In Indian system of medicine certain herbs are claimed to provide relief against liver disorders. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. One of the most versatile plant *Sphaeranthus indicus*, a member of the Asteraceae family was taken for anticancer evaluation on human liver cancer cell lines in-vitro and diethylnitrosamine (DEN) induced hepatocellular carcinoma in mice in-vivo.

### 5.1 Phytochemical screening of plant extracts

All the parts of the plant of *S. indicus* (leaves, flowers, stems and roots) were subjected to preliminary phytochemical analysis. There are many solvents of use in plant extraction (methanol, ethanol, chloroform, petroleum ether, and water). Of the four plant parts, the methanolic and aqueous extracts of *Sphaeranthus indicus* leaves revealed the presence of saponins, phenolic compounds, tannins, flavanoids and alkaloids. Our phytochemical screening revealed that both aqueous and ethanolic extracts contained secondary metabolites such as alkaloids, flavanoids, saponins, tannins, steroids, reducing sugars and phenolic compounds. However, higher tannin content was found in the methanolic extract than aqueous extract (Thamilmaraiselvi *et al.*, 2011).

According to Varadarajan *et al.* (2008), the secondary metabolites (phytochemicals) and other chemical constituents of medicinal plants account for their medicinal value. Saponins serve as natural antibiotics which help body
to fight infections and microbial invasions. They also enhance the effectiveness of certain vaccines, lower cholesterol and knock out some tumor cells, particularly lung and blood cancers (Okwu and Ndu, 2006). Some researchers have also reported that some saponins have anti-cancer and immune-modulatory properties (Kunle and Egharevba, 2009). The antioxidant activity of the extracts may be attributed to the presence of the identified phytochemicals. Flavonoids and tannins are phenolic compounds, and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Potterat, 1997). Current research has shown that polyphenols contribute to the prevention of cardiovascular diseases, cancers, osteoporosis and antioxidant character with potential health benefits (Arts and Hollman, 2005; Lambert et al., 2005 and Joseph et al., 2005). Similarly, terpenoids, act as regulators of metabolism and play a protective role as antioxidants (Soetan, 2008). A sesquiterpene lactone, 7-hydroxyfrullanolide isolated from *S. indicus* had antimicrobial activity (Atta-Ur-Rahman et al., 1989 and Perumalsamy et al., 1999). Sesquiterpene lactones from *Vernonia colorata*, possessed high antibacterial activity primarily against Gram positive and low activity against Gram negative species (Rabe et al., 2002).

The antioxidant property of the extracts may be a strong contributing factor to the applications of the plants in the management and treatment of various diseases. Antioxidants prevent oxidative stress, caused by free radicals, which damage cells and vital biomolecules. They terminate chain reactions triggered by free radicals by removing free radical intermediates and inhibit other oxidation reactions (Sies 1997).

Many medicinal plants exhibit antimicrobial activity by mechanisms of action characterized by cell membrane lyses and inhibition of protein synthesis,
proteolysis enzymes and microbial adhesions (Cowan, 1999). The medicinal value of plants lies in their constituent chemical substances that produce definite physiological actions on the human body (Iniaghe et al., 2009). The presence of some of these secondary metabolites suggests that the plant might be of medicinal importance and supports the bases for some of the ethno-uses. For instance, the presence of flavonoids suggest that the plant might have an antioxidant, anti-inflammatory, anti-microbial, anticancer activity. The plant might have Eudesmanolide type of sesquiterpene and was reported to have immunostimulating activity. It is reported to contain eudesmanoids, eudesmanolides, sesquiterpene lactone, sterol glycoside, flavanoids, and essential oil. Thamilmaraiseli et al. (2011), reported that the aqueous extract of the leaves of Clerodendrum phlomidis contains most of the phytochemicals and therefore it was found to be very effective against both Gram positive and Gram negative bacteria.

5.2 Pharmacognostical studies of plant extracts

Mean ash values (%) of S. indicus methanolic and aqueous extracts of leaves and flower was found to be relatively low, which may be due to low content of carbonates, phosphates, silicates and silica. Ash value is useful in determining authenticity and purity of drug and also these values are important quantitative standards. Ash values are helpful in determining the quality and purity of crude drugs in powder form. The percentage yield of the ethanolic extract and ethanol-extractable matter were greater than the percentage yield of aqueous extract and water-extractable matter. A higher content of total ash, acid-insoluble ash and water-soluble ash were found in the . Our phytochemical screening reveals the leaves of S.indicus contained secondary metabolites such as alkaloids, flavanoids, saponins, tannins, steroids, reducing sugars and
phenolic compounds. However, higher tannin content was found in the methanolic extract than aqueous extract.

The extractive values are useful in evaluating the constituent of crude drugs. It also indicates the nature of the constituents present in the drug. The percentage yield of the aqueous extract and water-extractable matter were greater than the percentage yield of alcohol extract and alcohol-extractable matter. Extractive value was highest in aqueous extract than alcohol extract indicating the possibility of considerable amount of polar compounds in *S. indicus* leaves and flower.

Fluorescence is an important phenomenon exhibited by various chemical constituents present in plant material. Some constituents show fluorescence in the visible range in day light. The ultra violet light produces fluorescence in many natural products (e.g. alkaloids like berberine), which do not visibly fluoresce in day light. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents hence some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation (Ansari, 2006). The pharmacognostical studies confirm the therapeutic value of *S. indicus* Linn.

The GC and MS running time for dried methanolic extracts of the dry leaves of *Sphaeranthus indicus* was 36 min. The total numbers of compounds identified in dry leaves methanolic extracts were 115. The GC-MS retention time (RT) and percentage peak of the individual compounds were presented in the Table 9. The plant is reported to contain deep cherry colored essential oil having methyl chavicol, d-cadinene, α-ionone, p-methoxycinnamaldehyde, α-terpinene, citral, geraniol, geranyl acetate, β-ionone, oscimene, eugenol,
sphaeranthene, sphaeranol, estragole, Indicusene (Baslas, 1959; Lodha, 2003). A novel isoflavone glycoside, 5,4′-dimethoxy-3′-prenylbiochanin 7-O-β-d-galactoside, has also been isolated from leaves (Yadava and Kumar, 1999).

Eudesmenolide type of sesquiterpene glycoside, sphaerananolide, with immunostimulant potential has been isolated from the flowers of *S. indicus* (Shekhani et al, 1990). Eudesmenolides such as frullanolide, 11-alpha-13-dihydro: 3, alpha-7-alpha-dihydroxy: frullanolide, 11-alpha-13-dihydro (Shekhani et al., 1991) and two sphaeranthus peptide alkaloids have been isolated from flowers (Chughtai et al., 1992). The alcoholic extract of powdered caputula contains stigmasterol and β-sitosterol (Gupta et al., 1967). A flavone glycoside, 7-hydroxy-3′,4′, 5, 6-tetramethoxy-flavone 7-O-β-d-(1-4)-diglucoside, has been isolated from the stem of *S. indicus* (Yadav and Kumar, 1998).

Eudesmanolide type of sesquiterpene from *S. indicus* was reported to have immunostimulating activity (Shekhani et al., 1990) and alkaloid sphaeranthine (Basu and Lamsal, 1946).

**HPTLC finger printing for *Sphaeranthus indicus* methanolic leaves extract**

The Mobile phase used was Dichloromethane : Methanol : Acetic acid (6.5:3.0:0.5). The Rf value of methanolic leaves extract was found to be 0.34 (Table 12), which is closer to 0.35. According to the literature reference, Rf value at 0.35 is a sesquiterpene glycoside (sphaerananolide). Eudesmanolide type of sesquiterpene glycoside, sphaerananolide and alkaloid sphaeranthine (Basu and Lamsal, 1946), from *S. indicus* was reported to have immunostimulating activity (Shekhani et al., 1990).
5.3 *In vitro* Cytotoxicity by MTT and tryphan blue dye exclusion assay method

The methanolic and aqueous leaf and flower extracts were subjected to in vitro cytotoxicity by both MTT and tryphan blue dye exclusion assay method using HEP-G2 cell lines (Human Liver Cancer cell lines). The ability of the cell to survive a toxic insult has been the basis of most cytotoxicity assays. Living cells did not allow the entry of dye due to cells permeability. Dead cells lose its permeability and allow dye to enter inside of the cells. Hence living cells are colourless and the dead cells are blue to violet coloured; which was counted and calculated. In the US NCI plant screening program, a crude extract is generally considered to have in vitro cytotoxic activity if the IC50 value (concentration that causes a 50% cell kill) in carcinoma cells, following incubation between 48 and 72 hours, is less than 20µg/ml. The methanolic leaves extract displayed to strongest cytotoxic effect on human liver cancer cell line (HePG2) with IC50 value of 15 µg/ml. The methanolic and aqueous leaves extracts of *Sphaeranthus indicus* was rich in tannins, flavonoids, phenols, and saponins, which may responsible for this cancer cell cytotoxic activity. Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work.

The result of the present study suggests methanolic and aqueous extracts obtained from *Sphaeranthus indicus* leaves exhibit excellent cytotoxic activity on HepG2 cell line.

5.4 *In vivo* studies

**Acute toxicity studies**

The toxicological studies of *S. indicus* was conducted for their safety and toxicity. The plant extracts were subjected to preliminary acute toxicity study in
mice at different dose levels. The extracts were administered intraperitoneally at doses of 250, 500, 1000, 1500, 2000 mg/kg body weight. They were observed for signs of toxicity continuously for 2 hrs and for mortality upto 24 hrs after injection. All extracts were found to be safe in the doses used and there was no mortality upto a dose of 2 g/kg. Therefore the crude extract is considered to be non-toxic and found to be safe for further clinical studies. The results showed no abnormal symptoms or any mortality in the test animals. The absence of conspicuous side effects suggests that plant extract is safe pharmacologically. Hence, the plant extract may prove fruitful for clinical application. However, further studies on its effect on immune status and biochemical pathways are warranted.

**Biochemical studies**

Liver is the typical detoxifying organ of the body and is profoundly affected by external chemicals. Diethylnitrosamine (DEN) is a hepatotoxin and a carcinogen Similarly PB is hepatotoxin and acts as promotor in hepatocarcinogenesis.

Cellular damage exhibits good correlation with the enzyme leakage (Sherawat and Sultana, 2006). Serum AST, ALT, ALP, γ-GT and bilirubin are the most sensitive markers employed in the diagnosis of hepatic damage (Sallie *et al.*, 1991). The increase in the activities of these enzymes in serum and subsequent fall in the tissue might be due to the leakage of these cytosolic enzymes into the in circulatory system resulting from hepatocellular damage during ethanol and DEN administration. This is indicative of the onset of hepatocellular damage due to liver dysfunction and disturbance of the biosynthesis of these enzymes, with alteration in the permeability of liver membrane. Treatment with MLE significantly reversed the alterations in the
status of these markers to normal levels, possibly by maintaining the hepatocellular membrane integrity. This is an indicator of possible hepatoprotective property offered by MLE.

Biochemical tumor markers are used to screen particularly tumor conditions for differential diagnosis, prognosis, monitoring the progress and for assessing the response to therapy (McIntyre and Rosalki, 1992). Aminotransferases (AST and ALT) and ALP have been used as markers of liver function. The increase in these activities by DEN seems to be due to the hepatotoxicity of the chemical. It has been previously reported that hepatotoxic responses to DEN result in increased levels of AST, ALT, and ketone bodies with nitroso compounds (Taniguchi et al., 1999).

Aminotransferases, alkaline phosphatase, lactate dehydrogenase and gamma glutamyl transpeptidase serve as markers of liver damage. Analysis of these marker enzymes in serum and liver reflects the mechanism of cellular damage and subsequent release of proteins, their extracellular turnover and mechanisms of neoplastic processes. The role of transamination in biological systems is well known. It is apparent from transaminase substrate, i.e. α-ketoglutaric, oxaloacetic and pyruvic acids on one hand and glutaric and aspartic acids on the other hand, that transamination is concerned with the interconversion of highly important metabolites.

Elevated aminotransferase activities levels were observed in cancer bearing animals. Clinical diagnosis of neoplastic patients show an eight times increase over normal control patients (McIntyre and Rosalki, 1992). Transaminase becomes gradually more pronounced towards the terminus, which indicates the severity of an advanced cancer condition. Increased transaminase
activities in HCC have been reported by Rocchi et al. (1997). A good correlation exists between the activities of ALT and AST of tumor masses during therapy. The stable clinical and enzymatic pattern of these enzymes is noticed in patients with hepatic malignancy after chemotherapy, while patients failing to respond to drug showed progressive increase in the level of these enzymes. Similar results were observed by in dimethyl hydrazine-induced colon cancer in rats. These enzymes are more unique and changes in their activities reflect the effect of proliferation of cells with growth potential and its metabolic turnover is dramatically different from those of normal cells. The rise in their activities is shown to be in good correlation with the number of transformed cells in cancer conditions.

An increase in AST, ALT activities in DEN induced animals correlate to the hepatotoxicity and carcinogenesis with the development of preneoplastic changes, increased severity and advanced stage of liver carcinoma. An increase in the serum LDH in malignant liver diseases depends upon the extent of metastasis. The lowering in the activities of AST, ALT and LDH on Sphaeranthus indicus treatment shows the hepatoprotective effect of plant extracts and inhibition of carcinogenesis.

Alkaline phosphatase (ALP) is involved in transport of metabolites across cell membrane, protein synthesis, secretory activities and glycogen metabolism. Alkaline phosphatase is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites. ALP was noticed in the serum and liver of hepatoma-bearing animals (Patel et al., 1994). Observed that the ALP activity was raised in the serum of cervical carcinoma patients. The rise in a activity of ALP in cancer bearing animals may be due to the disturbance in the secretory activity or in
transport of metabolites or may be due to altered synthesis of certain enzymes in these conditions. ALP is used as a specific tumor marker during diagnosis in the early detection of cancer (Kobayashi and Kawakubo, 1994). An increase in ALP activity on DEN administration may be due to altered synthesis of enzymes as in other hepatotoxic condition (Sharma et al., 1995).

Plasma Gamma Glutamyl Transpeptidase (GGT) activity is higher in hepatocellular carcinoma. An increase in GGT activity paralleled with an increase in alkaline phosphatase activity is frequent in hepatocellular carcinoma. (Induction of GGT during hepatocarcinogenesis is frequent. An increased GGT activity causes resorption of GSH by preneoplastic foci rich in GGT that enhances cell proliferation and increases tumor promotion and favors transformation of preneoplastic foci to neoplasia (Stark et al., 1993). The decrease in the levels of serum marker enzymes on Sphaeranthus indicus methanolic leaves extract treatment prevents the neoplastic growth and reduces hepatic disorder, indicating that it possesses anticarcinogenic properties. Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work.

The experimental data showed that DEN profoundly increased the level of lipid peroxidation. Free radicals, mostly ROS, cause cellular injury, the consequences of which are often exhibited and measured as lipid peroxidation (Spiteller, 1996). Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids to form radical intermediates that cause oxidative damage. Malondialdehyde, the major end-product of this reaction, is an index of lipid peroxidation and can be estimated as thiobarbituric acid reactive substances (Kohen and Nyska, 2002). Tremendous increases in malondialdehyde upon DEN-induced oxidative stress could be caused by increased production of or
decreased destruction of ROS. The lesser effect of methanolic aqueous extracts leaves of *S.indicus* on DEN-induced lipid peroxidation in various tissues may be due its antioxidant potential. Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work.

Administration of NDEA has been reported to generate LPO products in general. LPO can be prevented at the initiation stage by free radical scavengers and antioxidants. The observation suggests that MLE-treated group has a potential to significantly reduce the levels of LPO as compared to the positive controls. Free radical damage and oxidative stress are the major reasons for liver tissue damage that can progress to develop tumor and thus result in HCC. The antioxidant enzymes are therefore the first-line defense against such damage and thus provide protection against the deteriorating outcome (Gaurav *et al.*, 2008). Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals (Banakar *et al.*, 2004).

SOD scavenges the superoxide radical by converting it to H$_2$O$_2$, which in turn is converted to molecular oxygen by reactions catalyzed by peroxidase and catalase (Thomas, 2003). Catalase is ubiquitously present in all aerobic cells and is a major component of the primary antioxidant defense system. It is most abundant in liver and is responsible for catalytic decomposition of H$_2$O$_2$ to oxygen and water.

In our studies, catalase activity decreased significantly upon NDEA-induced oxidative stress in groupII animals and the effect being less in groups treated with plant extracts. Decreased catalase activity indicates that NDEA-induced oxidative stress interferes with the anti-oxygenic potential in the tissues,
thus resulting in increased generation of ROS. The $\text{H}_2\text{O}_2$ formed by SOD is decomposed by catalase. The decrease in catalase activity in animals administered NDEA may be caused by inactivation of catalase as superoxide anions have been shown to reduce catalase activity (Bartsch et al., 1989 and Bansal et al., 1996). Similarly, exposure of erythrocytes to NDEA inhibited catalase activity through excess superoxide anion formation (Bansal et al., 2005). The lesser effect on catalase in the group treated with during NDEA-induced oxidative stress is indicative of the protective effect of $S$. indicus. In an *in vivo* study, methanolic extract of $S$. indicus exhibited a significant antioxidant effect showing increasing levels of superoxide dismutase, catalase, and glutathione peroxides by reducing malondialdehyde levels in rats. (Tiwari and Khosa, 2009). Moreover, the methanolic extract of $S$. indicus enhanced the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and diminished the amount of lipid peroxides against acetaminophen-induced hepatotoxicity in these animals (Nayak et al., 2007). Thus, methanolic and aqueous extracts can offer useful protection against NDEA-induced oxidative stress.

$5'$-Nucleotidase has been reported to be altered in the sera of patients with solid tumors. In human lymphoid system, $5'$-nucleotidase is anchored to the plasma membrane and has been described as an important marker for differentiation of B-lymphocytes. In our study, $5'$-nucleotidase has been increased significantly in the serum, hepatoma, hepatoma bearing rats. Have demonstrated elevated activity of $5'$-nucleotidase in carcinoma of liver, gastrointestinal tract and pancreas. Rosi et al. (1998) also observed an increased activity of $5'$-nucleotidase in leukemia patients. During ST treatment the enzyme activity got decreased significantly on time dependent manner.
Lactate dehydrogenase (LDH), a cytoplasmic enzyme which catalyzes the oxidation of lactate to pyruvate and vice versa. LDH, a marker for membrane integrity and is a regulator of many biochemical reactions in the body tissues and fluids have reported that an increased activity of LDH in malignant cells spreading through the organs of tumor bearing rats. The elevated enzyme activity in the serum of patients with lung and ovarian cancer (Bose and Mukherjee, 1994). Depict the changes in permeability of cell membranes and the leakage to soluble enzymes. The possible reason for the elevated activity of LDH in cancer bearing rats may be due to enhanced glycolysis using the growth of tumor. The reduction in LDH activity on treatment with ST controlled the glycolysis and renders protection to membrane integrity.

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α - fetoprotein (AFP), is a serum protein that is detected in elevated concentration in conditions like hepatocellular carcinoma. AFP is a serum protein 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 hepatotoxic agents (or) hepatocarcinogens and are frequently associated with HCC. It is a 72 KDa α-1 globulin with an uncertain biological function, is synthesized during embryonic life by foetal yolk sac, liver and intestinal tract. AFP has high specificity for hepatocarcinoma. Its serum concentration can be used to confirm hepatocarcinoma and for the diagnosis of tumor response to therapy. More than 90% of patients with hepatic cancer have increased serum AFP levels.

Our data showed that DEN profoundly increased the level of lipid peroxidation. Free radicals, mostly ROS, cause cellular injury, the consequences
of which are often exhibited and measured as lipid peroxidation (Spiteller, 1996). Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids to form radical intermediates that cause oxidative damage. Malondialdehyde, the major end-product of this reaction, is an index of lipid peroxidation and can be estimated as thiobarbituric acid reactive substances (Kohen and Nyska, 2002). Tremendous increases in malondialdehyde upon NDEA-induced oxidative stress could be caused by increased production of or decreased destruction of ROS. The lesser effect of methanolic aqueous extracts leaves of *S.indicus* on NDEA-induced lipid peroxidation in various tissues may be due its antioxidant potential.

Administration of NDEA has been reported to generate LPO products in general. LPO can be prevented at the initiation stage by free radical scavengers and antioxidants. The observation suggests that MLE-treated group has a potential to significantly reduce the levels of LPO as compared to the positive controls. Other enzymatic and nonenzymatic antioxidants such as SOD, CAT and GPx, that were investigated are known to reduce the oxidative stress by reducing the production and accumulation of superoxide radicals (O2^\cdot)(Galanis *et al.*, 2009) GSTs are a family of detoxification enzymes involved in protecting the cells against cytotoxicity and carcinogenic chemicals by conjugating with GSH. Depletion in the activity of these antioxidant enzymes was observed in positive control group. Interestingly, AAE-treated group showed significant increase in the level of GST in grade dose level at 100, 200, and 400 mg/kg b.w., respectively. It is probable that the various phytoconstituents of the plant are involved in scavenging the free radicals from the tissues, thus, reducing oxidative stress. This, in turn, acts together for normalizing the levels of the antioxidant enzymes taken under study. The protective effect of *S.indicus* was also assessed by studying the histopathology of liver tissue. The biochemical findings are supported by histopathological observations of the liver. In this
study, noticeable changes were observed in the architecture of liver in HCC bearing animals. These indicate the presence of neoplastic conditions following NDEA and PB administration. In animals treated with methanolic and aqueous leaves extracts, the liver architecture was preserved. Hence, the regression of the tumors in liver may be due to the protective effect of *S. indicus*. 