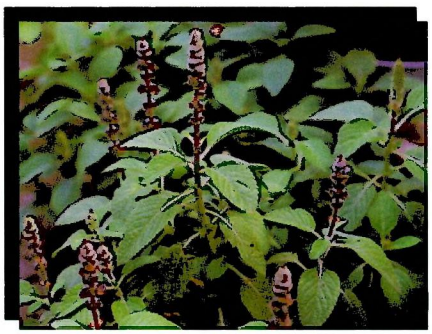


Experimental Procedure

structures for the development of novel therapeutic agents for the treatment of human diseases such as cancer and other infectious diseases (Mothana *et al.*, 2009). Nearly 25,000 effective plant based formulations are used in folk medicines by the rural communities in India (Ramakrishnappa, 2002).

Herbal medicines are popular remedies for diseases used by majority of the world's populations. Herbal formulations, which have attained widespread acceptability as therapeutic agents in India, include nootropics, antidiabetics, hepatoprotective agents and lipid-lowering agents. The pharmacological effects of many plants have been studied in various laboratories. But there are limitations regarding the safety and the efficacy of these preparations (Kuruvilla, 2002).

Coleus forskohlii briq.(Lamiaceae) grows wild in various parts of India; the roots have long been used in ayurvedic medicine for treating heart and lung diseases, intestinal spasms, insomnia and convulsions (Saleem *et al.*, 2005). The scope of the present study includes phytochemical analysis and pharmacological evaluation of the roots of the plant. The methodology adopted is as follows

PHASE I

3.1 TOXICOLOGICAL EVALUATION AND DETERMINATION OF ENZYMIC AND NONENZYMIC ANTIOXIDANTS IN DIFFERENT EXTRACTS OF ROOTS OF *Coleus forskohlii* IN MICE WITH DLA TUMOR

3.1.1 Collection, identification and processing of the roots of *Coleus forskohlii*

3.1.2 Acute toxicity evaluation of the root extracts of *Coleus forskohlii*

3.1.3 Treatment protocol for the mice study

3.1.4 Assessment of lipid peroxidation, enzymic and non enzymic antioxidants in DLA tumor mice treated different extracts

3.1.5 Estimation of serum marker enzymes in DLA tumor mice treated with different extracts.

3.1.6 Statistical analysis.

PHASE II

3.2 DETERMINATION OF THE EFFECT OF METHONOLIC EXTRACT OF *Coleus forskohlii* roots ON HEMATOLOGICAL PARAMETERS, BODY WEIGHT AND SURVIVAL TIME OF MICE WITH DLA TUMOR

3.2.1 Determination of hematological parameters.

3.2.2 Assessment of survival rate of mice

3.2.3 Determination of body weight of mice

3.2.4 Statistical analysis

3.2.5 Histopathological studies in liver of mice

PHASE III

3.3 IDENTIFICATION OF THE PHYTOCHEMICALS IN *Coleus forskohlii*

3.3.1 Free radical scavenging activity of the methanolic extract of *Coleus forskohlii*

3.3.2 Preliminary screening for phytochemicals in the methanolic extract

3.3.3 Fractionation of methanolic extract of *Coleus forskohlii* roots with different solvents

3.3.4 Determination of DLA cell viability against different fractions of methanolic extract of *Coleus forskohlii*

3.3.5 Identification of the constituents in the cytotoxic fraction of root of *Coleus forskohlii* by GC-MS

3.3.6 High performance thin layer chromatography for the identification of terpenoid profile

3.3.7 Identification of the phytochemicals present in the chloroform-methanolic fraction of *Coleus forskohlii* roots by TLC

3.3.8 Analysis of the active components by High Performance liquid Chromatography (HPLC)

PHASE I

3.1 TOXICOLOGICAL EVALUATION AND DETERMINATION OF ENZYMIC AND NONENZYMIC ANTIOXIDANTS IN DIFFERENT EXTRACTS OF ROOTS OF *Coleus forskohlii* IN MICE with DLA TUMOR

3.1.1 COLLECTION, IDENTIFICATION AND PROCESSING OF THE ROOTS OF *Coleus forskohlii*

The plant material *Coleus forskohlii* was collected from Tamil Nadu Agricultural University, Coimbatore and was duly authenticated by Dr.G.V.S.Murthy, Joint Director, Botanical Survey of India, Southern Regional Centre, Tamil Nadu Agricultural University, Coimbatore. The roots of the plants were cut into pieces and dried under shade for a week. The shade-dried roots were coarsely powdered and weighed. Extracted each 100 g of the powder in 500 ml each of of 70% petroleum ether, chloroform, acetone and methanol respectively using soxhlet apparatus. The extracts were concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature (40-50°C). The crude extracts yielded a dark brown solid, weighing approximately 40g. The extracts were preserved in a refrigerator at 4°C for further use.

Plate 1. *Coleus forskohlii*

Coleus forskohlii

Roots

Root powder

3.1.2 Acute toxicity evaluation of the selected medicinal plant

Evaluation of the toxicity and the adverse drug reaction of the herbal preparation has been a neglected area, as herbs are considered to be products of nature ane therefore safe. This lack of information makes it difficult to compare the benefit risk profile of herbal medicines. Further, the comparison of traditional medicines with modern drugs with comparative efficacy has not been conducted for most of the drugs.

Even if no adverse drug reaction has been reported, the acute toxicity studies were conducted to determine the safe dose as per OECD guidelines. Toxicity studies for herbal products should be conducted as per the regulatory requirements of the country to which they are marketed (Seth and Sharma, 2004).

In order to study any possible toxic effect or changes in the normal behaviour of experimental animals, the toxicity of the extracts was studied by checking the symptoms, the posture and the mortality.

Four groups containing six animals in each group were used. The extracts were administered orally. After administration, the animals were observed continuously for one hour frequently for the next four hours and then for 3days. The details of the test substance and the experimental protocol for acute toxicity are given in Table 1 and 2 respectively.

Table 1. Details of the Test Substance

Name of the test substance	*Coleus forskohlii* root extract
Colour of the test substance	Dark brown
Nature of the test substance	Sticky

Table 2. Experimental Protocol for acute toxicity

Name of the study	Acute toxicity
Guideline followed	OECD 423 method- acute toxic class method
Animals	Healthy young adult Balb/C , nulliparous.
Body weight	25-30 g
Sex	male
Administration of dose and volume	25, 100, 500,2000 mg/kg body weight, single dose in 0.5ml
Number of animals	Four groups each consist of 6 animals and treated with the minimal dose of *C. forskohlii* extracts orally. After 24h observation of the first group mice, the rest of the groups were given different *Coleus forskohlii* extracts and the groups were observed upto 3days for the toxicity sign.
Route of administration	Oral by using oral needle

Table 3. Housing and Feeding Conditions

Room Temperature	22°C ± 3°C
Humidity	40-60%
Light	12h : 12h (light: dark cycle)
Feed	Standard laboratory animal food pellets with *ad libitum*

Table 4. Study Period and Observation Parameters

Initial once observation	First 1 hour and periodically 24h
Special attention	First 1-4 h after drug administration
Long term observation	Upto 3 days
Direct observation parameters	Tremors, convulsions, salivation, urination, alertness.
Additional observation parameters	Skin and fur, eyes and mucus membrane, pain response, writhing reflex etc

The animals were closely observed for the first 12 hours for any toxic symptoms and for 72 hours for mortality rate (Joshi *et al.,* 2007). The mice were observed several times on the day of dosing and once daily therafter for abnormal behavioural signs (eg., respiratory effects such as labored breathings, evalution of skin, fur, eyes, and mucus membranes, circulatory effects, autonomic effects such as salivation, central nervous systems effects including tremors and convulsions, changes to the levels of motor activity, gait and posture, reactivity to handling or sensory stimuli or unusual behaviour. Observations also include bodyweight and food consumption. The various observations are recorded.

3.1.3 Treatment protocol for the mice study

Healthy male Balb/C mice of approximately with the same weight (25-30grams) were procured from N.G.P. College of Pharmacy, Coimbatore. The mice were fed with normal laboratory diet and water *ad libitum* and acclimatized for a week under laboratory conditions. The study protocol was approved by the IAEC.

The mice were divided into twelve groups of 6 each to determine the enzymic, nonenzymic antioxidants and liver marker enzymes of the different extracts of the root of *Coleus forskohli.*

Earlier studies of medicinal plants have revealed that the alcoholic extracts of these plants showed better antioxidant and anticancer effect than that of aqueous extracts. Most of the aqueous extracts do not show any notable anticancer activity or generally the aqueous extracts exhibit only weak cytotoxic effect (Mothana *et al.*, 2009). Hence, the present study was carried out with the alcoholic extract of the *Coleus forskohlii.* The petroleum ether, chloroform, acetone and methanolic extract of the root of *Coleus forskohlii* were prepared by continuous hot percolation of 100g of the sample with 2.5L of petroleum ether, chloroform, acetone and methanol respectively in Soxhlet apparatus. After the completion of the extraction, they were filtered and the solvent was removed by distillation under reduced pressure. A brown colored fumy residue was obtained. It was then dissolved in 0.3% carboxy methyl cellulose and was used for the study. The mice were given oral dose of extracts after the 1st day of induction of cancer. The treatment protocol for animal study is given in Table 5.

Propagation of tumor cells

The ascitic lymphoma bearing mice (donor) was taken 15 days after tumor transplantation. The asicitic fluid was drawn into a sterile syringe using a 22 gauge needle. A small volume was tested for microbial contamination. Tumor viability was determined by trypan blue exclusion test and cells were counted using haemocytometer. The ascitic fluid was suitably diluted in phosphate buffer saline to get concentration of 10^6 cells/ml of tumor cell suspension. This was injected intraperitoneally in to the experimental mice to develop ascitic tumor. The mice were weighed on the day of tumor inoculation and then for three subsequent days. Treatment was started 24 hours after tumor inoculation. All the treatments were given 24 hours after the tumor inoculation, daily once for 21 days. After the last dose and 24 hours fasting, the mice were killed for the study of biochemical parameter in liver and serum.

Selection of the dose

The dose selected for the extracts was about $1/10^{th}$ of the safe dose found in acute toxicity studies (John, 2008). 200mg/kg body weight was the dose selected for the present study.

Standard

The dose of methotrexate selected was 3.5mg/Kg body weight. This was calculated by using the body mass index and past experience with the drug (Dongre, 2008).

Table 5. Treatment protocol of mice for the determination of liver antioxidants and serum enzymes

Group	Designation	Treatment
1	UC	Untreated control (1.0 ml of distilled water)
2	VC	Vehicle control (1.0 ml of 0.3% carboxy methyl cellulose)
3	PE	1.0 ml of petroleum ether extract of *Coleus forskohlii* root (200mg/kg body weight/day)
4	CF	1.0 ml of chloroform extract of *Coleus forskohlii* root (200mg/kg body weight/day)
5	AC	1.0 ml of acetone extract of *Coleus forskohlii* root (200mg/kg body weight/day)
6	ME	1.0 ml of methanol extract of *Coleus forskohlii* root (200mg/kg body weight/day)
7	DC	DLA control (I.P. injection of $1x10^6$ cells of DLA on the 1^{st} day- 1.0 ml of distilled water)

Group	Designation	Treatment
8	DPE	DLA+CF (I.P. injection of $1x10^6$ cells of DLA on the 1st day) 1.0 ml of petroleum ether extract of *Coleus forskohlii* root (200mg/kg body weight/day)
9	DCF	DLA+CF (I.P. injection of $1x10^6$ cells of DLA on the 1st day) 1.0 ml of chloroform extract of *Coleus forskohlii* root (200mg/kg body weight/day)
10	DAC	DLA+CF (I.P. injection of $1x10^6$ cells of DLA on the 1st day) 1.0 ml of acetone extract of *Coleus forskohlii* root (200mg/kg body weight/day)
11	DME	DLA+CF (I.P. injection of $1x10^6$ cells of DLA on the 1st day) 1.0 ml of petroleum ether extract of *Coleus forskohlii* root (200mg/kg body weight/day).
12	Methotrexate	DLA+CF (I.P. injection of $1x10^6$ cells of DLA on the 1st day)1.0 ml of methotrexate (standard)

Administration of all the compounds and the vehicles were done using an intragastric tube for 21 days. Base line body weight, fluid intake, and food consumption pattern were established and monitored during the 21 days of oral treatment.

Collection of mice tissues and blood

The mice were made to fast overnight on the 21st day and were sacrificed on the next day after recording body weight by decapitation. Blood was collected by incision of jugular vein. Serum was prepared by centrifuging at 12000 rpm. Liver was excised, rinsed in ice cold normal saline, followed by cold 0.15M Tris-HCl buffer (pH 7.4) and blotted between filter paper to dry.

Preparation of Tissue Homogenate

A 10% w/v homogenate was prepared in 0.15M, Tris-HCl buffer with a homogenizer fitted with a Teflon plunger and centrifuged at 1500 rpm for 15 min at 4°C. The supernatants were used for the estimation of biochemical parameters.

3.1.4 Assessment of lipid peroxidation, enzymic and non enzymic antioxidants in DLA tumor mice treated with different extracts of *Coleus forskohlii*

The body possess defence mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defences and antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and Non-enzymatic antioxidants are ascorbic acid (vitamin C), glutathione (GSH), carotenoids, flavonoids, etc. All these act by one or more of the mechanisms like reducing activity, free radical-scavenging potential, complexing of pro-oxidant metals and quenching of singlet oxygen.

Lipid peroxidation

Lipid peroxidation (LPO) refers to the reaction of oxidative deterioration of poly unsaturated lipids. Peroxidation involves the direct reaction of oxygen and lipid to form radical intermideiates and to produce semistable peroxides, which in turn damage the enzymes, nucleic acids, membranes and proteins (Gupta *et al.,* 2003).

To 0.1 ml of the tissue homogenate added 20 per cent of 0.2M tris HCl buffer of pH 7.0 and incubated in a medium (total volume 0.5ml) containing 150mM KCl (0.1ml) at 37°C for one hour. To the reaction mixture added 1 ml of TCA (20%w/v) at the end of the incubation period. After thorough mixing, 2 ml

of TBA (0.67%w/v) solution was added and the mixture was heated in a boiling water bath for 15 minutes. After cooling the tubes to the room temperature, it was centrifuged (5000 rpm for 5 minutes) to remove suspended materials. The color of the supernatant was read at 535 nm using UV-Visible spectrophotometer. Appropriate blank and control tubes were run along with the test samples. The amount of lipid peroxide was expressed as nanomoles of MDA /mg protein.

Assessment of enzymic antioxidants in mice liver

Superoxide dismutases (Kakkar *et al.*, 1984)

In the enzymatic antioxidant defence system SOD is one of the most important enzymes and scavenges $O_2^{\cdot}$ anion to form H_2O_2, thus diminshes the toxic effects due to the radical or other free radicals dervied from secondary reactions. The assay mixture contained 1.2ml of sodium pyrophosphate (0.025M, pH 8), 0.1 ml of 186 µM phenazine methosulfate, 0.3 ml of 300 µM Nitro Blue Tetrazolium (NBT) and 0.2 ml of 780 µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3.0 ml. Reaction was started by the addition of NADH. After incubation at 30◦C for 90 seconds, the reaction was stopped by the addition of 1.0 ml glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes. Centrifuged and the butanol layer was collected. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against butanol. A system devoid of the enzyme served as the control. One unit of enzyme activity is defined as the enzyme concentration, which gave 50 per cent inhibition of NBT reduction in 1 min under the assay conditions and expressed as specific activity in units/mg protein.

Catalase (Sinha, 1972)

To 0.9 ml of 0.01M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 0.2 M H_2O_2 were added after 60 sec. Two ml of

dichromate acetic acid mixture (1:3 ratio of potassium dichromate was mixed with glacial acetic acid. From this, 1.0 ml was diluted again with 4.0 ml acetic acid) was added. The tubes were kept in the boiling water bath for 10 min and the colour developed was read at 620nm. Standards of H_2O_2 in the range of 2-10 µM were taken and preceded as test with blank containing reagent alone.

The activity is expressed as µM of H_2O_2 consumed/min/mg protein.

Glutathione peroxidase (Rotruck *et al.*, 1984)

To 2.0 ml of 0.4M Tris buffer (pH 7.0), 0.2 ml of 0.4mM EDTA, 0.1 ml of 10mM sodium azide and 0.5 ml of tissue homogenate were added to the mixture, 0.2 ml of 2 mM glutathione followed by 0.1 ml of 20mM hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 min along with a tube containing all the reagents except the sample. After 10 min the reaction was arrested by the addition of 0.5 ml of 10 per cent TCA, centrifuged and the supernatant was assayed for glutathione by the method of Moron *et al.*, (1979) . The activity is expressed as µg of GSH consumed/ min/ mg protein.

Glutathion-S –transferase (Habig *et al.*, 1974)

The enzyme was assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorption at 340 nm.

The enzyme activity was determined by monitoring the change in absorbance at 340 nm in a spectrophotometer. 0.1 ml of both substrates (1mM glutathione and 1mM 1-chloro-2,4-dinitrobenzene) were taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9 ml. The reaction was started by the addition of 0.1 ml of liver homogenate to this mixture. The readings were recorded against distilled water blank for a minimum of 3 minutes. The complete assay mixture without the enzyme served as the control. Care was

taken to ensure that the final concentration of ethanol in the mixture was always less than 4 per cent.

Estimation of glutathion reductase (GR)

Glutathione Reductase was estimated by the method of David. M. Goldberg (1985). Weighed sample of tissue was homogenized in a known volume of phosphate buffer 0.6ml of buffer, 0.1ml of EDTA and 0.1ml of GSSG acts as reagent mixture to which was added 0.1ml of the homogenised sample and waited for 5 minutes, then 0.05 ml of NADPH was added, mixed thoroughly and read at 340nm for 5min against a buffer blank.

Assessment of non enzymic antioxidants in the mice liver

The antioxidants belonging to the second line of defence include glutathione (GSH), Vitamin C, Vitamin E, carotenoids, flavonoids, and tannins. Glutathione is the most abundant non protein synthesized in the liver and acts as a substrate for glutathione peroxidase enzyme. This serves as a scavenger of different free radicals. Similarly, vitamin C is an important antioxidant scavenger, which cannot be synthesized by most mammals including human (Choudhari, 2002).

Estimation of reduced glutathione

The glutathione content was determined by the method of Moron *et al.* (1979). One gram of the sample was homogenized in 5 per cent TCA to give a 20 per cent homogenate. The precipitated protein was centrifuged at 1000 rpm for 10 minute. The homogenate was cooled on ice and 0.1 ml of the supernatant was taken for the estimation. The volume of the aliquot was made upto 1.0 ml with 0.2M sodium phosphate buffer (pH 8.0), two ml of freshly prepared DTNB solution (0.6 mM in 0.2M phosphate buffer, pH 8.0) was added to the tubes and

the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 minutes. The standards (2-19 nM GSH in 1.0 ml of 5 per cent TCA) were also treated in a similar manner.

Estimation of vitamin C (Omaye *et al.*, 1979)

Liver homogenate (0.5 ml) was mixed thoroughly with 1.5 ml of 6 per cent TCA and centrifuged for 20 min at 3500 g. The supernatant was shaken vigorously with a pinch of acid–washed Norit and filtered. To 5.0 ml of the filtrate, 0.5 ml of Dinitrophenyl hydrazine reagent (2.0 g of Dinitrophenyl hydrazine was dissolved in 100 ml of 9N sulphuric acid). To this, 4.0 g of thiourea was added and mixed well. The tubes were allowed to stand at room temperature for 3 hrs. Then they were placed in ice-cold water and added 2.5 ml of 85 per cent sulphuric acid and allowed to stand for 30 min. A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly with a blank. The absorbance was read at 530 nm.

3.1.5 Estimation of serum marker enzymes in DLA tumor mice treated with different extracts of *Coleus forskohlii*

The efficacy of any hepatoprotective drug is essentially dependent on its capacity of either reducing the harmful effects or maintaining the normal physilogic function which has been disturbed by hepatotoxic agents. Measurement of the activities of serum marker enzymes, like AST, ALT, ALP can give an assessment of the liver function (Venukumar and Latha, 2002; Ulican *et al.*, 2003; Porchezhian and Ansari, 2005).

Estimation of acid phosphatase (ACP)

Pipetted out 0.5 ml of the buffered substrate (5.5mM P-Nitro phenyl phosphate in 0.05M sodium citrate buffer) into four clean, dry test tubes labeled as control, test, standard and blank. The volume of the blank was made upto

1.0ml by adding 0.5ml of distilled water. Mixed well and incubated at 37°C for 3 min. Added 0.5 ml of the working standard phenol solution to the standard tube. 0.5 ml of the serum sample was added to the test. Mixed well and incubated at 37°C for 60 min. 0.5 ml of 0.5N sodium bicarbonate was added to all the test tubes. To the control 0.5 ml of the serum was added. Added 0.5 ml of 4-aminoantipyrine (0.6 %) and 0.5 ml of potassium ferricyanide (0.24%) to all the test tubes. Mixed well after the addition of each reagent and measured the O.D at 510 nm against blank.

Estimation of alkaline phosphatase (ALP)

Pipetted out 4.0 ml of buffered substrate (50 ml of 100mM disodium phenyl phosphate + 50 ml of 100mM sodium carbonate-bicarbonate buffer) in a test tube and placed in a water bath at 37°C for a few minutes. Added 0.2 ml of serum mixed stoppered and left in the water bath for 15 minutes exactly. Removed, added 1.8 ml of diluted (1:3 dilution) phenol reagent of Folin-Ciocalteau. Set up control containing 4.0 ml buffer substrate and 0.2ml of serum to which added 1.8 ml of diluted phenol reagent. Mixed and centrifuged. Took 4.0 ml of supernatant from each and added 2.0 ml of sodium carbonate (150g Na_2Co_3/L). To the standard (2-10 µg/ml) added 2.0 ml of sodium carbonate and varying concentrations of phenol reagent. Placed the tubes in a water bath at 37°C for 15 minutes and read at 700 nm against a blank. The King-Armstrong unit corresponds to the liberation of 1.0 mg of phenol by 100 ml of serum under the assay condition.

Estimation of serum aspartate transaminase (AST) and alanine transaminase (ALT) (Reitman and Frankel, 1957)

Added 2.0 ml of serum to 1.0 ml of buffered substrate (100mM phosphate buffer and 2 mM alpha-oxoglutarate with 100mM L-aspartate included for AST

or 200 mM DL-alanine for ALT). Dissolved 15g K_2HPO_4, 2.0g KH_2PO_4 and 300 mg alpha oxo glutatarate in 700-800 ml of water and (a) for AST added 15.7 g L-Aspartate, monosodium salt or (b) for ALT added 17.8g DL alanine. In both cases the pH was adjusted to 7.4 with sodium hydroxide, mixed and incubated for 60 min for AST or 30 min for ALT at 37°C in a water bath. Then added 1.0 ml of DNPH (1mM in 1M HCl), allowed to stand at room temperature for 20 min. Added 10 ml of 400 mM sodium hydroxide solution, mixed and after 5 min , read at 500-550 nm. For the blank took 0.2 ml of serum, 1.0ml of buffer substrate and 1.0 ml of DNPH, mixed and completed as for test. A set of pyruvate standards (10-100 µg) was run similarly and plotted the absorbance against concentration.

Estimation of Serum Lactate Dehydrogenase (LDH)

The serum Lactate dehydrogenase was determined by the method of Wrolewski *et al.* (1978). Measured 2.7ml of the phosphate buffer into a cuvette and added 0.1ml of serum and 0.1ml of NADH. Allowed to stand for 20minutes at 37°C to reduce any keto acids alerady present in the serum. Then added 0.1ml of sodium pyruvate. Read the extinction for 5 minutes at inervals of 15-30 seconds at 340nm.

3.1.6 Statistical analysis

The data given in the tables and figures are the mean of the values from the number of animals specified in the respective tables and figures. Statistical significance was determined by One - way Analysis of Variance (ANOVA). 'P' value of 0.05 or less was considered as significant.

PHASE II

3.2 DETERMINATION OF THE EFFECT OF METHANOLIC EXTRACT OF *Coleus forskohlii* ROOTS ON HEMATOLOGICAL PARAMETERS, BODY WEIGHT, MEAN SURVIVAL TIME AND HISTOPATHOLOGICAL STUDIES OF LIVER

In order to find out the effect of methanolic extract of *Coleus forskohlii* on hematological parameters and antitumor activity of DLA bearing mice, the mice were divided into 24 groups of six mice each. The comparison of hematological parameters such as hemoglobin, white blood cells, lymphocytes, monocytes and differential leucocytes like basophil and neutrophil was made amongst 6 groups of mice on the 0^{th}, 10th and 20^{th} day of DLA transplantation. The other 8 groups of mice were monitored for body weight and mean survival time. The rest of the group of mice were utilized for histopathological study. The treatment protocol for the animal study for all the above groups are given in Table 6.

STANDARD

The dose of methotrexate selected was 3.5mg/Kg body weight. This was calculated by using body mass index and past experience with the drug (Dongre *et al.*, 2008).

Table 6. Treatment protocols of mice for determination of hematological parameters, body weight and surivival time

Group	Designation	Treatment
1	UC	Untreated control(1.0 ml of distilled water)
2	VC	Vehicle control (1.0 ml of 0.3% carboxy methyl cellulose).
3	ME	1.0 ml of methanolic extract of *Coleus forskohlii* root (200mg/kg body weight/day)

Group	Designation	Treatment
4	DC	DLA control (I.P. injection of $1x10^6$ cells of DLA on the 1st day)
5	DME	DLA+CF (I.P. injection of $1x10^6$ cells of DLA on the 1st day) 1.0 ml of methanolic extract of *Coleus forskohlii* root (200mg/kg body weight/day)
6	DMT	DLA+MT (I.P. injection of $1x10^6$ cells of DLA on the 1st day) 1.0 ml of methotrexate (standard)

3.2.1 Determination of hematological parameters

The hematological parameters such as WBC, hemoglobin content, lymphocyte and monocytes, differential leukocyte count was estimated from the peripheral blood of untreated, DLA control, methanol, DLA and methanol and methotrexate (standard drug) treated mice by obtaining the blood from tail vein on 0^{th} ,10^{th} and 20^{th} day.

Estimation of hemoglobin (Drabkin and Austin, 1932)

The reaction mixture in a volume of 5.02 ml contained 5.0 ml of Drabkin's reagent and 0.02 ml of blood. Varying concentrations of cyanmethhemoglobin standards (16g/dl) were also treated similarly. The reaction mixture was kept at room temperature for 5 minutes to ensure the completion of the reaction and read at 540 nm against a reagent blank.

Enumeration of White Blood Corpuscles (Sanderson and Philips, 1981)

WBC dilution fluid or Turk's fluid (glacial acetic acid 5.0 ml + Gentian violet 1 per cent + water 95 ml) was used as the diluent, which can destroy

RBC's. One drop of diluted blood was released in to the groove of the Neubauer counting chamber. The chamber square is divided into 25 smaller squares by means of triple lines: counted the number of cells in 4 smaller squares (4 corner smaller square) including those touching the upper and left line in each square and avoiding those touching the lower and right lines. White blood cells were counted, after allowing the cells to settle for 2 min. in the 4 squares of the counting area of 1 mm square. The average number of cells in 4 squares is multiplied by a factor of 2500 to give the count/mm^3 of blood.

Determination of Differential Leucocyte Count (Sanderson and Philips, 1981)

The blood film was placed in a level position and the dry blood film was covered with Leishman's stain, which should be evenly distributed over the entire slide. At the end of one minute, the quantity of buffer solution or distilled water was doubled carefully and mixed with the stain by means of the clean pipette. The film was allowed to stain for 7 to 8 min. and the excess stain was removed by washing with distilled water for 2 min. The film was dried in air and then examined microscopically with out a coverslip under oil immersion objective. For differential leucocyte counts an area was chosen where the morphology of the cells was clearly visible. The counting is done by moving the slide in an area including the central and pheripheral of the smear. A total of 100 cells should be counted in which every white cell seen was recorded in a table under the following headings: Neutrophils, Basophils, Eosinophils, Monocytes and Lymphocytes.

3.2.2 Assessment of survival rate of mice

Total number of days an animal survived from the day of tumor inoculation was counted. Subsequently the mean survival time was calculated. The percentage increase in lifespan (% ILS) was calculated as follows.

$$\text{Percentage increase in lifespan (\%ILS)} = \frac{\text{MST of treated group-MST of control group}}{\text{MST of control group}} \times 100$$

An enhancement of lifespan by 25% or more over that of the DLA control was considered as effective antitumor response (John *et al.*, 2008).

3.2.3 Determination of body weight of mice

Upon weighing the mice on the day of tumor inoculation and after once in 3 days in the post inoculation period upto 21 days, the percentage increase in weight was calculated as follows

$$\text{Percentage increase in weight} = \frac{\text{Weight of the mice on respective day}}{\text{Weight of the mice on day 0}} - 1 \times 100$$

3.2.4 Statistical analysis

The data given in the tables and figures are the mean of the values from the number of animals specified in the respective tables and figures. Statistical significance was determined by Two- way Analysis of Variance (ANOVA). 'P' Value of 0.05 or less was considered as significant.

3.2.5 Histopathological studies in liver of mice

Three groups of mice were taken for the study. I group mice were kept as untreated control. II group of mice were given intraperitoneal injection of $1x10^6$ DLA cells on the 1st day of the study. III group of mice were given intraperitoneal injection of $1x10^6$ on the 1st day of the study and 200mg/Kg bodyweight of the test substance was given orally for 21 consecutive days. Throughout the experiment all the animals were provided with their regular diet and *ad* libitum. On the 21st day of the study the mice were sacrificed by cervical dislocation and an autopsy was carried out to obtain liver of the mice. Tissue

samples were taken and preserved in 10 per cent formalin solution for a minimum of one hour. Formalin was removed from the tissue samples with running water. Dehydration of the fixed tissue was done by giving three changes acetone (each 100 ml). Cleaning of tissue from acetone was followed by three changes of xylene (each 500 ml) in a total duration of three hours. Incubation of processed tissue in melted paraffin was done by two changes for 3-4 hours in an incubator maintained at 58-60°C. Embedding of the tissue in paraffin wax was then done by immersing the tissue in molten paraffin and then cooling it to harden the paraffin. Sections of the paraffin embedded tissue were done using a microtome adjusted to 1-3 μ thickness. The paraffin sections were carefully taken on glass slides. The sections were then cleaned by immersing in xylene. The sections were stained with hematoxylin and eosin stain and screened to evaluate the morphology and cellular composition.

PHASE III

3.3 IDENTIFICATION OF THE PHYTOCHEMICALS IN *Coleus forskohlii*

3.3.1 Free radical scavenging activity of methanolic extract of *Coleus forskohlii*

In order to consider any substrate or plant extract as an effective antioxidant, it should act as antioxidant under both *in vivo* and *in vitro* conditions by decreasing the levels of oxidative damage to biomolecules (Karuna *et al.*, 2009). Several methods are used to measure the antioxidant activity of a biological material. The most commonly used ones are those involving chromogen compounds of radical nature that stimulate the reductive oxygen species. These methods are popular due to their ease, speed and sensitivity. The presence of antioxidants leads to the disappearance of these radical chromogens.

DPPH radical scavenging activity

DPPH radical scavenging is considered to be a good in vitro model widely used to assess antioxidant efficacy within a very short period of time. In its radical form, DPPH disappears on reduction by an antioxidant compound or a radical species to become a stable diamagnetic moleucule resulting the color change from purple to yellow which could be taken as the indication of the hydrogen donating ability of the test samples. The DPPH (`1,1-diphenyl 2-dipicrylhydrazyl) radical scavenging activity was measured by the spectrophotometric method (Sreejayan *et al.*,1996).To a methanolic solution of DPPH (200μM), 0.05 ml of the plant extract dissolved in methanol were added at different concentration (100-500μg). An equal amount of methanol was added to the control. After 20 min, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the formula (Prasanthkumar *et al.*, 2006).

$$\text{Inhibiton \%} = \frac{\text{(Control-test)}}{\text{Control}} \times 100$$

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao (1990) by studying the competition between deoxyribose and plant extract for hydroxyl radical generated by Fenton's reaction. The reaction mixture contained deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA (0.1mM), H_2O_2 (1mM), Ascorbate (0.1mM), KH_2PO_4-KOH buffer (20mM, pH 7.4) and various concentrations of the plant extracts in a final volume of 1.0 ml. The reaction mixture was incubated for 1 hour at 37°C.

Deoxyribose degradation was measured as thiobarbituric acid reacting substances (TBARS) and the percentage inhibition was calculated.

$$\text{Inhibiton \%} = \frac{\text{(Control-test)}}{\text{Control}} \times 100$$

Superoxide radical scavenging activity

The superoxide radical scavenging activity was measured in terms of inhibition of generation of O_2 (Sanchezmoreno, 2002). The reaction mixture consisted of phosphate buffer (50mM, pH 7.6), riboflavin (20µg/0.2 ml), EDTA (12mM), NBT (0.1mg/3ml) and sodium cyanide (3 µg/0.2ml). Plant extract of various concentrations of100-500 µg/ml were added to a total volume of 3 ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15 min against a control. The percentage inhibition was calculated by using the same formula :

$$\text{Inhibiton \%} = \frac{\text{(Control-test)}}{\text{Control}} \times 100$$

Nitric oxide radical scavenging activity (Sreejayan and Rao, 1997)

Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction (Green *et al.*, 1982). Sodium nitroprusside (5mM) in standard phosphate buffer (0.1M pH 7.4) solution was incubated with different concentration (100-500 µg /ml) of the methanol extract dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 hours. Control experiments without the test compounds, but with equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubated solution was removed and diluted with 0.5ml of Griess' reagent

(1% sulphanilamide, 2% O-phosphoric acid and 0.1% napthylethylene diamine dihydrochloride) and absorbance read at 546 nm. The percentage inhibition was calculated using the following formula.

$$\text{Inhibiton \%} = \frac{\text{(Control-test)}}{\text{Control}} \times 100$$

3.3.2 Preliminary screening for phytochemicals in the mehtanolic extract of *Coleus forskohlii*

The methanolic extract of root of *Coleus forskohlii* was analyzed for the presence of phytochemicals such as alkaloids, flavanoids, terpenoids, tannins, and saponins.

Test for alkaloids (Akhilandeshwari *et al.*, 2001)

Wagner's reagent was prepared by dissolving 2.0 gram of potassium iodide and 1.2g of iodine in 5 ml sulphuric acid and the solution was diluted to 100 ml. 10ml of the alcoholic extract of the sample was acidified by adding 1.5% v/v of HCl and a few drops of Wagner's reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloid.

Test for flavanoids (Shinoda, 1928)

To the methanolic extract with magnesium ribbon added hydrochloric acid along the sides of the test tube. A deep blue colour shows the presence of flavanoids.

Test for Terpenoids

5.0ml of the methanol extract was mixed with 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was then added to form a layer. A reddish brown precipitate formed at the interface indicated the presence of terpenoids.

Test for Tannins ((Akhilandeshwari *et al.*, 2001)

The test solution of the extract was treated with a few drops of lead acetate solution. The appearance of white precipitate indicates the presence of tannins.

Test for saponins (Akhilandeshwari *et al.*, 2001)

Five ml of aqueous extract was taken in a test tube and a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 min. A honey comb like froth formed indicates the presence of saponins.

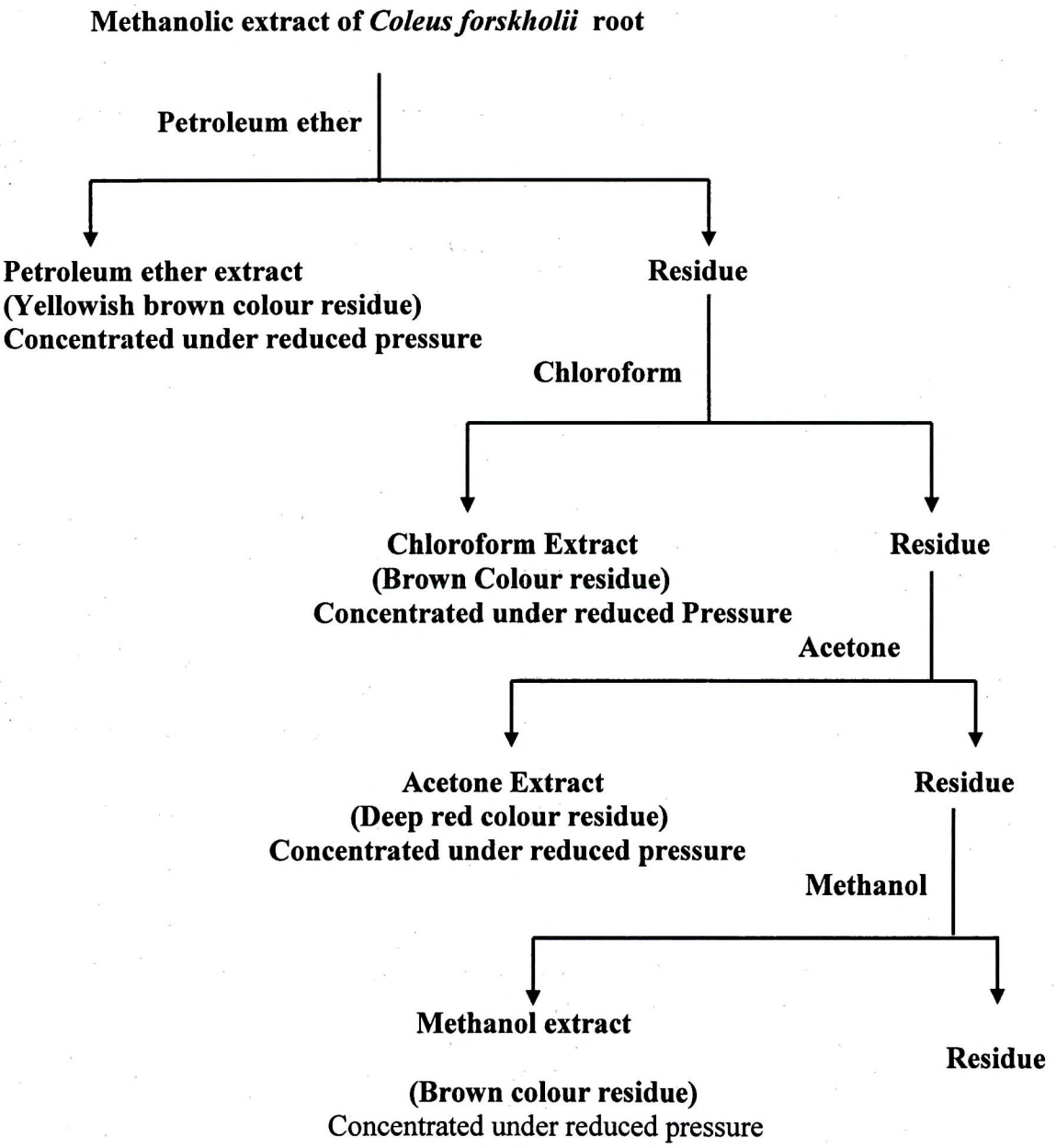
3.3.3 Fractionation of methanolic extract of *Coleus forksohlii* roots with different solvents

The methanolic extract was fractionated sequentially with petroleum ether, chloroform, acetone and methanol. Five gram of the dried methanol extract was macerated with 200ml of petroleum ether. The combined solvent portion was evaporated under reduced pressure to yield petroleum ether fraction. The residue was further macerated with 200ml of chloroform. The combined organic layer was evaporated under reduced pressure to yield chloroform fraction. The residue obtained was macerated with 200ml of acetone. The combined solvent portion was evaporated under redcued pressure to yield acetone fraction. The residue obtained was macerated with 200ml of methanol. The combined oraganic layer was evaporated under reduced pressure to yield methanol fraction.

Flow chart for the fractionation of the extract using organic solvents

The natural products chemistry has played a vital role in the development of modern harbal drugs, which are produced, standardized and clinically evaluated just like the conventional molecular drugs. These preparations which have known as phytomedicines or phytoparmaceuticals, are usually a single plant extract or an appropriate fraction thereof. Most important about these preparations is the need for standardization and reproducibility.

Figure 2. Fractionation of methanolic extract of *Coleus forskohlii* root



3.3.4 Determination of cell viability against different fractions of methanolic extract of *Coleus forskohlii*

Daltons Ascitic Lymphoma cell lines (DAL) was subcultured from stock and incubated with Blank (RPMI media) and different concentrations of an extract for 24 hour with RPMI media at 33°C. Varying concentrations (5.0-40µg) of an extract was prepared with a difference of 5 µg. Cell viability (Cell count in Lacs) was observed through haemocytometer.

MTT ASSAY

MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay was first proposed by Mossman in 1982. MTT is cleaved by mitrochondrial dehydrogenase in viable cells, yielding a measurable purple product formazan. This formazan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity.

Daltons Ascitic Lymphoma cell lines (DLA) was subcultured from stock and incubated with RPMI media. DLA cells were incubated with media (control) and with different concentrations of methanol extract of roots of *Coleus forskohlii* (treated) in humidified CO_2 (5%) incubator at 37^0C overnight. Treated cells along with control were treated with MTT dye (10µl/100µl per well of 96 well plate) and incubated at 37^0C for 3.0 hours. A stop mix (DMSO) solution was added in 100µl concentration and shook the plate for an hour at room temperature for formazan formation. Incubated for an hour to precipitate formazan. The precipitate was dissolved in acidified propanol and read the plate in plate reader at 570nm wavelength.

Percentage of live cells in each well was calculated using the formula given below.

$$\text{Percentage cell viability} = \frac{\text{Test absorbance}}{\text{Control absorbance}} \times 100$$

3.3.5 Identification of the anticancer constituents in the cytotoxic fraction of root of *Coleus forskohlii* by GC-MS

Sample analysis was performed essentially as described by Roessner *et al.* (2000) with DBMS -35 column. Sample volumes of 1µl were injected with a split ratio of 25:1 using a hot-needle technique. The GC-MS system consisted of an AS 2000 autosampler, a GC8000 gas chromatograph and a Voyager quadrupole mass spectrometer. Gas chromatography was performed on a 30m capillary column RTx-5 sil MS of 0.25µm film. Injection temperature was 230°C, the interface set to 250°C and the ion source adjusted to 200°C. The carrier gas used was helium set at a constant flow rate of 1ml/min. The temperature program was 6 min isothermal heating at 100°C followed by 1 min heating at 190°C.

The chromatograms and mass spectra were evaluated using the Masslab software

3.3.6 High performance thin layer chromatography for the identification of terpenoid profile

Sample application

2µl of test solution and 3µl of standard solanesol were loaded as 6mm band length in the 3x10 Silica gel $60F_{254}$ TLC plate using hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapor) with 1:1 n-hexane and ethyl acetate as mobile phase and the plate was developed in the 1:1 n-hexane and ethyl acetate phase up to 90mm.

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV 366 nm.

Derivatization

The developed plate was sprayed with Libermnn-Burchard spray reagent and dried at 120° C in hot air oven. The plate was photo-documented in UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Finally, the plate was fixed in scanner and scanning was done at UV 366nm. The reflected light was measured in absorbance mode. The spectral range is from 190-900 nm. Data step resolution is 25-200µm. The scanning speed was 1-100 mm/s. The spectra was recorded with a speed of 100nm/s.The peak table, peak display and peak densitogram were noted.

3.3.7 Identification of the phytochemicals present in the chloroform-methanolic fraction of *Coleus forskohlii* by thin layer chromatography

Thin layer chromatography technique is easy to perform and require simple apparatus. The mixture of compounds to be separated is placed near one of the TLC plate and allowed to dry. The plate is then placed with its end dipping in the solvent mixture, taking care that the sample spot is not immersed in the solvent. As the solvent moves towards the other end of the plate, the test mixture separates in to various components. This is known as development. The plate is removed after an optimal development time and dried and the spots are detected using a suitable location reagent. The silica gel acts as an inert support, the

interstices of which hold the more polar phase of the solvent mixture which thus acts as the stationary phase, the less polar phase acting as the mobile phase. Separation results from differences in partition equilibrium of the components in the mixture. However, the silica gel interacts with the components and these effects the separation.

Preparation of the plate

The precoated alumina silica gel G60 plates F254 obtained from EMerck. The aluminium sheets were activated by drying it in hot air oven at 110 °C for 30 minutes.

Application of the extracts for separation

The extract (3.0 µl) was taken in a capillary tube and it was spotted on a TLC plate, 2 cm above its bottom. The standard solution was prepared by dissolving accurately weighed forskolin (1 mg) in HPLC grade methanol (1ml). The solutions for application was between 0.1 to 1.0 per cent strength. The starting points were equally sized as far as possible and had a diameter ranging from 2 to 5 mm. They were then developed in solvent systems and the spots were identified. The solvent systems used for the identification of the compound is Toluene : ethylacetate (80:20,v/v) and anisaldehyde sulphuric acid was used as the spray reagent (Inamder,1984).

3.3.8 Analysis of active components by high performance liquid chromatography (HPLC)

The standard forskolin was obtained from Sigma Chemical Co. (Chromadex, USA). All HPLC analytical grade solvents were obtained from Fischer Scientific (Suwanee, GA). A quantity of 0.1ml of the standards were drawn from 250µg / ml of the standards and mixed with equal quantity of methanol and injected in LC- 10 AT HPLC system (Shimadzu, Kyota, Japan) for the elution of standard peaks.

Separation and identification of the compounds

To separate and identify the active compounds in the *Coleus forskohlii* extracts, reverse phase C_{18} high performance liquid chromatography (HPLC) was used. The Pinnacle II C_{18} column (150 x 4.6mm, 5 µm; Restek, PA) was connected to the LC- 10 AT HPLC system (Shimadzu, Kyota, Japan) and equilibriated with 0.05 percent aqueous triflouroacetic acid (TFA). 50µl of methanolic extracts were injected and eluted with 65:35 (pH 2.5) acetonitrile : water which was pumped isocratically at a flow rate of 1 ml/min. Collected 1.0 ml fractions of the eluant and the absorbance of the eluant was scanned from 200 to 500 nm by a SPD-M10V photodiode array detector (PDA).