and incubated for 3 hrs at 37°C. Then 1.5 ml of ice cold 65% H₂SO₄ was added, mixed well and the solution was allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520 nm. The concentration of ascorbic acid was expressed as mg/mg protein. The concentration of ascorbic acid in serum was expressed as mg/dl.

**Estimation of Vitamin E**

The level of vitamin E was estimated by the method of Desai, 1984.

**Reagents**

1. Ethanol
2. Petroleum ether
3. 0.2% bathophenanthroline in ethanol
4. 0.001 M Ferric chloride in ethanol
5. 0.001 M Ortho-phosphoric acid in ethanol
6. α-Tocopherol acetate
Procedure

To 1 ml of homogenate or serum, 1 ml of ethanol was added and thoroughly mixed. Then 3 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this 0.2 ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride was added followed by 0.2 ml of Ortho-phosphoric acid. The total volume was made up to 3 ml with ethanol. The colour developed was read at 530 nm. The concentration of vitamin E was expressed as mg/mg protein. The concentration of vitamin E in serum was expressed as mg/dl.

TUMOUR MARKER

Alpha fetoprotein (AFP)

AFP level in serum was estimated using ELISA kit supplied from Anogen (Mississauga, Ontario, Canada) following standard procedure provided in kit manual.

The level of AFP in serum was expressed as – 1U/ml.

HAEMATOLOGICAL PARAMETERS

Estimation of Haemoglobin (Hb)

Hb was measured by the method of Drabkin and Austin (1932)

Reagent

1. Drabkin’ Reagent
Dissolve 200 mg of potassium ferricyanide, 50 mg of KCN and 1 g of sodium bicarbonate in water and make up to a litre.

2. Cyanomethemoglobin Standard: 60 mg/ml (commercial)

**Procedure**

0.2ml of blood was diluted with 5 ml of Darbkin’s reagent. The diluted blood was mixed well and allowed to stand for 10 min to ensure the completion of the reaction. The solution was read at 540 nm together with standard solution of Cyanomethemoglobin prepared by mixing 0.2ml of standard with 5 ml of Darbkin’s reagent. The values were expressed as g/dl of blood.

**Enumeration of Red Blood Corpuscles**

The method involves an accurate dilution of a measured quantity of blood with a fluid which is isotonic with blood and which will prevent its coagulation. A dilution of 1 to 200 is usually necessary. The diluted blood is placed in a counting chamber and the number of cells in circumscribed volume in enumerated under a microscope (Chesbrough and Mc, Arthur, 1972)

**Materials Required**

1. Red cell pipette
2. Counting chamber: Neubaur counting chamber
3. Diluting fluid: A solution of 1% formalin in 31.3 g/L trisodium citrate
Procedure

Blood was drawn up to 0.5 mark in the RBC pipette. Then the diluting fluid was drawn up to 101 mark. After shaking this for 3 minutes the chamber was charged and the RBC was counted in Neubaur chamber. The values were expressed as m cells / cubic mm.

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were first introduced by Wintrobe in 1929 to define the size (MCV) and hemoglobin content (MCH, MCHC) of red blood cells. Red cell indices MCV, MCH and MCHC are calculated from hemoglobin, hematocrit, and red blood cell count as follows:

\[
MCV = \frac{\text{Volume of packed cells}}{1000 \text{ ml of blood}} \ (\text{fl or } \mu m^3)
\]

Red blood cell count in millions/ml

\[
MCH = \frac{\text{Hemoglobin in } g}{1000 \text{ ml of blood}} \ (\text{pg/cell})
\]

RBC count in millions/ml

\[
MCHC = \frac{\text{Hemoglobin in } g}{100 \text{ ml of blood} \times 100} \ (g/dl \ or \ %)
\]

Volume of packed cells/100ml of blood
Enumeration of White Blood Corpuscles

The cells were counted in a hemocytometer in the Neubaur chamber according to the method of Chesobrough and Mc. Arthur, 1972.

RESULTS AND DISCUSSION

Body weight, tumor incidence & Organ weight

All the five groups of wistar albino rats showing their body weight and tumor incidence is depicted in the (Table 2 and Figure1), Out of all the five groups the G2 group (ie) DEN induced liver cancer rats showed significant decrease in the body weight, when compared to the control rats (G1). In the treatment groups (G4) Solanum tuberosum treated rats showed marked increase in body weight compared to (G3) Ipomoea batatas treated rats and in (G5) rats which were treated with the standard drug Silymarin which showed slight increase in the body weight of the animals. The percentage of tumor incidence was markedly reduced in (G4) Solanum tuberosum treated rats, in case of (G3) Ipomoea batatas treated rats also showed reduced tumor incidence but when compared with (G4), it was not significant.

The present study demonstrated that administration of Ipomoea batatas and Solanum tuberosum has chemopreventive effect against DEN induced HCC in wistar albino rats. Both the plant extracts showed significant reduction in liver tumor incidence and tumor multiplicity and significant increase in body weight and organ weight.
Table 1 Experimental protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Normal rats</td>
<td>(n=6, the animals were given normal diet)</td>
</tr>
<tr>
<td>G2</td>
<td>Hepatocarcinoma induced rats</td>
<td>(n=6, the animals were given DEN 200 mg/kg bwt)</td>
</tr>
<tr>
<td>G3</td>
<td>Post-treated rats</td>
<td>(n=6, the animals were given DEN+ <em>Ipomoea batatas</em> 250mg/kg bwt)</td>
</tr>
<tr>
<td>G4</td>
<td>Post-treated rats</td>
<td>(n=6, the animals were given DEN + <em>Solanum tuberosum</em> 250 mg/kg bwt)</td>
</tr>
<tr>
<td>G5</td>
<td>Drug control rats</td>
<td>(n=6, the animals were given DEN+Silymarin 2.5 mg/kg bwt).</td>
</tr>
</tbody>
</table>

Table 2 Body Weight & Tumour Incidence in Experimental Animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>No of Animals in each group</th>
<th>Tumour incidence</th>
<th>Initial weight in gms</th>
<th>Final weight in gms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>6</td>
<td>Nil</td>
<td>180.8±9.08</td>
<td>190.3±3.4</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>6</td>
<td>6</td>
<td>182.8±8.05</td>
<td>146.2±5.6*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>6</td>
<td>1</td>
<td>181.8±7.43</td>
<td>165.2±4.5<em>a,b</em></td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>6</td>
<td>Nil</td>
<td>181.5±6.23</td>
<td>170.4±4.7<em>a,b</em></td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>6</td>
<td>Nil</td>
<td>180.1±4.54</td>
<td>171.2±3.9<em>a,b</em></td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD; n=6 animals,

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + *Ipomoea batatas* treated rats (250mg/kg b.w), Group IV- DEN+ *Solanum tuberosum* treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant.

a- as compared with Group I, b- as compared with Group II
Figure 1

Organ Weight in Experimental Animals

Liver
Pancreas
Kidney
Heart
Spleen
Brain

gms

GROUPS

a*
Values are expressed as mean ±SD; n=6 animals,

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + *Ipomoea batatas* treated rats (250mg/kg b.w), Group IV- DEN+ *Solanum tuberosum* treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant.

a- as compared with Group I, b- as compared with Group II
Bilirubin, Protein, Albumin and Globulin:

Table 3 represents the effect of plant extracts on the levels of Bilirubin and Protein in the serum of control and experimental rats. There was significant increase in the total bilirubin in DEN induced rats (G2) when compared to normal (G1) and it is decreased in (G3) *Ipomoea batatas* treated rats when compared to (G2), but it still remains higher when compared to (G1). (G4) *Solanum tuberosum* treated group showed significant decrease in bilirubin when compared to (G2). There was significant decrease in bilirubin in drug control group (G5) when compared to (G2), but in (G4) it is too near to normal when compared to (G1).

From the Table 3 it was found that there was significant decrease in the total protein level in DEN induced G2 group when compared to G1 control group. The level of protein was observed to get gradually increased in G3 *Ipomoea batatas* treated group, but there was marked increase in protein level in G4 *Solanum tuberosum* treated group, which was even near to the normal G1 group. The drug control group G5 also showed significant increase in the protein, compared to G2, G3, and G4 groups.

Table 3 shows the effect of the plant extract on albumin and globulin level in the control and experimental rats. The level of both albumin and globulin was found to be slightly (P < 0.001) increased in the DEN induced G2 group compared to control G1 group. The albumin was significantly (P < 0.001) increased in G3 *Ipomoea batatas*, G4 *Solanum tuberosum*, G5 drug control when compared to G2 animals.

Serum bilirubin is one of the most reliable tests employed in the diagnosis of hepatic diseases as it provides useful information on the functioning of the liver (Harper., 1961).
Bilirubin which is a chemical breakdown product of hemoglobin gets conjugated with glucuronic acid in hepatocytes to increase its water solubility. To evaluate chemically induced hepatic injury, Bilirubin concentration has been used as a reliable method. The breakdown product of hemoglobin namely bilirubin into bile is one of the normal function of the liver (Mahesh et al., 2010).

Althaf Faimum D and Sudaroli in 2012 studied the biochemical parameters of serum in control and treated animals. The group II (DEN+PB), group IV 200 mg/kg ethanolic extract of Vitex leucoxylon Linn (EVL), (p < 0.05) and group V 400 mg/kg ethanolic extract of Vitex leucoxylon Linn (EVL) (p < 0.001) rats exhibited significant increase in creatinine, serum bilirubin, and significant decrease in, total protein (p < 0.001). The group III (standard 5-FU) (p < 0.001) exhibited significant decrease in serum creatinine, serum bilirubin and significant increase in total protein.

Serum proteins have many functions, including the transport of other substances, immune defense, blood clotting, and inflammation defense. Serum protein levels are useful for evaluating nutritional status, infection, and various other disorders. Within the human body, albumin is an important component of life (Aiad, et al., 2004, Goyal and Soni 2011, Honarmand et al., 2011).

Arirudran et al., 2014, observed the change in total protein, albumin, globulin which are significantly decreased (p < 0.01) and A/G ratio is significantly increased (p < 0.001) in DEN induced HCC group II animals when compared with group I control animals. This significant decrease in total protein, albumin and globulin may be due to hepatotoxicity which leads to hepatocellular damage which in turn cause defective protein biosynthesis in liver. On treatment with ethyl acetate extract of R.tuberosa the level of globulin is significantly increased (p < 0.05)
and the level of total protein, albumin and A/G ratios are significantly increased (p < 0.001) in group III DEN + *R. tuberosa* (400mg/kg body weight) animals.

**Creatinine, Blood Urea, Cholesterol, Triglyceride and Blood Glucose**

*Table 4,* represents the changes in the creatinine, urea, Cholesterol, triglycerides and glucose in the serum of control and experimental rats. The biochemical parameters such as creatinine, urea, Cholesterol and triglycerides were significantly (P < 0.001) increased, and glucose was significantly to (P < 0.001) reduced in G2 DEN induced group when compared with G1 control group. The extract treated G3 (*Ipomoea batatas)* and G4 (*Solanum tuberosum*) treated groups showed marked decrease (P < 0.001) in creatinine, urea, Cholesterol and triglycerides when compared to G2 group, but G4 was more effective than G3. In G5 the creatinine and urea level were significantly decreased (P < 0.001). The blood glucose was found to be increased in both the extracts treated groups namely G3 *Ipomoea batatas* treated group and *Solanum tuberosum* treated group compared to the G2 DEN induced cancer rats.

It was found that creatinine levels significantly decreased in EPE treated rats compared with the DEN, control and EPE (aerial parts of *Echinacea purpurea* extract (EPE) groups almost completely suppressed. DEN induced the increases in creatinine levels (Annahita Rezaie *et al.*, 2013).

Hwang *et al.*, in 1990, carried out their experiments on Hepatotoxicity induced by diethylnitrosamine rats and observed a remarkable remarkable ability of homeostatic mechanisms to preserve normal plasma glucose and glucose tolerance in spite of dramatic
### Table 3 Levels of bilirubin, total protein, albumin and globulin in Serum of experimental animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Total bilirubin (mg/dl)</th>
<th>Total protein (g/dl)</th>
<th>Albumin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>0.57±0.04</td>
<td>6.35±0.31</td>
<td>4.28±0.35</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>1.8±0.10*</td>
<td>3.88±0.52</td>
<td>2.45±0.18*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>1.4±0.06a,b*</td>
<td>4.33±0.18a,b***</td>
<td>2.76±0.10a,b***</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>0.63±0.02aNS,b*</td>
<td>5.31±0.14a,b*</td>
<td>4.10±0.17aNS,b*</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>0.90±0.06a,b*</td>
<td>5.98±0.47a**<em>b</em></td>
<td>3.85±0.18a**,b*</td>
</tr>
</tbody>
</table>

**Values are expressed as mean ±SD; n=6 animals,**

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + *Ipomoea batatas* treated rats (250mg/kg b.w), Group IV- DEN+ *Solanum tuberosum* treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant.

a- as compared with Group I, b- as compared with Group II

### Table 4 Levels of creatinine, blood urea, blood glucose, cholesterol and triglycerides in experimental animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Creatinine (mg/dl)</th>
<th>Blood urea (mg/dl)</th>
<th>Blood glucose (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>0.50±0.01</td>
<td>41.3±1.4</td>
<td>115.33±10</td>
<td>87.6±7.3</td>
<td>67.30±1.33</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>1.81±0.17*</td>
<td>53.6±2.5a*</td>
<td>84.5±7.4*</td>
<td>120.9±6.0*</td>
<td>114.71±2.10a*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>1.59±0.10a,b*</td>
<td>46.9±1.3aNS,b*</td>
<td>97.41±6.03a,b***</td>
<td>101.0±2.3a,b*</td>
<td>104.09±0.95a,b*</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>0.60±0.02aNS,b*</td>
<td>42.5±1.4aNS,b*</td>
<td>108.5±3.3a**,b*</td>
<td>89.3±5.9aNS,b*</td>
<td>74.61±1.67a,b*</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>0.70±0.02a**,b*</td>
<td>45.7±2.9aNS,b*</td>
<td>103.5±4.3a**,b*</td>
<td>90.7±3.6aNS,b*</td>
<td>70.04±1.53a**,b*</td>
</tr>
</tbody>
</table>
changes in insulin and glucagon which means it showed no significant disturbances of systemic glucose homeostasis in rats.

The biochemical parameters of serum in control and treated animals were studied by Althaf Faimum D and Sudaroli in 2012. The group II (DEN+PB), group IV 200 mg/kg ethanolic extract of *Vitex leucoxyylon* Linn (EVL), (p < 0.05) and group V 400 mg/kg ethanolic extract of *Vitex leucoxyylon* Linn (EVL) (p < 0.001) rats exhibited significant increase in total cholesterol, TGL. The group III (standard 5-FU) (p<0.001) exhibited significant decrease in total cholesterol and TGL (Althaf Faimum D and Sudaroli in 2012).

**Liver marker enzymes**

Figure 2 demonstrates the effect of *Ipomoea batatas* and *Solanum tuberosum* extracts on the levels of Marker enzymes in the serum of control and experimental rats. The activities of SGOT, SGPT, ALP, γ – GT were increased significantly (P < 0.001) in the serum of DEN induced liver cancer rats (G2) compared to G1 control rats. The activities of SGOT, SGPT, GGT were decreased significantly (P < 0.001) in G4 (*Solanum tuberosum*), and in G3 (*Ipomoea batatas*) treated rats the activities of SGPT, GGT decreased significantly (P < 0.001) and SGOT, ALP were decreased significantly (P < 0.01) compared to G2 rats. In G4 *Solanum tuberosum* treated group the ALP level reached near normal. G5 drug control rats showed significant (P < 0.001) decrease in all the four marker enzymes activities such as SGOT, SGPT, ALP and γ – GT compared to G2.

The experiments on evaluating the chemopreventive effect of bark extracts of *Bombax malabaricum* against DEN induced hepatocellular carcinoma revealed that SGOT, SGPT, ALP
activities were increased in DEN induced cancerous rats whereas the treatment with the ethanolic and aqueous extract of *Bombax malabaricum* reverse these levels more are less to the normal level (Surender *et al.*, 2011).

**Lipid peroxides**

Table 5 represents the level of lipid peroxides in the liver tissue homogenate of control and experimental rats. There was significant (P < 0.001) increase of lipid peroxides in G2 group compared to G1 control group. Both G3 *Ipomoea batatas* and G4 *Solanum tuberosum* groups showed significant (P < 0.001) decrease in Lipid peroxides compared to G2. Drug control G5 and G4 *Solanum tuberosum* treated group showed more or less similarity to G1.

LPO has been suggested to be responsible for numerous deleterious effects observed in biological systems, especially after initiation, it concurrently proceeds by a free radicals reaction mechanism and it was regarded as one of the basic mechanism of cellular damage caused by free radical (Mori., 1994).

Naina Mohamed Pakkir Maideen *et al.*, 2011 determined the level of LPO in liver tissues of control and experimental animals. They found an increase in LPO in group II (p < 0.001) cancer bearing rat when compared to control animals. These significant effects were reversed in MPP (200 and 400 mg/kg) treated group III (200 mg/kg) methanol extract of *Phyllanthus polyphyllus* (MPP) and IV (400 mg/kg) methanol extract of *Phyllanthus polyphyllus* (MPP) (p < 0.001) on dose dependent manner.

Methanolic leaf extract of *Phyllanthus polyphllus* of Euphorbiaceae family when treated on various carcinoma cell lines like human breast cancer (MCF7), colon cancer (HT29) and liver cancer (HepG2) cell lines decreases the levels of lipid peroxides (Lpo), glutathione peroxidase
Table 5 Level of Lipid Peroxides in Liver Tissue of Experimental Animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>LPO(nm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>2.92±0.28</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>4.42±0.20*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>3.10±0.09aNS,b*</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>2.96+0.13aNS,b*</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>2.89±0.15aNS,b*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD; n=6 animals,

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + *Ipomoea batatas* treated rats (250mg/kg b.w), Group IV- DEN+ *Solanum tuberosum* treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant. 

a- as compared with Group I, b- as compared with Group II
(GPx), glutathione Stransferase (GST) and increases the levels of superoxide dismutase (SOD) and catalase (CAT) (Rajkapoor et al., 2007).

**Antioxidants Level in Liver Tissue**

The changes in enzymatic antioxidants such as superoxide, catalase both in serum & liver and glutathione peroxidase activity in serum of control and experimental rats were represented in the Table 6. All the antioxidants level were significantly (P < 0.001) reduced in the G2 animals when compared with G1 animals. Both *Ipomoea batatas* and *Solanum tuberosum* treated (G3 and G4) animals showed significant increase in all the enzyme activity compared with G2. Superoxide, catalase and glutathione peroxide activity in serum and SOD, Catalase activity in liver tissues increased with (P<0.001) significance, compared to G2. Glutathione peroxide increased with (P<0.05) significance and catalase with (P < 0.001) significance in G3 *Ipomoea batatas* treated group. In *Solanum tuberosum* treated G4 group and G5 Drug control group, all the four antioxidant level increased (P< 0.001) significantly, compared with G2 group.

The levels of catalase both in serum and liver tissue, and Glutathione peroxidase in serum reversed more or less to normal in G4, *Solanum tuberosum* treated group compared to G1 control rats. Benzo (a) pyrene induced experimental lung cancer in Swiss albino mice the Methanolic leaf extract of *Smilax zeylanica* belonging to Smilacaceae family showed increase in the activities of enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase) and non-enzymatic antioxidants (reduced glutathione, vitamin C and vitamin E) and decrease in the extent of lipid peroxidation levels (Venugopalan Rajesh and Perumal Perumal., 2013).
**Clerodendrum serratum** Verbenaceae Methanolic leaf extract in 7, 12- dimethylbenz [a] anthracene induced skin carcinogenesis in Swiss albino mice significantly curtailed tumor development and counteracted all the biochemical effects. It increased the body and testis weight, DNA, RNA, protein, glycogen, GSH level, SOD, CAT and GST activities. It decreased the cholesterol content, LDH, ACP activities and TBARS level (Jayaraj et al., 2012).

**Bauhinia variegata** Caesalpiniaceae Ethanolic extract N-nitrosodiethylamine induced experimental liver tumor in rats and human cancer cell lines, human epithelial larynx cancer (HEp2) and human breast cancer (HBL-100) cell lines. Suppressed levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin, gamma glutamate transpeptidase (GGTP), lipid peroxidase (LPO), glutathione peroxidase (GPx) and glutathione S-transferase (GST). Increases in enzymatic antioxidant (superoxide dismutase and catalase) levels and total proteins (Rajkapoor et al., 2006).

Supporting our findings with EGB, Naik and Panda (Naik et al., 2007) reported that EGB significantly increased the activities of free radical scavenging enzymes, SOD, GPx, GR and catalase and the non-enzymatic antioxidant, GSH in CCl4-treated rats compared with CCl4 treatment alone. Ginkgo biloba extract inhibits lipid peroxidation by scavenging free radicals and ROS maintaining the integrity and permeability of cell membranes thereby protecting cells and tissues against oxidative stress induced by the free radicals (Marcocci L and Packer L., 1994).
Non enzymatic antioxidants

Table 7, represents the changes in Non enzymatic antioxidants in serum of control and experimental rats. The non-enzymatic antioxidants such as vitamin C, vitamin E and reduced Glutathione were significantly (P < 0.001) reduced in G2 animals when compared with G1 animals. Both Ipomoea batatas and Solanum tuberosum treated (G3 and G4) groups showed increase in vitamin C, vitamin E and reduced glutathione when compared to G2 animals. G3, G4 and G5 showed (P < 0.001) significant increase of vitamin E, Vitamin C and reduced glutathione compared to G2.

The non-enzymatic antioxidants such as vitamin C and vitamin E in Liver tissue is represented in the table (9). Vitamin C and Vitamin E were significantly (P < 0.001) reduced in G2 animals when compared with G1 animals. Both Ipomoea batatas and Solanum tuberosum treated (G3 and G4) groups showed increase in vitamin C and vitamin E when compared to G2 animals. G4 showed (P < 0.001) significant increase of vitamin C and vitamin E, G3 showed (P < 0.01) significant increase of Vitamin C compared to G2. Drug control group G5 vitamin C & E, was too near to that of G1.

Balamurugan and J. Karthikeyan in (2012) observed that DEN induced treated group showed significantly decreased levels of Vitamin-C, Vitamin-E, GSH and MDA. The luteolin treated group possessed slightly elevated levels of non-enzymatic antioxidants than the control group-I untreated rats. The non enzymatic antioxidants in the DEN induced luteolin treated group IV rats were found to be similar to that of control (normal) rats (Balamurugan and Karthikeyan, 2012)
Table 6  Activities of Enzymatic Antioxidants in serum and liver tissues of experimental animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>In Serum of experimental animals</th>
<th>In Liver of experimental animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOD units/mg protein</td>
<td>CAT µmoles of H_{2}O_{2} utilised/min/mg protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Group I</td>
<td>6.26±0.22</td>
<td>30.2±1.21</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>3.19+0.07a*</td>
<td>11.3±0.60a*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>3.46+0.10a*,b***</td>
<td>20.2±1.6a*,b***</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>5.89+0.07a**,b***</td>
<td>29.6±0.9a**,b***</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>4.71+0.31a*,b*</td>
<td>21.5±1.2a*,b*</td>
</tr>
</tbody>
</table>

Table 7  Activities of Non-Enzymatic Antioxidants in serum and liver tissues of experimental animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>In Serum of experimental animals</th>
<th>In Liver of experimental animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vitamin C (mg/dl)</td>
<td>Vitamin E (mg/dl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Group I</td>
<td>1.91±0.17</td>
<td>1.87±0.01</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>0.89±0.05a*</td>
<td>1.01±0.02a*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>1.03±0.07a**,b***</td>
<td>1.24±0.01a*,b*</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>1.85±0.06a NS,b*</td>
<td>1.84±0.02a**,b**</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>1.5±0.04a*,b*</td>
<td>1.43±0.01a*,b*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD; n=6 animals,

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + Ipomoea batatas treated rats (250mg/kg b.w), Group IV- DEN+ Solanum tuberosum treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant.

a- as compared with Group I, b- as compared with Group II
Table 8, represents the changes in AFP level in the serum of control and experimental rats. AFP showed, significantly (P < 0.001) increase in G2 animals when compared with G1 animals, it showed tenfold increase compared to G1 animals. Both Ipomoea batatas and Solanum tuberosum treated (G3 and G4) groups showed significant (P < 0.001) decrease in AFP when compared to G2 animals. Drug control group G5, also showed (P < 0.001) decrease when compared to G2. But the effect of Solanum tuberosum was comparatively much effective than drug control group too.

AFP is a serum protein that is detected in elevated concentration in conditions like hepatocellular carcinoma. It is similar in size, structure and amino acid composition to serum albumin, but it is detectable only in minute amounts in the serum of normal adults. Elevated serum concentrations of this protein can be achieved in the adult by exposure to hepatocarcinogenic agents. AFP has the high specificity for hepatocarcinoma. Its serum concentration confirms hepatocarcinoma and the diagnosis of tumor response to therapy.

α-fetoprotein (AFP) an oncofetal serum protein, was observed to be progressively lost during development, such that it is virtually absent from the healthy adult (Seckin et al., 1993). It has long been identified that exposure of rats to certain carcinogens like DEN causes an elevation of circulating AFP levels (Sell et al., 1983). Carcino embryonic antigen (CEA), a member of the immunoglobulin supergene family, is a 180–200kDa heavily glycosylated protein used clinically as a tumor marker to detect recurrence of many types of tumors (Becker and Sell., 1979).
### Table 8  Levels of AFP in serum of experimental animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Alfa feto protein (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>35.5±3.05&lt;sup&gt;a*&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>16.23±0.9&lt;sup&gt;a*,b*&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>6.16±0.2&lt;sup&gt;a*,b*&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>8.1±0.3&lt;sup&gt;a*,b*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD; n=6 animals,

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + <i>Ipomoea batatas</i> treated rats (250mg/kg b.w), Group IV- DEN+ <i>Solanum tuberosum</i> treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant.

a- as compared with Group I, b- as compared with Group II
**Serum -Feto Protein** (AFP) in the serum of the control and experimental animals. The group II showed the highest level of AFP (2.86±0.18) IU/ml. The other groups III (200mg/kg) methanol extract of *Phyllanthus polyphyllus* (MPP) and IV (400 mg/kg) methanol extract of *Phyllanthus polyphyllus* (MPP) were significantly lowered AFP levels (p < 0.001) in dose dependent manner when compared to the cancer bearing animals of group II. Among the cancer treated animals, the groups IV (400 mg/kg) showed pronounced activity than group III (200 mg/kg). They suggested that MPP could reduce back the level of AFP in the blood of cancerous rats to near normal value (Naina Mohamed Pakkir Maideen *et al.*, 2011)

**Haematology**

Table 9, represents the changes in Haematological parameters in blood of control and experimental rats. There was significant (P < 0.001) decrease in RBC, Hb, HCT, MCV, MCH, MCHC levels in DEN induced G2 rats compared to G1 control rats. In case of WBC there was significant (P < 0.001) increase in G2 compared with G1 group. All the values were normal on treatment with *Ipomoea batatas* (G3), *Solanum tuberosum* (G4), and Drug control (G5) groups.

*Cleome gynandra* Cleomaceae Methanolic extract Swiss albino mice against Ehrlich Ascites Carcinoma Shows significant decrease in tumor volume, viable cell count, tumor weight and elevated the life span mice. RBC, hemoglobin, WBC and lymphocyte count reverted to normal level (Asis Bala *et al.*, 2010).

White blood cells, or leukocytes, play the main role in immune responses. These cells carry out the many tasks required to protect the body against disease-causing microbes and abnormal cells. Some types of leukocytes patrol the circulation, seeking foreign invaders and
Table 9  Haematological Parameters in experimental animals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>RBC (million cells/cu.mm) X10⁶ cells/dl</th>
<th>HB (g/dL) X10³ cells/dl</th>
<th>HCT (%)</th>
<th>MCV fL</th>
<th>MCH Pg</th>
<th>MCHC g/dL</th>
<th>WBC (thousand cells/cu.mm) X10³ cells/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>9.3±0.39</td>
<td>13.6±0.44</td>
<td>30.6±0.55</td>
<td>34.53±0.56</td>
<td>17.00±0.32</td>
<td>37.56±0.90</td>
<td>8.4±0.6</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>5.39±0.25 b*</td>
<td>9.4±0.58 a*</td>
<td>24.22±1.09 a*</td>
<td>18.35±0.50 a*</td>
<td>12.37±0.21 a*</td>
<td>23.28±1.3 a*</td>
<td>15.0±0.10 a*</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>6.7±0.3 a,b*</td>
<td>9.8±0.3 a,bN</td>
<td>28.5±1.7 a,b*</td>
<td>23.14±0.79 a,b</td>
<td>13.05±0.77 a,b</td>
<td>25.62±0.67 a,b</td>
<td>14.9±1.18 a,bNS</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>8.4±0.7 a,b*</td>
<td>12.3±0.7 a,b</td>
<td>29.5±1.13 a,bN</td>
<td>27.70±0.21 a,b</td>
<td>15.83±0.72 a,b</td>
<td>29.40±0.73 a,b</td>
<td>11.5±0.9 a,b*</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>7.39±0.55 a,b*</td>
<td>10.4±0.83 a,b</td>
<td>29.0±1.6 aNS,b</td>
<td>24.61±1.25 a,b</td>
<td>14.50±0.28 a,b</td>
<td>26.80±0.71 a,b</td>
<td>11.01±0.72 a,b*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD; n=6 animals,

Group I- Control rats, Group II- DEN induced liver cancer rats, Group III- DEN + *Ipomoea batatas* treated rats (250mg/kg b.w), Group IV- DEN+ *Solanum tuberosum* treated rats (250mg/kg b.w), Group V- Drug control rats

Statistical significance: *p<0.001, **p<0.01, ***p<0.05, NS- Non Significant.

a- as compared with Group I, b- as compared with Group II
**HISTOPATHOLOGICAL STUDIES OF LIVER**

(Magnification at 40X)

**Group 1**: Section shows normal liver tissue with hepatocytes, portal triad showing prominent central vein

**Group 2**: Section shows liver tissue with ballooning degeneration of the hepatocytes, nucleomegaly, nuclei with prominent nucleoli, kupffer cell activity, regular nuclear membrane and focal collection of inflammatory cells around portal triad with fibrosis.

**Group 3**: Section shows liver tissue with widening of portal triad, focal kupffer cell activity, prominent nucleoli and widening of sinusoids.

**Group 4**: Section shows liver tissue with mild central vein dilatation and mild inflammation.

**Group 5**: Section shows liver with mild inflammation, degeneration, congested sinusoids and kupffer cell activity.
Group 1: Section shows kidney with mild hypercellular glomeruli with normal PCT and DCT. Intersitium appears normal.

Group 2: Section shows mild hypercellular glomeruli, congestion and thick walled blood vessels.

Group 3: Section shows mild hypercellular glomeruli with congestion, and normal PCT and DCT.

Group 4: Section shows normal glomeruli with normal tubules.

Group 5: Section shows kidney with mild sclerosis of the glomeruli and congestion of the tubules.
HISTOPATHOLOGICAL STUDIES OF SPLEEN

(Magnification at 40X)

Group 1: Section shows spleen with thin capsule, prominent red and white pulp

Group 2: Section shows spleen with congestion.

Group 3: Section shows spleen with congestion.

Group 4: Section shows spleen with normal architecture.

Group 5: Section shows spleen with thinned out capsule, collection of histiocytes in the sinusoids and congestion of the red pulp
HISTOPATHOLOGICAL STUDIES OF HEART
(Magnification at 40X)

Group 1: Section shows cardiac myocytes with normal nucleoli, homogenous sarcoplasm

Group 2: Section shows heart with cardiac myocytes peripherally placed nuclei and mild inflammation

Group 3: Section shows heart with cardiac myocytes, intra muscular thick walled blood vessels and centrally placed plump oval nuclei

Group 4: Section shows cardiac muscle with normal thin walled blood vessels.

Group 5: Section shows heart with muscle fibres with diffuse inflammation and normal nuclei.
**HISTOPATHOLOGICAL STUDIES OF BRAIN**

(Magnification at 40X)

- **Group 1**: Section shows brain tissue with glial cells.
- **Group 2**: Section shows brain tissue with proliferation of neuroglial tissue, oedema, and prominent vessels.
- **Group 3**: Section shows brain tissue with reactive gliosis, perivascular oedema and congestion.
- **Group 4**: Section shows normal brain tissue.
- **Group 5**: Section shows brain tissue with reactive gliosis with oedema.
HISTOPATHOLOGICAL STUDIES OF PANCREAS

(Magnification at 40X)

Group 1: Section shows mainly islands of adipose tissue with mild lymphocytic infiltrate in the septae. No pancreatic duct and acini identified.

Group 2: Section shows pancreatic acini, congestion and normal islet cell population.

Group 3: Section shows lobules of pancreatic acini, dilated ducts, preserved islet cells and congested thick walled blood vessels.

Group 4: Section shows normal picture of pancreas

Group 5: Section shows normal pancreas with islet cell preservation and congestion.
diseased, damaged, or dead cells. These white blood cells provide a general—or nonspecific—level of immune protection (Bianchini and Wild., 1994, Lee et al., 2010)

The haematological parameters of blood collected on day of sacrifice in control and treated rats showed much significance. The group II rats (DEN+PB) exhibited significant decrease in Hb, RBC, differential WBC, lymphocytes, monocytes and PCV (p < 0.001) and showed significant increase in total WBC and ESR (p < 0.001). The group III (standard 5-FU) (p < 0.001), group IV 200 mg/kg ethanolic extract of Vitex leucoxylon Linn (EVL) (p < 0.05) and and group V 400 mg/kg ethanolic extract of Vitex leucoxylon Linn (EVL) (p < 0.001) exhibited significant increase in Hb, RBC, differential WBC, lymphocytes, monocytes and PCV, While WBC and ESR showed significant decrease (Althaf Faimum D and Sudaroli., 2012).


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language book society and Churchill Livingston; 145.


Chapter 2


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INTRODUCTION

Medicinal plants are of great importance to the health of individuals and communities (Hill AF., 1952). Koduru et al., in 2006 stated that in developing countries, people mostly rely on traditional herbal medicines, to meet their primary health care needs. Herbal medicines have gained popularity in many industrialized countries as alternative and complimentary therapies. Some of the plants were used as food or medicine. A broad range of biological and pharmacological activities were observed to be exhibited in these plants possessing effects such as anti-cancer, anti-inflammatory, diuretic, laxative, antispasmodic, antihypertensive, anti-diabetic, and anti-microbial functions. Hill AF in 1952 year stated that chemical substances present in the plants were responsible for their medicinal properties that produce a definite physiological action on the human body.

Plants are a rich source of secondary metabolites with interesting biological activities. In general, secondary metabolites play a key role as a source with a variety of structural arrangements and properties (de-Fatima et al., 2006). A number of biological active products which has been used extensively as drugs, foods, flavors, insecticides, fragrances, additives, colorants, and chemicals were provided by the secondary metabolites of plants for mankind (Koduru et al., 2006). Examples for secondary metabolites include compounds like flavonoids, phenols, phenolic glycosides, saponins and cyanogenic glycosides (Shahidi et al., 2008). Numerous plant derived therapeutic agents obtained from the medicinal plants have been used in our modern medicine now days. A number of varieties of phyto pharmaceuticals were found to possess very important applications in the fields of agriculture, human and veterinary medicine. Thus a dominant role in the development of novel drug leads for the
treatment and prevention of diseases were obtained from natural plant products. (Shahidi et al., 2008, Baliga MS et al., 2003, Wagner and Ulrich-Merzenich, 2009).

Natural products namely plant extracts, either as pure compounds or as standardized extracts provide unlimited opportunities for new drug discovery due to the availability of chemical diversity (Cosa et al., 2006). Most bioactive compounds of natural origin are secondary metabolites. A typical protocol to isolate a pure chemical agent from natural origin is bioassay-guided fractionation, meaning step-by-step separation of extracted compounds based on differences in their physicochemical properties and assessing the biological activity.

The crude plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. The separation and determination of active components in the plant extracts provide solutions in studying their pharmacological properties. Mostly the isolation of these bioactive compounds are performed by different separation techniques such as TLC, column chromatography, Sephadex chromatography and HPLC, to obtain pure compounds. The pure compounds are then used for the structural determination and biological activity.

Isolation of pure, pharmacologically bioactive constituents from plants remains a long and tedious process. Peter (2004) stated that it is necessary to have methods available for efficient separation from plant extracts, which are typically mixtures of thousands of different molecules. There are several ways to identify these molecules from these extracts. The common approach is to set up a fractionation scheme and to screen the fractions for the presence of the desired bioactive properties. Active fractions are subfractionated and tested, until the molecules responsible for the bioactivity could be identified. Column
chromatography and Thin-layer chromatography (TLC) are the simplest and cheapest method of detecting plant constituents as it is easy to run, reproducible and requires little equipment (Jothy et al., 2011, Devi and Thangam., 2010, Battu and Reddy., 2009, Prachayasittikul et al., 2009).

Column chromatography is one of the most frequently used techniques in isolation of natural constituents. In principle, plant constituents are distributed between the solid phase (for example: silica gel, alumina, sephadex) mobile phase, which comprises an eluting solvent. In silica gel, the separation of compounds from each other in an extract is based on a number of factors including the polarity of compounds; hence compounds were eluted from the column with solvent systems of differing polarity. Silica gel constitutes polar ends which interact strongly with polar compounds and they are eluted from the column.

TLC was used to support the identity of a compound in a plant extract when the $R_f$ of a compound is compared with the $R_f$ of a known compound. Other tests involve the spraying of phytochemical screening reagents, which cause color changes according to the phytochemicals existing in plants extract or by viewing the plate under the UV light. This has also been used for confirmation of purity and identity of isolated compounds.

Cell death is of two types namely apoptosis and necrosis. Apoptosis is described as an active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation. Necrosis is characterized as passive, accidental cell death with uncontrolled release of inflammatory cellular contents. The term apoptosis was proposed by Kerr colleagues et al., in 1972. Apoptosis is considered to be a regulated and controlled process. It is characterised by a series of typical morphological events, such as shrinkage of the cell,
fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells (Kerr et al., 1972). The occurrence of apoptosis in tissues is typically a rare event, under physiological conditions. Only a small number of apoptotic cells could be seen at a time. Lazebnik et al., (1993) and Solary et al., (1993) stated that researches with cytosolic extracts of cells, have been induced to study the process of apoptosis, and it was observed that it occur in a synchronous manner. They showed that apoptosis could be divided into biochemically and morphologically distinct phases (Lazebnik et al., (1993) and Solary et al., 1993). The onset of apoptosis was characterised by shrinkage of the cell and the nucleus as well as condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membranes. Finally it results in breaking up the nucleus (karyorrhexis) Kerr et al., (1972) and Kerr et al., 1994.

**Objective**

The aim of the present study was to perform bioactivity guided fractionation of active principle from the tubers of *Ipomoea batatas* and *Solanum tuberosum* and to study the anticancer activity using HepG2 cell lines (liver cancer cells). There was very good anti hepatocarcinogenic effect in rat model as discussed in chapter 2, in order to identify the actual compound responsible for this property further studies were carried out. The bioactivity guided fractionation and anticancer effect on cell lines was investigated.
MATERIALS AND METHODS

Preparation of the Extract

Crude aqueous extract of *Ipomoea batatas* and ethyl acetate extract of *Solanum tuberosum* was prepared and subjected to column chromatography.

Column chromatography

The extract was subjected to column chromatography using different solvent systems. The fractions collected were pooled according to the polarity of the eluted solvent. Silica gel (100–200 mesh) was used as stationary phase. Column chromatography was done by using a glass column. The height of the column was 50 cm. The column was packed with silica gel by wet packing method where a padding of cotton was placed at the bottom of the column. Slurry was made with the required amount of stationary phase (silica gel) in the solvent of slowest polarity (n-hexane) and poured into the column to form a bed of silica. The extract was made into admixture (1:3 ratio) of silica gel (100–200 mesh) then poured on to the top of silica gel making a slurry, a layer of cotton was covered again and allowed to percolate. The column was then eluted gradiently with solvents of increasing polarity. The fractions were collected and the solvent was recovered by simple distillation. All the concentrated fractions were subjected to TLC and similar fractions were combined. Totally 5 fractions were obtained after pooling based on TLC patterns of the extracts.
Anticancer activity of fractions

All the 5 fractions of each plant extracts were subjected to cytotoxicity assays by MTT assay using HepG2 cell lines (Liver cancer cell lines) to identify the best active fraction which had the highest anticancer effect.

MTT Assay for cell viability

Reagents

Minimal Essential Medium (MEM)

Fetal bovine serum (FBS)

Trypsin

Methylthiazolyl Diphenyl- Tetrazolium bromide (MTT)

Dimethyl sulfoxide (DMSO) were purchased from Hi media & Sigma Aldrich Mumbai.

Maintenance of cell lines

HepG2 cell lines were procured from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented (MEM) with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 50 μg/ml CO₂ at 37°C.
MTT Assay for cell viability

The MTT assay (Mossman, 1983) is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. This assay was performed on Human liver cancer cell lines (HepG2).

The cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2 X 10^4 cells/ well and allowed to attach overnight at 37°C. The medium was then discarded and cells were incubated with different concentrations of all the 5 fractions for 24 hrs. After the incubation, medium was discarded and 100 µl fresh medium was added with 10µl of MTT (5mg/ml). After 4 hrs, the medium was discarded and 100 µl of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570nm in a microtitre plate reader. Cell survival was calculated by the following formula:

Viability % = (Test OD/Control OD) x100

Cytotoxicity % = 100 – Viability %

Determination of bioactive constituents of the active fractions

GC-MS Analysis

GC-MS technique was used in this study to identify the phyto components present in *I. batatas* active Fraction III (ethylacetate ethanolic) and fraction V (ethanolic) of *Solanum tuberosum*. GC-MS analysis of the two fractions of the extracts was performed using a Perkin-Elmer GC Clarus 680 system and gas chromatograph interfaced to a mass spectrometer (clarus 600) equipped with an Elite-5 MS, fused silica capillary column (30 mm × 0.25 mm 1D × 250 µm df, composed of 100% dimethyl polysiloxane). For GC-MS
detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate of 1 ml/minute and an injection volume of 1 μl was employed (split ratio of 10:1); injector temperature 250°C; Ion-source temperature 230°C. The oven temperature was programmed from 60°C (isothermal for 2 minutes), with an increase of 10°C/minute to 200°C, then 250°C/minute, ending with a 6 minutes isothermal at 300°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 50 to 600 Da. Solvent delay = 2.00 minutes, transfer temperature = 230°C, source temperature = 230°C, total GC running time was 32 minutes. The relative % of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a GC-MS solution ver. 2.53.

**Identification of components**

Interpretation of the mass spectrum GC-MS was done using the database of National Institute Standard and Technique (NIST08s), WILEY8 having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 library. The name, molecular weight, molecular formula, and structure of the component were ascertained.

**High performance thin layer chromatography (HPTLC) fingerprint profile of *I. batatas***

The standardization of plant extract is an integral part of establishing its correct identity. The results of this investigation could therefore, serve as a basis for proper identification of the plant. Chromatographic fingerprint profile of Fraction III of *Ipomoea batatas* and Fraction V of *Solanum tuberosum* were studied by HPTLC.
Instrumentation

A Camag HPTLC system (Muttenz, Switzerland) equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner 2, CATS Software and Hamilton (Reno, Nevada, USA) syringe (100 μl).

Chromatographic conditions

Sample Preparation and Application

5 mg/ml concentration of extracts were prepared in respective solvents of chromatographic grade and then filtered by whatman filter paper No. 1. Prepared samples of extracts were applied on TLC aluminium sheets silica gel 60 F 254 (E. Merk Ltd., Darmsladt, Germany) 07 μl each with band length of 5 mm using Linomat 5 sample applicator set at a speed of 150 nl/sec, with the help of Hamilton Microsyringe (Switzerland), mounted on a Linomat V applicator, at the distance of 15 mm from the edge of the plates. A number of solvent systems were tried, for each extract for better resolution and maximum number of spots, and the satisfactory resolution was obtained in the solvent Toluene: Ethyl acetate (3:7v/v). The plates were developed up to a distance of 80 mm (distance to the lower edged was 10 mm) and chromatography was performed at room temperature in a camag twin trough chamber, previously equilibrated with mobile phase for 20 minutes.

The air dried plates were viewed in ultraviolet radiation to mid-day light. Spots were visible without derivatization at 254 and 366 nm Scanning was performed by CAMAG HPTLC Densitometer (Scanner 3) in absorbance mode at both 254 and 366 nm, the extracts were also scanned at 350-600 nm using deuterium and tungsten lamp with slit dimension 6.0
X 0.45. The Rf values and colour of the resolved bands were noted.

RESULTS AND DISCUSSION

As discussed in chapter II since both the plant extracts namely *Ipomoea batatas* and *Solanum tuberosum* showed anticancer effect on the DEN induced hepatocellular carcinoma rat model, it provoked our interest to identify the exact anti hepatocarcinomic compound present in the extracts, so we carried on further studies with fractionation using column, and GCMS chromatographic techniques. The results are discussed below.

**Fractionation of Aqueous extract of *Ipomoea batatas* by Column Chromatography**

Totally 75 fractions of 50 ml each was eluted using various solvents in the order of increasing polarity. The fractions were subjected to TLC to combine the similar fractions and finally 5 fractions were obtained as follows Table 1.

**Fraction I**

The fraction 1 to 15 of column (100% hexane + 75% hexane: 25% ethyl acetate + 50% hexane: 50% ethyl acetate) showed similar TLC profiles, then it was pooled and concentrated to form a light yellow paste.

**Fraction II**

The fraction 16 to 25 of column (25% hexane: 75% Ethyl acetate + Ethyl acetate100%) showed similar TLC profile, on concentration it formed light yellowish brown paste.
Fraction III

The fraction 26 to 43 of column (Ethyl acetate75%: Ethanol 25% + Ethyl acetate 50% : Ethanol 50% + Ethyl acetate 25% : Ethanol75% + Ethanol 100%), on concentration formed brown paste.

Fraction IV

The fraction 44 to 64 of column (Methanol 100%), on concentration formed blackish brown paste.

Fraction V

The fraction 65 to 75 of column (Aqueous100%), on concentration formed black paste.

After performing column chromatography we got 75 fractions. Then by performing TLC the fractions were pooled into 5 Active Fractions. 87.4% of total extract was obtained and 12.6% of extract was lost by binding into silica & collecting tubes.

Fractionation of Ethyl acetate extract of Solanum tuberosum by Column Chromatography

Totally 70 fractions of 50ml each was eluted using various solvents in the order of increasing polarity. The fractions were subjected to TLC to combine the similar fractions and finally 5 fractions were obtained as follows Table 2.
### Table 1: Percentage yield of different fractions of *Ipomoea batatas*- aqueous extract

*(Total weight of extract – 50g)*

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Solvent</th>
<th>Obtained weight of fractions</th>
<th>Percentage of yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>Hexane:ethyl acetate</td>
<td>2000mg</td>
<td>9.2.0%</td>
</tr>
<tr>
<td>Fraction II</td>
<td>Ethyl acetate, ethylacetate: ethanol</td>
<td>4300mg</td>
<td>13.8.6%</td>
</tr>
<tr>
<td>Fraction III</td>
<td>Ethyl acetate: ethanol</td>
<td>5700mg</td>
<td>11.4%</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>Methanol</td>
<td>6500mg</td>
<td>13%</td>
</tr>
<tr>
<td>Fraction V</td>
<td>Distilled water</td>
<td>20,000mg</td>
<td>40%</td>
</tr>
</tbody>
</table>

87.4% of total extract was obtained and 12.6% of extract was lost by binding into silica and collection tubes.

### Table 2: Percentage yield of different fractions of *Solanum tuberosum*- ethylacetate fraction

*(Total Weight of Extract – 50g)*

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Solvent</th>
<th>Obtained weight of fractions</th>
<th>Percentage of yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>Hexane, Hexane: Ethyl acetate</td>
<td>4350mg</td>
<td>18.41%</td>
</tr>
<tr>
<td>Fraction II</td>
<td>Hexane:Ethyl acetate, Ethyl acetate</td>
<td>4800mg</td>
<td>13.6%</td>
</tr>
<tr>
<td>Fraction III</td>
<td>Ethyl acetate: Ethanol</td>
<td>5800mg</td>
<td>16.6%</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>Ethyl acetate: Ethanol</td>
<td>8500mg</td>
<td>24.6%</td>
</tr>
<tr>
<td>Fraction V</td>
<td>Ethanol</td>
<td>6500mg</td>
<td>16.4%</td>
</tr>
</tbody>
</table>

89.6% of total extract was obtained and 10.4% of extract was lost by binding into silica and collection tubes.
Fraction I

The fraction 1 to 37 of column (100% hexane + 75% hexane : 25% ethyl acetate + 50% hexane : 50% ethyl acetate) showed similar TLC profiles, then it was pooled and concentrated to form a light brown paste.

Fraction II

The fraction 38 to 50 of column (50% hexane : 50% ethyl acetate + hexane 25% : ethyl acetate 75% + ethyl acetate 100%) showed similar TLC profile and on concentration it formed brown paste.

Fraction III

The fraction 51 to 60 of column (ethyl acetate 75% : ethanol 25% + ethyl acetate 50% : ethanol 50%), on concentration formed dark brown paste.

Fraction IV

The fraction 61 to 64 of column (ethyl acetate 25% : ethanol 75%), on concentration formed blackish brown paste.

Fraction V

The fraction 65 to 70 of column (ethanol 100%), on concentration it formed black paste.

After performing column chromatography we got 70 fractions. Then by performing TLC Chromatography the fractions were pooled into Fraction I, II, III, IV, and V.
89.6% of total extract was obtained and 10.4% of extract was lost by binding into silica and collection tubes.

**MTT Assay**

Cytotoxicity and proliferation profiles were determined by MTT (3-[4,5-dimethylthioazol-2-yl]-2,5-diphenyltetrazolium bromide) assay whereby cell viability of treated cell population was compared to the control cell population. The cell viability was determined by the formation of blue formazan crystals of MTT in which the crystals were dissolved in DMSO and the absorbance was read with Elisa reader at 570nm and references wavelength of 630 nm. Human tumor cell lines serves as models for pre clinical drug screening. It allows estimating the potential activity of a drug in a certain tumor type by taking the preclinical IC50 value (Voigt *et al.*, 2000).

Lovy *et al* in 1999 stated that, he worked on four human cancer cell lines which included HL-60 (human promyelocytic leukemia), MCF-7 (human breast cancer), HT-29 (human colon cancer), and HeLa (human cervical cancer) cell lines, and tested against crude extracts and pure compounds isolated from edible mushroom. The cytotoxic index used was IC50, which is the concentration that yields 50% inhibition of the treated cell compared with untreated control. Extracts that show IC50 < 30 µg/mL are considered to have significant cytotoxic activity whilst pure compounds that show cytotoxicity IC50 < 20 µg/mL are qualified for further cytotoxicity investigations (Lovy *et al.*, 1999).

The cytotoxicity of Solanum Nigrum on HeLa cell was evaluated by MTT assay. Solanum Nigrum methanolic extract has significant cytotoxicity effect on HeLa Cell Line in concentration range between 10 mg/ml to 0.0196 mg/ml by using MTT assay. The IC50 value of Solanum Nigrum on HeLa cell was 265.0mg/ml by MTT (Sanjay Patel *et al.*, 2009). IC50
value of Solanum Nigrum on Vero cell was 6.862 by MTT assay. Thus Solanum Nigrum showed anticancer activity.

The antiproliferative property of Solanum tuberosum extract was determined using MTT assay using HepG2 and normal 3T3 cell lines. Solanum tuberosum extract was able to exert antiproliferative effect on HepG2 cell line in dose dependent manner. The results showed that HepG2 cell lines treated with 100μg/ml of Solanum tuberosum extract showed 62% cytotoxicity and 37% viability. In non malignant 3T3 cells (Fibroblast) treated with 100μg/ml of Solanum tuberosum still retained >50% viability, (68% viability) (Nazareth Arockiamary and Vijayalakshmi., 2014).

All the ten chromatographic fractions and of Ipomoea batatas and Solanum tuberosum were examined for cytotoxicity properties on HepG2 cells. All the fractions showed dose dependent cytotoxic activity by killing liver cancer cells. Among the five fractions (I, II, III, IV and V) of Ipomoea batatas, fraction IV and fraction V showed greater cytotoxicity than the remaining 3 fractions. At IC50 concentration of 29.5 μg/ml fraction IV (ethylacetate: ethanolic fraction) of Ipomoea batatas showed marked cytotoxicity on Hep G2 cells. In the same way five fractions (I, II, III, IV and V) of Solanum tuberosum when subjected to MTT assay proved to show cytotoxicity towards the Hep G2 cells. Out of all the five fraction of Solanum tuberosum, Fraction V showed significant cytotoxic effect on Hep G2 cells and its IC50 concentration was 28.08 μg/ml (Table 3 and 4, Figure 3.1 to 3.5, 4.1 to 4.5). Thus Fraction V was found to be the best among rest of the four fractions namely (I, II, III, and IV).
Table 3: Effect of *Ipomoea batatas* on human Liver cancer cell lines (HEP-G2)

**MTT Cytotoxicity Assay**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Concentration (µg/mL)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>83.23±0.60</td>
<td>63.51±0.54</td>
<td>93.4±0.85</td>
<td>76.55±0.64</td>
<td>87.66±0.56</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>67.9±0.98</td>
<td>58.84±0.52</td>
<td>84.3±0.74</td>
<td>7.53±1.2</td>
<td>83.34±0.14</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>54.78±0.46</td>
<td>55.56±0.43</td>
<td>80.07±54</td>
<td>68.41±1.2</td>
<td>78.76±0.42</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
<td>43.75±0.36</td>
<td>47±0.36</td>
<td>72.69±0.54</td>
<td>54.44±0.42</td>
<td>66.19±0.46</td>
</tr>
<tr>
<td>5</td>
<td>62.5</td>
<td>35.43±0.16</td>
<td>42.87±0.33</td>
<td>65.86±0.56</td>
<td>51.36±0.24</td>
<td>61.06±0.51</td>
</tr>
<tr>
<td>6</td>
<td>31.25</td>
<td>28.1±0.12</td>
<td>36.66±0.25</td>
<td>52.51±0.32</td>
<td>46.54±0.14</td>
<td>39.24±0.84</td>
</tr>
<tr>
<td>7</td>
<td>15.625</td>
<td>20.39±0.11</td>
<td>33.19±0.2</td>
<td>33.5±0.22</td>
<td>30.45±0.42</td>
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<tr>
<td>8</td>
<td>7.8125</td>
<td>16.63±0.1</td>
<td>25.44±0.13</td>
<td>28.5±0.12</td>
<td>24.15±0.57</td>
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<tr>
<td>9</td>
<td>Cell control</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>100</td>
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</table>
Figure 3.1: Fraction III- 1000ug

Figure 3.2 Fraction III- 500ug

Figure 3.3 Fraction III- 250ug

Figure 3.4 Fraction III- 62.5ug

Figure 3.5 Fraction III- 31.25ug
Table 4: Effect of *Solanum tuberosum* on human Liver cancer cell lines (HepG2)-

MTT Cytotoxicity assay

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration µg/ml</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
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<td>52.1±0.42</td>
<td>34.3±0.25</td>
<td>73.4±0.15</td>
<td>84.4±0.62</td>
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<tr>
<td>3</td>
<td>250</td>
<td>57.4±0.61</td>
<td>38.6±0.21</td>
<td>56.3±0.42</td>
<td>64.5±0.12</td>
<td>74.6±0.54</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
<td>54.1±0.84</td>
<td>31.3±0.24</td>
<td>51±0.24</td>
<td>44.8±0.36</td>
<td>64.3±0.31</td>
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</tr>
<tr>
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<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 4.1  Fraction V - 1000ug

Figure 4.2  Fraction V - 500ug

Figure 4.3  Fraction V - 250ug

Figure 4.4  Fraction V - 62.5ug

Figure 4.5  Fraction V - 31.2ug