3

Experimental Procedure

Cancer is the leading cause of death in developing countries like India. As there is an enormous increase in the population day by day, the alternative therapy in the market is getting its glimpse. The cheap herbal drug treatment may highly be recommended to the rural and poor people to treat effectively the cancers of various type is an ideal choice. Based on that the siddha medicines are coming up in combination with metals and other essential supplements to improve the immune status of the cancer patients in India. This reveals the role of Indian medicinal plants and the various phytochemicals may be used effectively for cancer treatment. The isolation and identification of active principles and pharmacological studies of the active phytoconstituents may be considered and studied elaborately to treat effectively the various types of cancer (Dhanamani *et al*., 2011).

The search and use of drugs and dietary supplements from plants have been intensified in recent years. Medicinal plants are good sources of great economic value in the Indian subcontinent. Because of the presence of bioactive compounds, plant materials are used as drugs for most of the diseases (Sirappuselvi and Chitra, 2012).

An extensive survey of literature revealed that *Cassia* is an important source of many pharmacologically and medicinally important chemicals. Presence of wide range of chemical compounds such as alkaloids, flavonoids, glycosides, triterpenoids and sterols in different species of *Cassia* indicates that the active constituents isolated from this species could serve as a "lead" for the development of novel agents having good efficacy in various pathological disorders. Although many studies have claimed the use of some species of *Cassia* for the treatment of various diseases but still the pharmacological potential of the other species of this genus are required to be explored. In traditional medicine *Cassia* species have been well known for their laxative and purgative properties and for the treatment of skin diseases. However, *Cassia* invites attention of researchers worldwide for its phytochemistry and pharmacological activities ranging from antidiabetic to antiviral (Deshpande and Bhalsing, 2013).

The experimental procedure pertaining to the present study entitled, "Evaluation of antioxidant and anticancer potential of *Cassia senna* L. using *in vitro* and *in vivo* methods**"** is discussed under the following headings and the study was carried out in three phases.

PHASE I

3.1. Preliminary investigation of antioxidants and phytochemicals in the selected parts of *C. senna*

3.1.1. Collection of the plant sample

3.1.2. Preparation of the different extracts from the leaf and pod of *C. senna*

3.1.3. Antioxidant assays in leaf and pod of *C. senna*

3.1.3.1. Determination of enzymic antioxidant activities

3.1.3.2. Determination of nonenzymic antioxidant levels

3.1.3.3. Determination of total antioxidant activity

3.1.4. Preliminary phytochemical screening of different solvent extracts of leaf and pod of *C. senna*

3.1.5. Quantitative estimation of selected phytochemicals in the various solvent extracts of leaf and pod of *C. senna*

PHASE II

3.2. Screening of effective extract of *C. senna* leaves by radical scavenging effect, chromatographic analysis and *in vitro* cytotoxic activity.

3.2.1. Evaluation of free radical scavenging activity in the various solvent extracts of *C. senna* leaves

3.2.1.1. Determination of DPPH radical scavenging activity

3.2.1.2. Determination of ABTS radical scavenging activity

3.2.1.3. Determination of Hydroxyl radical scavenging activity

3.2.1.4. Determination of Inhibition of Super oxide radical generation

3.2.1.5. Determination of Inhibition of Nitric oxide radical generation

3.2.1.6. Determination of Hydrogen peroxide scavenging activity

3.2.2. Analysis of selected phytoconstituents in the leaf extracts of *C. senna* using HPTLC and HPLC methods

3.2.2.1. HPTLC analysis for alkaloid and flavonoid profiles in the different solvent extracts of *C. senna* leaves

3.2.2.2. HPLC analysis for alkaloids and flavonoids in the ethanolic extracts (DEE-Direct ethanolic extract and SEE-Successive ethanolic extract) of *C. senna* leaves

3.2.4. Assessment of *in vitro* cytotoxic activity of the two ethanolic extracts(DEE and SEE) of *C. senna* leaves in selected cancer and noncancer cell lines

PHASE III

3.3. Evaluation of *in vivo* anticancer activity and characterization of the active principles of DEE (Direct Ethanolic Extract) of *C. senna* leaves

3.3.1. Assessment of *in vivo* anticancer activity of DEE(Direct Ethanolic Extract) of *C. senna* leaves in Ehrlich Ascites Carcinoma(EAC) induced mice

3.3.1.1. Assessment of tumour growth response

3.3.1.2. Assessment of tumour markers in serum

3.3.1.3. Determination of antioxidant status in liver tissue

3.3.1.4. Assessment of lipid peroxidation(LPO) in liver tissue

3.3.1.5. Estimation of liver marker enzyme activities in serum

3.3.1.6. Estimation of lipid content in serum and liver tissue

3.3.1.7. Evaluation of histopathological studies on liver tissue

3.3.2. Characterization of active principles of DEE of *C. senna* leaves by Spectroscopic analysis.

3.3.2.1. Gas Chromatography-Mass Spectrum(GC-MS)

3.3.2.2. Fourier Transform Infrared spectroscopy(FT-IR)

PHASE I

3.1. Preliminary investigation of antioxidants and phytochemicals in the selected parts of *C. senna*

3.1.1 Collection of the plant sample

The fresh plant of *C. senna* was collected from Madurai district, Tamilnadu. The plant was identified and authenticated in Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2012). The plant material was cleaned. The leaves and pods of the plant were separately shade dried and were coarsely powdered using a mechanical grinder. The powdered samples were stored in air tight and light resistant containers to be used for further analyses.

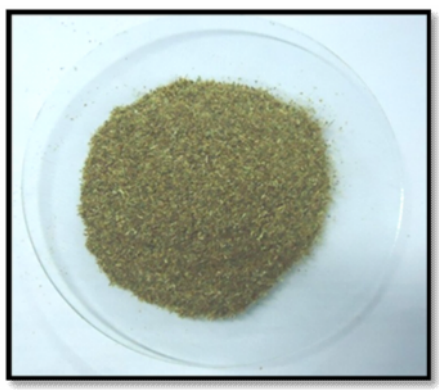
Plate 1

Fresh plant sample of *Cassia senna*

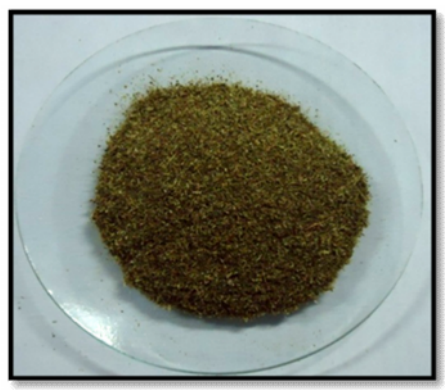
Leaf

Pod

Leaf Powder



Pod Powder



3.1.2. Preparation of the different extracts from the leaf and pod of *C. senna*

The most frequently used technique for the isolation of plant antioxidants is solvent extraction that includes maceration and percolation. Conversely, the extract yields and resulting antioxidant activities of the plant materials are strongly reliant on the type of extracting solvent, due to the presence of different antioxidant compounds of diverse chemical characteristics and polarities that may or may not be soluble in a particular solvent. Usually polar solvents are used for the recovery of polyphenols from a plant matrix. The most frequently used solvents include Acetone, Ethanol, Methanol, n-Hexane, Petroleum ether, Ethyl acetate and Hydroalcoholic mixtures (Khan *et al*., 2012a; Bushra *et al*., 2009). Extraction with solvents of different polarities should give different substances (Graidist *et al*., (2015).

Preparation of extracts for antioxidant assays

The powdered samples of leaf and pod of *C. senna* were subjected to extraction with appropriate buffers and the extracts were analyzed for the various enzymic and nonenzymic antioxidants and total antioxidant activity.

Preparation of organic solvent extracts by successive solvent extraction using Soxhlet apparatus

The powdered samples of leaf and pod of *C. senna* were subjected separately to successive solvent extraction from non-polar to polar solvents namely petroleum ether, benzene, chloroform, ethyl acetate and ethanol. Fifteen gms of each of the powdered samples of leaf and pod was subjected to Soxhlet extraction for 8 hrs with 250ml of the selected solvents successively. The extracts obtained were then evaporated at the respective boiling points of the selected solvents. The residues were stored in a cool dry place and were used for the analyses (Vadivel *et al*., 2012).

Preparation of Direct Ethanolic Extract (DEE)

Leaf powder of *C. senna*(10 gm) was taken in 100 ml of ethanol and macerated in stopper flask for 48 hours, shaking frequently at room temperature. Next day the mixture was filtered by using Whattmann no.1 filter paper and it was dried on water bath until the constant weight with dry mass was obtained (Kokate, 2005).

Preparation of the aqueous extract

Ten gms of each of the powdered samples of leaf and pod of *C. senna* was mixed with distilled water and boiled on slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume to be one-fourth of the original volume (Parekh *et al*., 2005).

3.1.3. Antioxidant assays in leaf and pod of *C. senna*

A series of enzymic and nonenzymic detoxification systems are present in plants to counteract ROS and protect the cells from oxidative damages (Sairam and Tyagi, 2004). The extracts of leaf and pod samples of *C. senna* prepared using appropriate buffers were analyzed for the various enzymic and nonenzymic antioxidants and total antioxidant activity.

3.1.3.1. Determination of enzymic antioxidant activities

The extracts of leaf and pod samples of *C. senna* prepared using appropriate buffers were analyzed for the various enzymic antioxidants namely superoxide dismutase(SOD), catalase(CAT), peroxidase(POD), glutathione S-transferase(GST) and polyphenol oxidase(PPO).

Estimation of superoxide dismutase(SOD) activity in plant tissue

Superoxide dismutase activity was determined by the method of Misra and Fridovich, (1972) and the detailed procedure is given in Appendix 1.

Estimation of catalase(CAT) activity in plant tissue

Catalase, an enzyme which catalyses the decomposition of hydrogen peroxide to water and oxygen. The activity of this enzyme was determined by the method of Luck, (1974) and the detailed procedure is given in Appendix 2.

Estimation of peroxidise(POD) activity in plant tissue

Peroxidase converts hydrogen peroxide into water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine). Peroxidase activity was estimated by the method of Reddy *et al*., (1995) and the detailed procedure is given in Appendix 3.

Estimation of glutathione S-transferase(GST) activity in plant tissue

Glutathione S-transferase was assessed by the method of Habig *et al*., (1974) and the detailed procedure is given in Appendix 4.

Estimation of polyphenol oxidase(PPO) activity in plant tissue

The polyphenol oxidase activity was estimated by the method of Esterbauer *et al*., (1997) and the detailed procedure is given in Appendix 5.

Determination of nonenzymic antioxidant levels

The sample solutions of leaf and pod of *C. senna* prepared using appropriate buffers were analyzed for the various nonenzymic antioxidants such as ascorbic acid, α-tocopherol, polyphenols, flavonoids, and reduced glutathione.

Estimation of ascorbic acid (vitamin C) in plant tissue

Ascorbic acid has an ability to scavenge a wide variety of reactive oxygen species and acts as a chain breaking antioxidant. Ascorbic acid was estimated by the method of Roe and Kuether, (1953) and the detailed procedure is given in Appendix 6.

Estimation of α-tocopherol (vitamin E) in plant tissue

α-Tocopherol was estimated by the method of Rosenberg, (1992) and the detailed procedure is given in Appendix 7.

Estimation of flavonoids in plant tissue

The procedure in Appendix 8 explained by Cameron *et al*., (1943) was followed for the determination of flavonoids.

Estimation of polyphenols in plant tissue

Polyphenols were estimated by the method of Malick and Singh, 1980 and the detailed procedure is given in Appendix 9.

Estimation of reduced glutathione in plant tissue

Reduced glutathione content was estimated by the method of Moron *et al*., 1979 and the detailed procedure is given in Appendix 10.

3.1.3.3. Determination of total antioxidant activity

The total antioxidant activity was measured by the reduction of phosphomolybdenum Mo(VI) to Mo(V) by the plant extract and subsequent formation of green phosphate/Mo(V) complex at acid pH. It evaluates both water-soluble and fat-soluble antioxidants(total antioxidant capacity) (Aliyu *et al*., 2013). The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid (Prieto *et al*., 1999).

The total antioxidant activity was measured in the leaf and pod powder of *C. senna* by the method of Prieto *et al*., (1999) and the detailed procedure is given in Appendix 11.

3.1.4. Preliminary phytochemical screening of different solvent extracts of leaf and pod of *C. senna*

Phytochemical investigations of crude plant extracts shows the presence of active principles in the plant parts like bark, leaves, flowers, roots, fruits and seeds. Phytochemicals are nonnutritive plant chemicals that have protective or disease preventive properties. Plant produces these chemicals to protect itself but research work demonstrates that many phytochemicals can protect humans against diseases. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances (Krishnaiah *et al*., 2009).

The different solvent extracts of leaf and pod of *C. senna* were screened for the presence of phytochemicals such as carbohydrates, glycosides, proteins, alkaloids, flavonoids, steroids, terpenes, diterpenes, triterpenes, phytosterols, tannins, saponins and phenols. Preliminary phytochemical analysis was performed using methods of Harborne, (1984), Kokate *et al*., (1990), Siddiqui and Ali, (1997) and Basset *et al*., (1985) and the details of the procedures are given in Appendix 12.

3.1.5. Quantitative estimation of selected phytochemicals in the various solvent extracts of leaf and pod of *C. senna*

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body and these chemical substances are called phytochemicals. The most important of these phytochemicals are alkaloids, flavonoids, tannins and phenolic compounds.

Quantitative estimation of alkaloids, flavonoids, phenols, steroids and tannins was carried out for different solvent extracts of leaf and pod of *C. senna*.

Estimation of total alkaloids

Alkaloids were quantified by the method of Singh *et al*., (2004) and the detailed procedure is given in Appendix 13.

Estimation of total flavonoids

Flavonoids were estimated by the method of Zhishen *et al*., (1999) and the detailed procedure is indicated in Appendix 14.

Estimation of total phenols

Phenols were quantified by the method of Eberhardt *et al*., (2000) as detailed in Appendix 15.

Estimation of total tannins

Tannins were quantified by the method of Shanmugam *et al*., (2010) and the detailed procedure is given in Appendix 16.

Estimation of total steroids

Estimation of steroids was done by the method of Sabir *et al*., (2003) as given in Appendix 17.

PHASE II

3.2. Screening of effective extract of *C. senna* leaves by radical scavenging effect, chromatographic analysis and *in vitro* cytotoxic activity.

3.2.1. Evaluation of free radical scavenging activity in the various solvent extracts of *C. senna* leaves

The various solvent extracts of leaf of *C. senna* were analysed for various free radical scavenging activities namely DPPH radical, hydroxyl radical, ABTS radical and hydrogen peroxide scavenging activity and inhibition of radical generation such as super oxide and nitric oxide radical.

3.2.1.1. Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was carried out by the method of Mensor *et al*., (2001) and the detailed procedure is given in Appendix 18.

3.2.1.2. Determination of ABTS radical scavenging activity

ABTS radical scavenging activity was carried out by the method of Shirwaikar *et al*., (2006) and the detailed procedure is given in Appendix 19.

3.2.1.3. Determination of hydroxyl radical scavenging activity

Hydroxyl radical($OH^{\bullet}$) scavenging activity was carried out by the method of Apak *et al*., (2007) as given in Appendix 20.

3.2.1.4. Determination of inhibition of superoxide radical generation

Inhibition of super oxide anion($O_2^{\bullet -}$) radical generation was measured by the method of Winterbourn *et al*., (1975) and the detailed procedure is given in Appendix 21.

3.2.1.5. Determination of inhibition of nitric oxide radical generation

Inhibition of nitric oxide(NO) radical generation was measured by the method of Green and Hill (1984) as detailed in Appendix 22.

3.2.1.6. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was carried out by the method of Ruch *et al*., (1989) and the detailed procedure is given in Appendix 23.

3.2.2. Analysis of selected phytoconstituents in the leaf extracts of *C. senna* using HPTLC and HPLC methods

3.2.2.1. HPTLC analysis for alkaloid and flavonoid profiles in the different solvent extracts of *C. senna* leaves

HPTLC study was performed for alkaloid and flavonoid profiles in the different solvent extracts of *C. senna* leaves namely petroleum ether extract(PEE), benzene extract (BE), chloroform extract(CE), ethyl acetate extract(EAE), successive ethanolic extract(SEE), direct ethanolic extract(DEE) and aqueous extract(AE) along with the standard marker compound colchicine for alkaloids and quercetin for flavonoids. The detailed procedure is given in Appendix 24 for alkaloids and flavonoids.

3.2.2.2. HPLC analysis for alkaloids and flavonoids in the ethanolic extracts (DEE-Direct ethanolic extract and SEE-Successive ethanolic extract) of *C. senna* leaves

Inorder to screen and identify the selected phytoconstituents HPLC analysis was carried out in the ethanolic extracts namely DEE and SEE of *C. senna* leaves using a

Shimadzu HPLC system with RP C18 column, temperature control module and UV detector. The reference standards used were caffeine for alkaloid and quercetin and kaempferol for flavonoids. The detailed procedures are given in Appendix 25 alkaloids and flavonoids respectively.

3.2.4. Assessment of *in vitro* cytotoxic activity of the two ethanolic extracts(DEE and SEE) of *C. senna* leaves in selected cancer and noncancer cell lines

MTT reduction into formazan crystals as a cell viability measurement is now extensively chosen as the most advantageous end point (Wahab *et al.,* 2009). MTT[(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide)] assay measures the metabolic activity of the viable cells and it is non-radioactive and can be performed entirely in a microtiterplate(MTP). It is suitable assay for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and "mitochondrial dehydrogenase" produce water-insoluble formazan salt. This method involves culturing the cells in a 96-well microtiterplate and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number (Scudiero *et al*., 1988).

MTT assay in cancer and noncancer cell lines

The percent viability of cancer cell lines namely EAC(Ehrlich ascites carcinoma), MCF7(Breast cancer cell lines) and HCT116(Colon cancer cell lines) and noncancer cell line, L929 with different concentrations of DEE and SEE of *C. senna* leaves were analyzed by MTT dye reduction assay by the method of Scudiero *et al*., (1988) as per Appendix 26.

PHASE III

3.3. Evaluation of *in vivo* anticancer activity and characterization of the active principles of DEE (Direct Ethanolic Extract) of *C. senna* leaves

3.3.1. Assessment of *in vivo* anticancer activity of DEE(Direct ethanolic extract) of *C. senna* leaves in Ehrlich ascites carcinoma(EAC) induced mice

Inorder to ensure the promising antioxidant and anticancer potential of DEE under *in vivo* conditions, the present study has been focused on the various biochemical

parameters such as tumour growth response, tumour markers, antioxidants, lipid peroxidation, liver marker enzymes, lipid content and histopathological studies in the Swiss albino mice challenged with the EAC cells and it was compared with the standard anticancer drug, methotrexate.

Experimental animals

Adult male swiss albino mice weighing approximately 20-25g were used as experimental model. The animals were kept in well ventilated cages and were fed with commercial pelleted mice chow and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before the commencement of the experiments. The mice were divided into six groups of six each. Institutional Animal ethical clearance (RegNo.623/02/b/CPCSEA19.06.2002-AUW.IAEC.2013-14.BC:07) was obtained before starting the experiment.

Cell lines

Experimental tumors have great importance in modeling and Ehrlich ascites carcinoma (EAC) is one of the most common tumors. EAC is referred to as an undifferentiated carcinoma and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also does not have tumor-specific transplantation antigen (TSTA). Frequently, tumor virulence increases via repetitious passages, while the proliferating rate of such tumors increases gradually. However, the differentiation gradually disappears, while the cells get free growth control mechanisms, gain hetero-transplantability and at the end, they are converted to the ascites form. EAC resembles human tumors which are highly sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid growth rate (Ozaslan *et al*., 2011).

Plant extract

Direct ethanolic extract prepared by simple maceration technique was dissolved in acacia gum. The dose of DEE was selected from the results of *in vitro* cytotoxicity study and also based on previously published reports.

Standard anticancer drug

Methotrexate(4-amino-4-deoxy-10-methylfolic acid) was used as standard anticancer drug and it was dissolved in saline solution. Methotrexate (MTX) is used as a chemotherapeutic agent used to treat many cancer types (Tousson *et al*., 2014b).

Tumour induction in experimental animals

Ehrlich ascites carcinoma (EAC) cells were procured from Amala Cancer Research centre, Thrissur, Kerala and were propagated in Swiss albino mice by intraperitoneal transplantation of $1X10^6$ cells in 100µl of PBS. After 10 to 15 days, the cells were drawn from the intraperitoneal cavity of the mice and were injected into experimental groups of mice(Table no) intraperitoneally at concentration of $1X10^6$ cells/mouse. After 24 hour of the tumor cell induction treatment with DEE and standard anticancer drug(methotrexate) was started through peritoneal cavity. Group I and Group II animals received saline only. Group III was administered with the standard drug and the remaining groups (IV, V and VI) were treated with various concentration of DEE of *C. senna* leaves for 14 days.

Table 4 : Experimental design for *in vivo* studies

Groups (6 animals)	Treatment Groups
Group – I	Untreated group – Negative control
Group – II	Tumor control - Positive control (Ehrlich Ascites Carcinoma cells($1X10^6$cells/mouse)
Group - III	Ehrlich Ascites Carcinoma cell line ($1X10^6$cells/mouse)+Standard anticancer drug-Methotrexate 10mg/Kg b.w.
Group – IV	Ehrlich Ascites Carcinoma cell line ($1X10^6$cells/mouse)+DEE 100 mg /Kg b.w.
Group – V	Ehrlich Ascites Carcinoma cell line ($1X10^6$cells/mouse)+DEE 200 mg /Kg b.w.
Group –VI	Ehrlich Ascites Carcinoma cell line ($1X10^6$cells/mouse)+DEE 300 mg /Kg b.w.

After the experimental period, the animals were sacrificed by cervical decapitation, the blood was collected from the controls and the experimental groups of mice and the serum was separated out for the biochemical analysis. The liver and kidney samples were washed with ice cold saline. After washing, samples from each group were homogenized with phosphate buffer for the analysis of antioxidants and lipid content while few samples were fixed with 10% formalin for histopathological studies.

3.3.1.1. Effect of DEE *C. senna* leaves on Tumour growth response

Body weight (g)

The body weight of the control and treated group animals was taken at 0^{th}, 5^{th}, 10^{th} and 14^{th} day.

Mean survival Time(MST) **and Increased Life Span (ILS %)**

Mean Survival Time and percentage increased life span of each group containing 6 mice was daily monitored by recording the mortality for 6 weeks and they were calculated using following formulae.

- MST = (Day of first death + day of last death)/2
- ILS (%) = [(Mean survival time of treated group/ mean survival time of control group)-1] X100

3.3.1.2. Assessment of Tumour markers in serum

Tumour Markers such as Gamma glutamyl transferase(GGT) and Nitric oxide(NO) were analysed in serum of experimental group mice.

Estimation of gamma glutamyl(GGT) transferase activity in serum

The Gamma glutamyl transferase was activity estimated by the method of Persijn and van der Slik, (1978) using kit procured from Span Diagnostics Limited, Sachin, India and the procedure is given in Appendix 27.

Estimation of nitric oxide level in serum in serum

The level of nitric oxide(NO) was measured by the method of Green *et al*., (1982) as per the procedure given in Appendix 28.

3.3.1.3. Assessment of the antioxidant status in liver tissue

After sacrifice, the liver of different experimental group animals were washed with ice cold saline, homogenized with phosphate buffer and used for the assays of enzymic antioxidants namely superoxide dismutase (SOD), catalase (CAT) and glutathione Peroxidase (GPx).

Determination of enzymic antioxidant activities in liver tissue

Estimation of superoxide dismutase(SOD) activity in liver tissue

The methodology adopted for assessing the activity of SOD was the same method (Misra and Fridovich 1972), which was explained in Appendix 1 of the phase I study. Liver homogenate was used as enzyme source for the assay instead of plant extracts.

Estimation of catalase(CAT) activity in liver tissue

The methodology adopted for assessing the activity of CAT was the same method (Luck 1974), which was explained in Appendix 2 of the phase I study. Liver homogenate was used as enzyme source for the assay instead of plant extracts.

Estimation of glutathione peroxidise activity in liver tissue

The method of Rotruck *et al*. (1973) was followed for the assessment of GPx in liver (Appendix 29).

Determination of nonenzymic antioxidant levels in liver tissue

After sacrifice, the liver of different experimental group animals were washed with ice cold saline, homogenized with phosphate buffer and used for the estimation of nonenzymic antioxidants namely Vitamin C, Vitamin E and reduced gluahione.

Estimation of Vitamin C in liver tissue

The procedure for the estimation of Vitamin C was the same method (Roe and Keuther, 1953) as that used in Appendix 6 of the phase I study, except that the liver homogenate was the sample used for the assay.

Estimation of Vitamin E in liver tissue

The procedure for the estimation of Vitamin E was the same method (Rosenberg 1992) as used in Appendix 7 of the phase I study, except that the liver homogenate was the sample used for the assay.

Estimation of Reduced glutathione (GSH) in liver tissue

The procedure for the estimation of reduced glutathione was the same method (Moron *et al*., 1979) as used in Appendix 10 of the phase I study, except that the liver homogenate was the sample used for the assay.

3.3.1.4. Assessment of lipid peroxidation(LPO) in liver tissue

The rate of lipid peroxidation was evaluated by the assessment of the levels of lipid peroxides in the liver homogenate of different groups of mice using the method of Nichans and Samuelson, (1968). The detailed procedure is given in Appendix 30.

3.3.1.5. Estimation of liver marker enzyme activities in serum

The marker enzymes for hepatic damage, namely aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were estimated in the serum

of experimental group mice. All these enzymes were assayed using kits procured from Span Diagnostics Limited, Sachin, India.

Estimation of aspartate transaminase(AST) activitiy in serum

Necrosis or membrane damage releases this enzyme into circulation; therefore, it can be measured in serum. High levels of AST in serum indicate liver damage. AST activity were determined by the method of Bergmeyer *et al.*, (1978) as shown in Appendix 31.

Estimation of alanine transaminase(ALT) activitiy in serum

ALT catalyses the conversion of alanine to pyruvate and glutamate and is released into circulation due to necrosis or membrane damage of liver cells ALT is more specific to the liver, and is thus a better parameter for detecting liver injury (Williams, 1996). ALT activity were determined by the method of Bergmeyer *et al.*, (1978) as given in Appendix 31.

Estimation of alkaline phosphatase(ALP) activitiy in serum

Serum ALP level is related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis of the enzyme, in presence of increasing biliary pressure (Moss and Butterworth, 1974). ALP activity was assayed by the method of Schlebusch *et al.*, (1974) as in Appendix 32.

3.3.1.6. Estimation of lipid content in serum and liver tissue

Estimation of lipid content in serum

The levels of cholesterol, triglycerides and free fatty acids were estimated in the serum samples of mice. The kits used for these assays were purchased from Span Diagnostics Ltd., Sachin, India.

Estimation of serum cholesterol

Serum cholesterol was estimated by Allain *et al.*, (1974) method as shown in Appendix 34.

Estimation of serum triglycerides

Triglycerides were estimated in serum by the method of Bucolo and David, (1973) as given in Appendix 35.

Estimation of serum free fatty acids

Free fatty acids were estimated by the method of Falholt *et al.*, (1973) as shown in Appendix 36.

Estimation of lipid content in liver tissue

Extraction of lipids from liver tissue

The lipids were extracted from liver homogenate of mice by the method of Folch *et al*., (1970) as given in Appendix 37 and the lipid extracts were redissolved in 3ml of chloroform : methanol(2:1) mixture and aliquots were taken for the estimation of cholesterol, triglycerides, phospholipids and free fatty acids

Estimation of liver cholesterol

Liver cholesterol were estimated by Zak *et al*., (1953) method as shown in Appendix 38

Estimation of liver triglycerides

Liver triglycerides were estimated by the method of Foster and Dunn, (1973) as given in Appendix 39.

Estimation of liver phospholipids

Phospholipids were estimated by the method of Fiske and Subbarow, (1925) as shown in Appendix 40.

Estimation of liver free fatty acids

Free fatty acids were estimated by the method of Falholt *et al*., (1973) as shown in Appendix 41.

3.3.1.7. Evaluation of histopathological observations on liver tissue

The sample of the liver and kidney from the controls and the experimental groups of mice were fixed in 10 per cent formalin and then embedded in paraffin. Microtome sections of 6 Bm thicknesses were prepared from each portion of the liver and stained with haemotoxylin-eosin for pathological observation using the method of Culling, (1974) as shown in Appendix 42.

3.3.2. Characterization of active principles of DEE of *C. senna* leaves by Spectroscopic analysis

3.3.2.1. GC-MS

Separation of the bioactive compounds of DEE using GC-MS analysis was performed by the method of Maciejewicz *et al.*, (2007) as seen in the Appendix 43.

3.3.2.2. FT-IR

Functional groups of the bioactive constituents of DEE by FT-IR procedure was carried out by the method of Nasir *et al*., (2006) as seen in Appendix 44.

Statistical analysis

The data presented in Phase I and II are means ± SD(n=3) and the data presented in vivo studies of Phase III are means ± SD of 6 mice in each group. All the parameters studied were analysed statistically using SigmaStat statistical package (Version 3.1). The parameters studied in Phase I were subjected to "t" test and the parameters studied in Phase II and Phase III were subjected to One way ANOVA.

The results obtained for the various parameters analyzed during the different phases of the study are presented and discussed in the next chapter.