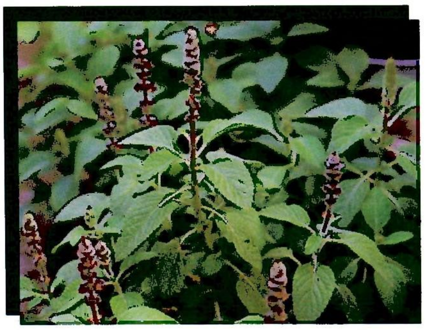


Results and Discussion

4 RESULTS AND DISCUSSION

Cancer accounted for 7.6 million deaths globally in 2008, it is more than the deathes due to AIDS, malaria, and tuberculosis combined. The increasing magnitude of cancer mortality throughout the world, claiming over six million lives each year and the failure of conventional chemotherapy of advanced invasive diseases to effect major reductions in cancer mortality rates indicate that new approaches are critically needed to control cancer (Sengupta *et al.*, 2004).

There is a growing interest in the pharmacological evaluation of various plants used in the traditional Indian systems of medicine. The plant kingdom plays a major role in the life of human beings and animals. The plants are one of the important medicinal sources, used in the treatment of various diseases, including tumors. Quite a number of studies have been carried out on the ethnomedicinal plants (indigenous system of medicine) of India; and a few of theses plants have attracted the interest of scientists so as to investigate them as a remedy for tumors. Such plants may promote host resistance against infection by restabilizing body equilibrium and conditioning body tissues. Ethnomedicinal plants are easily available, cheap and possess no toxicity (Sivakumar *et al.*, 2008).

At present, the scientific community is interested in elucidating the role of several therapeutic modalities, considered as elements of complementary and alternative medicine in the control of certain diseases. Plant derived natural products such as polyphenols, terpenoids and steroids have received considerable attention in recent years due to their diverse pharmacological activity (De feudis *et al.*, 2003, Takeoka and Dao, 2003).

One of the main reasons for the rapid progression of human cancer is the ability of tumor cells to escape from the immune surveillance mechanism of the body. Cancer cells may secrete immunosuppressive factors to modify the host's immune responses, thereby impairing the inflammatory responses, chemotaxis of macrophages and the complementary cascade (Guruvayoorappan and Kuttan, 2007).

Oxidative stress occurs in a cellular system when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system. Oxidative stress plays an important contributory role in the process of aging and pathogenesis of numerous diseases like cancer. Improved antioxidant status helps to minimize the oxidative damage and thus delays or decreases the risk for developing many chronic age related, free radical induced diseases (Karuna *et al.*, 2009).

The generation and the subsequent involvement of free radicals in cancer have prompted the researcher to study the antioxidant potential of *Coleus forskohlii.* In order to consider a plant extract as an effective antioxidant, it should act as such under both *in vivo* and *in vitro* conditions and it should render lymphocytes more resistant to oxidative challenges. Therefore, the present study has been designed to explore the effect of the different extracts of the root of *Coleus forskohlii* on plasma antioxidant status and hematological parameters and also to evaluate cell viability of DLA cells against the plant extract.

The results pertaining to the study **"The antioxidant and the antitumor potential of roots of *Coleus forskohlii* in *Balb/C* mice with DLA tumor"** are presented under the following headings:

PHASE I

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

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

4.1.2 Effect of *Coleus forskohlii* root extracts on the liver of DLA induced mice

a. Lipid peroxidation

b. Enzymic antioxidants

c. Non enzymic antioxidants

4.1.3 Effect of *Coleus forskohlii* root extracts on serum marker enzymes

PHASE II

4.2 EFFECT OF METHONALIC EXTRACT OF THE ROOTS OF *Coleus forskohlii* ON HEMATOLOGICAL PARAMETERS IN DLA TUMOR INDUCED MICE

4.2.1 Hemoglobin

4.2.2 White blood cell

4.2.3 Lymphocyte count

4.2.4 Monocyte count

4.2.5 Differential count

4.2.6 Survival rate

4.2.7 Body weight of DLA induced mice

4.2.8 Histopathology of liver

4. IDENTIFICATION OF THE PHYTOCHEMICALS IN THE METHANOLIC EXTRACT ROOT OF *Coleus forskohlii*

4.3.1 Free radical scavenging activity of the methanolic extract of the roots *Coleus forskohlii*

4.3.2 Qualitative detection of phytochemicals in the methanolic extract of the roots of *Coleus forskohlii*

4.3.3 Determination of DLA cell viability against chloroform fraction of the methanolic extract of the roots of *Coleus forskohlii* by cell viability and MTT assay

4.3.4 Identification of the phytochemicals in the chloroform methanolic fraction of roots of *Coleus forskohlii* by HPTLC

4.3.5 Identification of the anticancer constituents in chloroform fraction of the methonolic extract of *Coleus forskohlii* roots by by GC-MS

4.3.6 Identification of the constituents present in chloroform fraction of the methanolic extract of *Coleus forskohlii* roots by TLC and HPLC

PHASE I

4.1 TOXICOLOGICAL EVALUATION AND DETERMINATION OF ENZYMIC AND NON ENZYMIC ANTIOXIDANTS IN DIFFERENT EXTRACTS OF THE ROOTS OF *Coleus forskohlii*

One of the best approaches in the search for anticancer agents from plant resources is the selection of the plants based on the ethnomedicinal leads and testing the selected plant's efficacy and safety in the light of modern sciences (Gupta *et al.*, 2004).

Plants contain a large number of biologically active chemicals. Some of them have been found to be extremely useful for treating various human diseases (e.g. digitoxin, colchicines and atropines). However, some plant constituents produce adverse health effects. The onset of these adverse effects can be quite sudden or take some time to develop. Fortunately, among the thousands of plants in the environment, relatively a few cause life- threatening illnesses when ingested (Bnouham *et al.*, 2006).

The different extracts viz., petroleum ether, chloroform, acetone and methanol were subjected to acute toxicity test to find out the lethal effect, if any, of the extracts and the LD_{50}.

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

Acute toxicity is usually defined as the adverse changes occurring immediately or in a short time following a single or short period of exposure to a substance or substances or as adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given within 24 hours. An adverse effect is "any effect that results in the impairment of function and / or biochemical lesions that may affect the performance of the whole organism or that reduces the organ's ability to respond to an additional challenge". Consequently, a chemical that enters the organism *via* the oral route during a restricted time and produces any adverse effect with little delay is orally and acutely toxic. However, the term acute oral toxicity is most often used in connection to lethality and LD_{50} determinations. In screening drugs, determination of LD_{50} is usually an initial step in the assessment and the evaluation of the toxic characteristics of a substance (Akhila, 2007).

If the dose dependent lethality incidence is determined in a precise manner, it is usually expressed as an LD_{50}. This is defined as the statistically derived dose that, when administered in an acute toxicity test is expected to cause death in 50% of the treated animals in a given period. The acute toxicity studies were conducted as per the Organization for Economic Co-operation and Development guidelines (OECD, 2001) where the limit test dose of 2000 mg/kg was used. The following chemical labeling and classification of acute systemic toxicity based on oral LD_{50} values are recommended by the OECD : very toxic-5 mg/kg body weight; toxic -5 to 50 mg/kg body weight; harmful- 50 to 500 mg/kg body weight and no label - 500 to 2000 mg/kg body weight.

As per the procedure OECD (2001), the test substance may be given at four fixed dose levels to animals under the study. The objective is to identify a dose that produces clear signs of toxicity but no mortality. Depending on the results of the first test, either no further testing is needed or a higher or a lower dose is tested. If mortality occurs, retesting at a lower dose level is necessary (except if the original dose chosen is 5mg/kg body weight). If no signs of toxicity occurs at the initial dose, it is necessary to retest at a higher dose level. The results are thus interpreted in relation to animal survival and evident toxicity and it becomes possible to assign the chemical to one of the OECD classification categories. The absolute LD_{50} value for a compound varies among different laboratories and these variations have been attributed to differences in protocol details, animal strains, caging and test chemical source. The adverse effects and the number of deaths were recorded within 24 hours. The results of the acute toxicity studies conducted for the dose levels 25, 50, 500, 2000mg/kg body weight of the mice were presented in the Table 7.

Signs recorded during acute toxicity studies

The signs recorded during 24 hrs of acute toxicity were alertness, grooming, restlessness, touch response, torch response, pain response, tremors, convulsions, writhing reflexes, gripping strength, corneal reflex, lacrimation, urination, skin colour and salivation. With the dosage of 25 mg/kg, the animals were restless and died at the 11^{th} hour which indicated that the dose was toxic at 25 mg/kg. Mortality is the main criteria for assessing the acute toxicity (LD_{50}) of any drug. The extracts administered at a concentration of 25 mg/kg to the hind limb of mice resulted in the death of this group which is significant to the study. There was no mortality recorded at a dose of 50 and 500 mg/kg body weight as represented in the Table 7. The test substances were tried again with 2000mg/kg body weight. The LD_{50} of the test substance as per OECD guideline falls under category 5 (LD_{50} 500 to 2000 mg/kg). High concentration of the substance was not found to be toxic to the mice.

TABLE 7. EFFECT OF *Coleus forskohlii* AT THE DOSAGE 25mg, 50mg, 500mg and 2000mg/Kg BODY WEIGHT

S.No	Response	OBSERVATION							
		25 mg/kg b.w		50 mg/kg b.w		500 mg/kg b.w		2000 mg/kg b.w	
		BD	AD	BD	AD	BD	AD	BD	AD
1.	Alertness	N	N	N	N	N	N	N	N
2	Grooming	Ab	Ab	Ab	Ab	Ab	Ab	Ab	Ab
3	Restlessness	Ab	P	Ab	Ab	Ab	Ab	Ab	Ab
4	Touch response	N	N	N	N	N	N	N	N
5	Torch response	N	N	N	N	N	N	N	N
6	Pain response	N	N	N	N	N	N	N	N
7	Tremors	Ab	Ab	Ab	Ab	Ab	Ab	Ab	Ab
8	Convulsions	Ab	Ab	Ab	Ab	Ab	Ab	Ab	Ab
9	Writhing reflex	N	N	N	N	N	N	N	N
10	Gripping strength	N	N	N	N	N	N	N	N
11	Corneal reflex	N	N	N	N	N	N	N	N
12	Lacrimation	Ab	Ab	Ab	Ab	Ab	Ab	Ab	Ab
13	Urination	N	N	N	N	N	N	N	N
14	Skin colour	N	N	N	N	N	N	N	N
15	Salivation	N	N	N	N	N	N	N	N

NOTE : BD: Before the oral Dosage AD: After the oral Dosage

bw: body weight

N : Normal Ab : Absent P : Present

It is evident from the above study that *Coleus forskohlii* at the doses of 50, 500 and 2000mg/Kg body weight administered to the mice has caused no visible toxicity. In an acute oral toxicity study by Adenaye and Agbaje (2008) *Morinda lucida* leaf extract was documented to be non-lethal in rats at a dose of 2000mg/kg body weight.

The LD_{50} of *Cinnomomum iners* leaves extract was estimated to be more than 5000mg/kg. As the highest dose used in the analgesic study was 10 fold lesser than the dose used in acute toxicity study, the doses of 100, 200 and 500mg/kg were given to the rats in the analgesic study which were found to be safe (Mustaffa *et al.*, 2010).

According to OECD guidelines for acute oral toxicity, an LD_{50} dose of 2000mg/Kg and above is categorized as unclassified and hence the drug is found to be safe (Joshi *et al.*, 2007).

This finding is in agreement with the fact that no side effects have been reported while using this plant in treating various ailments.

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.

4.1.2 Effect of *Coleus forskohlii* root extract on the liver of DLA induced mice

The presence of tumor in the humans or experimental animals is known to affect many functions of the vital organs especially the liver, even when the site of the tumor does not interfere directly with the organ functions (De wys, 2000). Liver plays an important role in the modulation of the process of carcinogenesis, as it is the primary site for biotransformation of xenobiotics including carciogens and anticancer drugs (Koul, 2006).

4.1.2a. Lipid peroxidation (LPO)

One of the indices of oxidative stress is lipid peroxidation which can be described as the degeneration of polyunsaturated fatty acids in cell membranes through ROS (Reactive Oxygen Species) attacks. The end products of LPO include 4-hydroxy nonenal and malondialdehyde which have been investigated by using various techniques (Bindu *et al.*, 2004, Gonene *et al.,* 2005, Centinkaya *et al*., 2005). Human body has an array of enzymatic antioxidant defence mechanisms that protect cells and tissues from free radical induced lipid peroxidation. Measurement of plasma or serum lipid peroxidation helps to assess the extent of tissue damage.

Considerable attention has recently been focused on the interrelationship of lipid peroxidation processes, free radical-related reactions and the development of a variety of pathological events. It is well established that lipid peroxidation is the deleterious result of free radical reactions, leading to the disruption of biomembranes and the dysfunctions of cells and tissues. Therefore, lipid peroxidation is a crucial step in the pathogenesis of free radical related disease states, including inflammatory injury, gastrointestinal disease, cardiovascular disease, nervous system disorder, diabetes mellitus and cancer (Cho *et al.,* 2003). The levels of lipid peroxidation of mice bearing DLA cells and mice treated with *Coleus forskohlii* extracts are presented in Table 8.

TABLE 8

LEVELS OF LIPID PEROXIDATION IN THE LIVER OF MICE

Groups	Treatment		Lipid peroxidation#
1	Untreated control	UC	0.83 ± 0.03^{c}
2	Vehicle control	VC	0.85 ± 0.01^{c}
3	Petroleum ether	CPE	0.90 ± 0.01^{c}
4	Chloroform	CCF	0.80 ± 0.05^{c}
5	Acetone	CA	0.86 ± 0.04^{c}
6	Methanol	CM	0.95 ± 0.01^{c}
7	DLA Control	DC	4.58 ± 0.43^{a}
8	DLA+Petroleum Ether extract	DPE	3.31 ± 0.03^{b}
9	DLA+Chloroform extract	DCF	3.83 ± 0.20^{b}
10	DLA+Acetone extract	DA	3.67 ± 0.14^{b}
11	DLA+Methanol extract	DM	0.89 ± 0.07^{c}
12	DLA+Methotrexate extract	DM	0.95 ± 0.06^{c}
	CD (5%)		**0.31**

Values are mean±SD of six mice
Means followed by common superscripts donot differ significantly at 5% level.
n moles of MDA/mg protein.

The level of lipid peroxidation in liver tissue was significantly higher (P<0.05) in DLA control mice, (4.58±0.43 n moles of MDA/ mg protein) compared to the untreated control mice (0.83±0.034 n moles of MDA/mg protein). Treatment with methanolic extract of *Coleus forskohlii* significantly decreased (P<0.05) the lipid peroxidation levels (0.89±0.07 n moles MDA/mg protein) of liver tissue, whereas treatment with petroleum ether extract

(3.31±0.03), chloroform extract (3.83±0.02) and acetone extract(3.67±0.014) showed a decrease in LPO levels which was not significant compared to methanolic extract treated mice. The methotrexate standard treated mice showed a significant ($P<0.05$) reduction in lipid peroxidation level (0.95±0.06 n moles of MDA/mg protein) which is similar to that for methanolic extract treated animals. Several authors suggested that MDA can be regarded as an important biomarker of chemoprevention in experiment rodent model of carcinogenesis.

MDA, the end product of lipid peroxidation, was also reported to be higher in carcinomatous tissue than in normal organs (Sivakumar, 2008). Malondialdehyde, a major end product and index of lipid peroxidation, cross links DNA, proteins and nucleotides on the same and opposite strands thereby promoting carcinogenesis. Elevation in the level of lipid peroxide observed in the present study is in accordance with the earlier report by Shan *et al.* (2001) showing an increased level of super oxide dismutase, catalase levels and lipid peroxide in Aflotoxin B_1 injected animals. Pretreatment with *Coleus forskohlii* extract effectively controlled the rate of lipid peroxidation, which suggests the beneficial effect of the extract against DLA mediated free radical formation.

In the present study, ROS might have induced significant lipid peroxidation in the mice liver mitochondria as measured by LOOH, an unstable intermediate, which further breaks down to stable aldehydes and reacts with thiobarbituric acid (TBA) to form TBARS, the final stable end product (Tilak and Devasagayam, 2003).

It has been observed that the activities of enzymatic antioxidants tend to decline to the minimum point when the TBARS values are at peak or vice versa. This indicates that the activities of enzymatic antioxidants and TBARS are inversely correlated.

4.1.2b. Enzymic antioxidants

The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. These act on different oxidants as well as in different cellular compartments. One important line of defence is a system of enzymes including glutathione peroxidases, superoxide dismutases and catalase which will decrease the concentrations of most harmful oxidants in the tissues.

The highly reactive free radicals and oxygen species (ROS) that are present in the biological systems from a wide variety of sources may oxidize proteins, lipids or DNA and can initiate degenerative disease (Bhaskar, 2008). To protect against the toxic effects of ROS and to modulate the physiological effects of ROS, the cell has developed an intricately regulated antioxidant defence system. The antioxidant system is very complex, being composed of small molecular weight antioxidant compounds (vitamins E,C and A, glutathione, glutaredoxin, and thioredoxin systems); primary (manganese, copper and zinc superoxide dismutase, catalase and glutathione peroxidase) and secondary antioxidant enzymes (glutathione reductase and glucose-6-phosphate dehydrogenase) (Oberley, 2002).

Superoxide Dismutase (SOD)

Living cells have numerous defence systems to counteract the deleterious effect of ROS/RNS. In defence systems, superoxide dismutase, is an enzyme that repairs cells and reduces the damage done to them by breaking the anion into oxygen and hydrogen peroxide. Catalase and glutathione peroxidase remove H_2O_2 by converting it to water and oxygen (Zelco *et al.,* 2002).

The effect of different extracts of the roots of *Coleus forskohlii* on superoxide dismutase and catalase activities in the liver of mice induced with DLA cells is given in Table 9.

TABLE 9

ACTIVITIES OF SUPEROXIDE DISMUTASE AND CATALASE IN THE LIVER OF MICE

Groups	Treatment		Superoxide dismutase[#]	Catalase [$]
1	Untreated Control	UC	4.42 ± 0.28[a]	50.56 ± 1.88[a]
2	Vehicle Control	VC	4.39± 0.19 [a]	49.43±0.10 [a]
3	Petroleum ether	CPE	4.29±0.02 [a]	48.76±0.03 [a]
4	Chloroform	CCF	4.04±1.50 [a]	49.75±0.05 [a]
5	Acetone	CA	3.18±0.20 [a]	50.00±0.90 [a]
6	Methanol	CM	4.40±0.34 [a]	47.45±0.75 [a]
7	DLA Control	DC	2.10 ± 0.20[e]	20.87 ± 1.13[c]
8	Petroleum Ether extract	DPE	2.45 ± 0.04[c]	21.99 ± 1.65[c]
9	Chloroform extract	DCF	2.29 ± 0.02[d]	22.83 ± 1.76[c]
10	Acetone extract	DA	2.19 ± 0.02[e]	21.44 ± 0.57[c]
11	Methanol extract	DM	3.96 ± 0.12[b]	48.46 ± 2.86[a]
12	Methotrexate	DMT	4.5 ± 0.20[a]	42.00 ±1.21[b]
	CD (5%)		**0.19**	**2.20**

Values are mean±SD of six mice

Means followed by common superscripts donot differ significantly at 5% level.

[#] values are expressed for 50% inhibition of Nitroblue tetrazolium /min/mg protein

[$] values are expressed in µmoles of H_2O_2 consumed/min/mg protein

The activity of SOD was significantly decreased in DLA bearing mice (2.10 NBT/min/mg protein) when compared to the untreated control mice (4.42 NBT/min/mg protein). Among the different extracts of *Coleus forskohlii,* the methanolic extract showed significant increase ($P<0.05$) in SOD activity, compared to chloroform, acetone and petroleum ether extracts.

SOD is a ubiquitous chain breaking antioxidant found in all aerobic organisms. It is a metalloenzyme, widely distributed in all cells and plays an important protective role against ROS induced oxidative damage. It has been demonstrated both *in vivo* and *in vitro* that the antioxidant enzyme activities are altered in cancer. The manganese superoxide dismutase (MnSOD), a mitochondrial antioxidant enzyme, is lowered in most types of primary cancers and cancer cell lines (Oberley and Oberley, 2000).

Due to increased lipid peroxidation the level of free radicals overcomes the saturation point. The decreased SOD activity in the present study may be due to highly reactive oxygen metabolites (ROM) produced. Over production of O· or $OH^{\cdot}$ free radicals during oxidative stress due to cancer causes membrane damage. The results of the present study are in accordance with the finding of Isabel *et al* (2002) that the membrane damage due to over production of free radicals possibly causes conformational changes and hence inactivates enzymes such as SOD.

Catalase (CAT)

Catalase is an enzyme that catalyzes the conversion of hydrogen peroxide to water and oxygen using either an iron or manganese cofactor (Chelikani *et al.*, 2004). This protein is localized to peroxisomes in most eukaryotic cells. Catalase is an unusual enzyme since hydrogen peroxide is its only substrate, it follows a ping pong mechanism. Here its cofactor is oxidized by one molecule for hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Hiner, 2002).

The catalase activity was decreased significantly ($P<0.05$) in DLA control mice (20.87 µmoles of H_2O_2 consumed/min/mg protein) when compared with untreated control mice (50.56 µmoles of H_2O_2 consumed/min/mg protein) .The treatment with methanolic extract brought back the catalase activity to normal level 48.46 µmoles of H_2O_2 consumed/min/mg protein) compared to other extracts which was also found to be higher than the standard drug (42 µmoles of H_2O_2 consumed/min/mg protein).

Catalase is a free radical scavenging system which is present in all the major organs of animals and human beings and is especially concentrated in the liver and the erythrocytes. Both SOD and catalase play an important role in the elimination of ROS derived from the redox process of xenobiotic in liver tissue. It has been suggested that catalase and SOD are easily inactivated by lipid peroxides and ROS. It has been reported that DLA bearing mice showed decreased levels of SOD activity and this might be due to loss of Mn^{++} SOD activity in the liver (Sivakumar *et al.*, 2008).

The decreased catalase activity observed in the present study might be due to high reactive oxygen molecules production especially $O_2^{\cdot -}$ which itself affects directly the CAT activity. Under high rate of free radicals input, the enzyme inactivation prevails and the enzymatic activities are reduced, leading to autocatalysis of oxidative damage process.

Lowered activities of enzymatic antioxidants (SOD, CAT and GPx) have been reported in various malignancies including mammary gland carcinomas. Low activity of antioxidant enzymes cause the accumulation of hydrogen peroxide and superoxide anion radical in tumor cells. The lowered activity of SOD and catalase in DLA induced mice was due to inactivation of both these enzymes by the singlet oxygen generated. Furthermore, the toxic oxygen radicals might react with intrinsic or extrinsic radical scavenger forming a secondary free radical that by itself can produce injury. SOD reacts with superoxide radicals and converts them to hydrogen peroxide. Excessive amounts of these metabolites start lethal chain reactions, which oxidize and disable structures that are required for cellular integrity and survival (Sampson *et al.,* 2001).

Tumor cells have increased rate of metabolism compared to normal cells which would typically lead to increased amount of reactive oxygen species. Alternatively, it could be due to post translational modification of the enzymes by free radicals (Halliwell, 2003).

Glutathione peroxidases (GPx)

GPx plays a critical role in maintaining redox status during acute oxidative stress. This protective role of GPx is coordinated with other antioxidant enzymes (Cheng *et al.*, 1999).

The utilization of GSH might be mediated by GST or GPx system. Unavailability of GSH caused a reduction in the activity of GPx and GST has been reported. GST also possesses peroxidase activity and can directly attack the peroxides (Mari *et al.,* 2001).

The effect of different extracts of the roots of *Coleus forskohlii* on Glutathione peroxidase, Glutathione S transferase and Glutathione reductase in DLA induced mice is shown in Table 10.

Glutathione peroxidase activity was reduced to 4.26 µg of glutathione/min/mg protein in control DLA mice when compared to untreated control mice which was 7.26 µg of glutathione/min/mg protein. The petroleum ether, chloroform and acetone extracts treated mice recorded a non significant GPx activity when compared to the untreated control mice. The methanolic extract treated mice showed a significant ($P<0.05$) increase in GPx activity (6.53 µg of glutathione/min/mg protein). The methotrexate standard treated mice also showed a significant raise (6.95 µg protein of glutathione /min/mg of protein) in GPx activity.

TABLE 10

ACTIVITIES OF GLUTATHIONE PEROXIDASE AND GLUTATHIONE S-TRANSFERASE AND GLUTATHIONE REDUCTASE IN THE LIVER OF MICE

Groups	Treatment		Glutathione peroxidase#	Glutathione s-transferase$	Glutathione reductase$
1	Untreated Control	UC	7.26 ± 0.19[a]	41.74 ± 0.50[d]	35.64 ± 0.21[a]
2	Vehicle Control	VC	7.14 ± 0.09[a]	41.50 ± 0.70[d]	33.63 ± 2.18[a]
3	Petroleum ether	CPE	7.21 ± 0.02[a]	40.89 ± 0.06[d]	34.17 ±1.36[a]
4	Chloroform	CCF	7.13 ± 0.05[a]	42.35 ±1.05[d]	34.33 ±1.32[a]
5	Acetone	CA	7.24 ± 0.01[a]	41.4 7± 0.05[d]	34.50 ±0.41[a]
6	Methanol	CM	6.95 ± 0.21[a]	42.35 ± 2.00[d]	35.60 ±1.10[a]
7	DLA Control	DC	4.26 ± 0.15[f]	73.44 ± 3.03[a]	20.63 ± 2.18[d]
8	Petroleum Ether extract	DPE	4.42 ± 0.13[e]	71.40 ± 1.03[a]	33.05 ±1.36[b]
9	Chloroform extract	DCF	4.71 ± 0.17[d]	69.52 ± 2.22[b]	25.12 ± 0.56[c]
10	Acetone extract	DA	4.40 ± 0.45[e]	70.38 ± 1.27[b]	26.08 ± 0.72[c]
11	Methanol extract	DM	6.53 ± 0.16[c]	45.36 ± 1.28[c]	34.67 ±0.52[a]
12	Methotrexate	DMT	6.95 ± 0.21[b]	47.56 ± 2.85[c]	35.90 ± 1.51[a]
	CD (5%)		**0.20**	**2.51**	**1.53**

Values are mean±SD of six mice

Means followed by common superscripts donot differ significantly at 5% level.

# µg of glutathione /min/mg of protein
$ n moles of CDNB complexed/min/mg/protein

GPx catalyses the reduction of H_2O_2 at the expense of reduced GSH there by protecting mammalian cells against oxidative damage. The decreased activity in the present study might be due to the less availability of the substrate GSH. GSH could directly scavenge and eliminate the toxicity, thereby minimizing the toxic effects which definitely indicates the antioxidant potency of the plant extract due to the presence of the flavonoids and the terpenoids.

The major H_2O_2 removing enzyme in mammalian cells is GPx. In addition, GPx acts as regulatory enzyme in the metabolism of lipid peroxides. GSH, the substrate of GPx, may additionally exert direct antioxidant effects. Its reaction with superoxide anion and H_2O_2 are slow. But it reacts fairly fast with peroxy radicals ($ROO^{\cdot}$) and is a powerful scavenger of certain RNS such as peroxy nitrite ($ONOO^{\cdot}$) (Halliwel, 1996).The treatment with the extract of *Coleus forskohlii* afforded the maximum protection to the antioxidant enzymes such as SOD, CAT and GPx.

Glutathione-S- Transferase (GST)

Reactive oxygen species are tumorigenic by virtue of their ability to increase cell proliferation, cellular migration and also by inducing DNA damage leading to genetic lesions that initiate tumorogenicity and sustain subsequent tumor progression. A decrease in serum GST in early cancer can trigger the initiation and progression of cancer (Tew, 2005). The enhanced antioxidant capacity made the tumor tissue less susceptible to oxidative stress conferring special growth advantage (Kolangiappan, 2003).

GST was increased in the DLA control mice (73.44 n moles of CDNB complexed/min/mg protein) significantly ($P<0.05$), compared to the untreated control mice (41.74± 0.50 n moles of CDNB complexed/min/mg protein). The petroleum ether (71.40 n moles of CDNB complexed/min/mg protein), chloroform (69.52 n moles of CDNB complexed/min/mg protein) and acetone (70.38 n moles of CDNB complexed/min/mg protein) extracts treated mice recorded a non significant GST activity, compared to the control mice ($P<0.05$). The methanolic extract treated mice showed a significant decrease ($P<0.05$) in GST activity (45.36 n moles of CDNB complexed/min/mg/protein) which was comparable with the standard, methotrexate treated mice (47.56 n moles of CDNB complexed/min/mg protein).

The methanolic extract could activate GPx and GST activities. Many investigators have suggested that GST offers protection against lipid peroxidation by promoting the conjugation of toxic electrophiles with GSH (Jokoby, 1988). An increased activity of GST was also reported in the methanolic extract of *Ipomea obscura* treated animals. Alteration in circulating oxidants and free radical scavengers like GST has been involved in the treatment of various malignancies (Nagini *et al.*, 1998). An increase in GST in oral tumor tissue has been reported by Saroja *et al.* (1999) and Subapriya *et al.* (2002).

GST also plays a critical role in detoxification mechanism that functions primarily in conjugating functionalized P_{450} metabolites with endogenous ligand (GSH) favouring their elimination from the organism. There are convincing evidences to support induction of GST and protection against a wide spectrum of cytotoxic, mutagenic and carcinogenic agents (Reeves, 2001).

Glutathione reductase (GR)

GR activity was significantly ($P<0.05$) reduced to 20.63 n moles of CDNB complexed/min/mg protein in the DLA control mice compared to the untreated contol mice. The methanol extract treated mice showed a significant ($P<0.05$) increase in GR activity followed by petroleum ether extract. The standard methotrexate has shown a significant increase in GR activity to 35.90 n moles of CDNB complexed/min/mg protein.

4.1.2c Non enzymic antioxidants

Non enzymatic antioxidants play a crucial role in scavenging or disposing lipid peroxidation byproducts when they are excessively generated in the body. Tumor tissues sequester nutrients and antioxidants from circulation to combat the deleterious effects of reactive oxygen species and for their abnormal growth (Kolangiappan *et al.*, 2003). Ascorbic acid, the most important antioxidant in the plasma, scavenges a variety of free radicals. It protects cell membranes against lipid peroxidation mediated oxidative stress both by free radical scavenging and by membrane stabilizing mechanisms (Manoharan *et al.*, 2002). Reduced glutathione is the most powerful intracellular antioxidant and the molar ratio of reduced glutathione to oxidized glutathione serves as an important marker of the antioxidative capacity of the cell (Kavitha and Manoharan, 2006; Vijayamalini and Manoharan, 2004). Decreased levels of vitamin E, vitamin C and GSH were reported in both the human and the experimental carcinogenesis. Lowered levels of vitamin C and reduced glutathione in plasma and erythrocytes were probably due to their utilization by malignant tumor (Manoharan et al., 2009).

Glutathione

GSH acts as substrate for the antioxidant enzymes GPx, GST and GR which are involved in the termination of peroxidation by removing peroxides generated within the cell. The three enzymes GPx, GR and GSH act in concerted way with Glucose 6 Phosphate Dehydrogenase (G6PD) supplying reducing equivalent needed for GR activity which in turn maintains adequate concentration of GSH required for GPx activity. GSH, being the most important biomolecule against chemically induced toxicity can participate in the elimination of reactive intermediates by reduction of hydro peroxides in the presence of GPx (Guruvayoorappan *et al.*, 2007). GSH also functions as a free radical scavenger and helps in the repair of radical caused biological damage. It can react chemically with singlet oxygen, super oxide and hydroxyl radical and therefore function directly as a free radical scavenger. GSH stabilizes membrane structure by removing acyl peroxide formed by lipid per oxidation.

The effect of administration of the root extracts of *Coleus forskohlii* on non enzymic antioxidants glutathione and ascorbic acid in the control and the DLA induced mice is depicted in Table 11.

TABLE 11

LEVELS OF GLUTATHIONE AND ASCORBIC ACID IN THE MICE LIVER

Groups	Treatment		GSH mg/g tissue	Ascorbic acid mg/100g tissue
1	Untreated control	UC	4.47 ± 0.03[a]	99.61 ± 4.05[a]
2	Vehicle Control	VC	4.53± 0.02 [a]	98.42 ± 3.35 [a]
3	Petroleum ether	CPE	4.41±0.05 [a]	99.01± 5.41 [a]
4	Chloroform	CCF	4.49±0.01 [a]	100.00± 3.98 [a]
5	Acetone	CA	4.51±0.05 [a]	101.41± 8.45 [a]
6	Methanol	CM	4.40±0.04 [a]	97.79± 1.55 [a]
7	DLA Control	DC	0.7 ± 0.04[e]	64.71 ± 3.40[b]
8	Petroleum Ether extract	DPE	1.43 ± 0.01[d]	68.22 ±2.02[b]
9	Chloroform extract	DCF	1.92 ±0.02[c]	66.52 ±1.47[b]
10	Acetone extract	DA	1.22 ± 0.01[d]	65.98 ± 2.11[b]
11	Methanol extract	DM	3.75 ± 0.09[b]	97.51 ±2.56[a]
12	Methotrexate	DMT	4.26±0.02[a]	96.34 ± 3.72[a]
	CD (5%)		**0.28**	**3.72**

Values are mean±SD of six mice
Means followed by common superscripts donot differ significantly at 5% level.

The GSH content in the liver tissues of normal mice was found to be 4.47mg/g tissue. Inoculation of DLA drastically decreased the GSH content to 0.7mg/g tissue. Among the different extracts of *Coleus forskohli*, only methanolic extract had increased the GSH to near normal (3.75 mg/g tissue), whereas the petroleum ether, chloroform and acetone extracts did not show a significant increase which indicated that the methanolic extract of *Coleus forskohlii* root has pronounced effect on GSH levels compared to other extracts.

Glutathione is an endogenous intracellular thiol containing tripeptide is an important cellular antioxidant and has been the focus of interest in cancer chemotherapy. The active role of GSH against cellular lipid peroxidation has been well recognized. GSH can act either to detoxify or activate oxygen species such as H_2O_2 or reduce lipid peroxides. It is also involved in many cellular functions i.e., bioreductive reactions, maintenance of enzyme activity, amino acid transport, protection against oxidative stress, radiation and chemotherapy, detoxification of xenobiotics and drug metabolism. GSH also controls the onset of tumor cell proliferation by regulating protein kinase C (Rosangkima and Prasad, 2004).

Ascorbic acid

Ascorbic acid appears to trap the peroxy radicals in the aqueous phase with a rate large enough to intercept virtually all these radicals before they can diffuse into plasma lipids. Those peroxy radicals that escape the antioxidants in the aqueous phase diffuse into the plasma lipids where they initiate lipid peroxidation. The propagation is inhibited by the lipophilic chain breaking antioxidants.

As seen in Table 14, the level of ascorbic acid, the nonenzymatic antioxidant shows a significant ($P<0.05$) decrease (64.71 mg/100g tissue) in the DLA control mice compared to the level in the untreated control mice

(99.61 mg/100g tissue). Among the extracts, the methanolic extract caused significant ($P<0.05$) increase in ascorbic acid (97.51mg/100g tissue) which is on par with the standard drug, methotrexate (96.34mg/100g tissue). The other extracts did not improve the ascorbic acid content to a greater extent.

The decreased ascorbic acid in the DLA induced animals observed might be due to excessive utilization of these antioxidants for quenching the enormous amounts of free radicals produced. The antioxidants react cooperatively *in vivo* so as to provide greater protection to the organs against radical damage that could not be provided by any single antioxidant. The decreased levels of GSH and vitamin C in the DLA injected animals in the present study indicated the increased rate of lipid peroxidation with a concomitant decrease in the activities of SOD, CAT and GPx. The treatment with *Coleus forskohlii* extract might have effectively controlled the loss of GSH and Vitamin C, thereby maintaining the activities of SOD, CAT and GPx.

4.1.3 EFFECT OF *Coleus forskohlii* ROOT EXTRACTS ON SERUM MARKER ENZYMES

Changes in plasma enzyme activity are used as indicators of tissue injury, environmental stress or a diseased condition. The rate of increase of plasma enzyme activity depends on the concentration of an enzyme in the cells, the rate of leakage caused by injury and the rate of clearance of the enzyme from plasma (Siakperi *et al.*, 2010).

In the assessment of liver damage by any hepatotoxin, the determination of enzymes such as AST and ALT is largely used (Dobbs *et al.,* 2003). Because of their intracellular location in the cytosol, toxicities affecting the liver pass subsequently to the membrane architecture of the cells leading to their spillage into plasma and their concentration gets increased (Jagadeesan, 2006).

Acid phosphatase and Alkaline phosphatase

The marker enzymes namely acid phosphatase, alkaline phosphatase, aspartate transaminase, alanine transaminase and lactate dehydrogenase were assessed in the serum.

The influence of *Coleus forskohlii* root extracts on serum acid phosphatase and alkaline phosphatase in the DLA induced mice is given in Table 12.

TABLE 12

ACTIVITIES OF ACID PHOSPHATASE AND ALKALINE PHOSPHATASE IN SERUM OF MICE

Treatment		Acid phosphatase#	Alkaline phosphatase#
Untreated control	UC	4.60 ± 0.45[e]	5.41 ± 0.21[f]
Vehicle control	VC	4.53 ±0.03[e]	5.20 ±0.02[f]
Petroleum ether	CPE	4.93 ± 0.02[e]	5.65 ±0.75[f]
Chloroform	CCF	5.14 ±0.01[e]	5.44±0.04[f]
Acetone	CA	4.43 ± 1.01[e]	5.55±1.02[f]
Methanol	CM	4.82 ±0.01[e]	5.01±0.05[f]
DLA Control	DC	11.52 ± 0.81[a]	9.92 ± 0.31[a]
Petroleum Ether extract	DPE	11.47 ± 0.51[a]	9.87 ± 0.72[a]
Chloroform extract	DCF	11.03 ± 0.27[b]	9.25 ± 0.89[b]
Acetone extract	DA	11.35 ± 0.66[a]	8.70 ± 0.96[c]
Methanol extract	DM	7.695 ± 0.57[c]	7.12 ± 0.45[d]
Methotrexate	DMT	6.48 ± 0.52[d]	6.53 ± 0.46[e]
CD (5%)		**0.28**	**0.48**

Values are mean±SD of six mice

Means followed by common superscripts donot differ significantly at 5% level.
KA Units/100 ml serum

As shown in Table 12, acid phosphatase and alkaline phosphatase activities in the DLA induced mice increased in the serum significantly compared to the untreated control mice indicating their leakage from the marker tissues to the serum due to stress and membrane damage created by the DLA cells. The petroleum ether, chloroform and acetone extracts of *Coleus forskohlii* showed a less significant decrease in the activity of ALP and ACP when compared to the methanolic extract. Methotrexate standard treated mice showed a more significant decrease ($P<0.05$) in acid phosphatase activity.

The increase in the enzyme activities is likely due to the leakage of enzyme from the cytosol of liver that might have entered into the blood stream resulting in high levels of activities of these enzymes. They have proved to be excellent indicators of DLA cells induced hepatocellular necrosis in mice (Robert *et al.*, 2002). The increased activity of alkaline phosphatase in the DLA cells induced mice could be due to the damage to the cell membrane of tissues, where these enzymes are firmly attached. The damage releases these enzymes from the membrane, joining the binary canalicules and the sinusoidal border of parenchyma cells (Garry *et al.*, 2002). Alkaline phosphatase is a membrane bound enzyme found at the bile pole of hepatocytes and also found in the pinocytic vesicle and golgi complex. It is present on all cell membranes where active transport occurs. It is often employed to assess the integrity of plasma membrane, since it is localized predominantly in the microvilli of the bile canaliculi, located in the plasma membrane. Decrease in ALP activity may be taken as an index of hepatic parenchymal damage and hepatocytic necrosis (Siakperi *et al.*, 2010).

Aspartate transaminase, Alanine transaminase and Lactate dehydrogenase

Aspartate aminotransferase (AST) is a mitochondrial enzyme involved in the transfer of an amino group from a 2-amino- to a 2-oxoacid found in the heart, liver, skeletal muscle and kidney and is normally present in the plasma. The activities of these enzymes in the serum are raised when any of these tissues are damaged and are clinically useful in the diagnosis of hepatobiliary disease, myocardial infarction, skeletal muscle necrosis and pulmonary infarction (Whitehead *et al.,* 1999).

Serum glutamate oxaloacetate transaminase (SGOT or AST) and serum glutamate pyruvate transaminase (SGPT or ALT) activities are known toxicity markers in the study of hepatotoxicity of chemicals. An increase in the activity of these enzymes is termed as the early recognition of toxic hepatitis (Jyotsana *et al.*, 2008).

The influence of *Coleus forskohlii* root extracts on serum asparate transaminase, alanine transaminase and lactate dehydrogenase activity in the DLA induced mice is given in Table 13.

Asparte transaminase and alanine transaminase activities showed a marked increase ($P<0.05$) in the DLA control mice compared to the untreated control mice. The high levels of AST and ALT in serum are usually indicative of liver damage in animals. Among all the extracts, methanolic extract caused the most significant decrease in these serum marker enzymes showing the efficiency of this extract for healing the membrane damage. Sedeghi *et al.* (2008) observed the antihepatotoxic effect of *Cichorium intybus* extract against CCl_4 toxicity in albino rats. He observed the tendency of the the extract to serum levels of ALT, AST and ALP to return towards near normal level in CCl_4 intoxicated rats.

The reduction in AST and ALT activities towards normal might be due to the regeneration of hepatic cells by active constituents like flavonoids, alkaloids and terpenoids in the plant extract. Effective control of ALP activity indicated an early improvement in the secretory mechanism of the hepatic cell (Ojo *et al.*, 2006).

TABLE 13

ACTIVITIES OF ASPARTATE TRANSAMINASE, ALANINE TRANSAMINASE AND LACTATE DEHYDROGENASE IN SERUM OF MICE

Treatment		Aspartate transaminase#	Alanine transaminase#	LDH#
Untreated control	UC	30.30 ± 0.90[f]	34.57 ±1.79[e]	20.40 ± 0.92[e]
Vehicle control	VC	35.55± 0.41[f]	33.45 ±0.07[e]	23.15 ±0.75[e]
Petroleum ether	CPE	33.23±3.00[f]	33.05±0.05[e]	21.57±0.08[e]
chloroform	CCF	29.01±3.03[f]	35.12±0.03[e]	23.74±0.93[e]
Acetone	CA	34.02±0.02[f]	34.13±0.07[e]	23.15±0.14[e]
Methanol	CM	32.43±0.05[f]	33.14±0.02[e]	20.20±0.10[e]
DLA Control	DC	55.51 ± 3.64[b]	51.48 ± 2.65[a]	46.81 ± 5.53[b]
Petroleum Ether extract	DPE	59.76 ± 1.46[a]	47.30 ± 1.85[b]	43.14 ± 0.56[c]
Chloroform extract	DCF	52.23 ± 3.23[c]	51.73 ± 0.49[a]	49.02 ± 3.32[a]
Acetone extract	DA	39.62 ±0.68[d]	40.08 ± 1.84[c]	41.23 ± 0.08
Methanol extract	DM	34.02 ± 2.03[e]	31.99 ± 1.69[f]	20.19 ± .89[e]
Methotrexate	DMT	35.62 ± 3.47[e]	37.96 ±1 .53[d]	24.06 ± .08[d]
CD (5%)		**2.94**	**1.68**	**3.52**

Values are mean ± SD of six replicates
Means followed by common superscripts donot differ significantly at 5% level.

#IU/100 ml of serum

LDH is an enzyme associated with the soluble portion of the cell. The increase in serum LDH activity (46.81 IU/100ml serum) in the control mice supported the suggestion of Ayorinde *et al.* (2008) of possible leakage of cytosolic enzyme from the tissues into the serum.

Administration of the methanolic extract of *Coleus forskohlii* attenuated the increased activities of LDH in serum (20.19 IU/100ml of serum). A subsequent recovery towards normalization of these hepatic markers strongly suggests the possibility of the extract being able to protect the hepatocyte against membrane fragility and maintaining the functional status of the liver.

The antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase, glutathione reductase and nonenzymic antioxidants such as glutathione and ascorbic acid were found to be decreased in the DLA cells induced mice. Treatment with the extracts of *Coleus forskohlii* plant increased these enzymes and antioxidant levels. The methanolic extract caused a significant increase, bringing back the enzyme activities to normal . Lipid peroxidation was found to be increased in cancer. But, the treatment with *Coleus forskohlii* extract could counter this increase. The serum enzymes, acid phosphatase, alkaline phosphatase, aspartate transaminase, alanine transaminase and LDH activities were found to be increased in the DLA treated mice and the activities reverted to almost normal in animals treated with the methanolic extract of roots of *Coleus forskohlii.* As the methanolic extract showed better anticancer activity, it was used further to study the hematological parameters in cancer

PHASE II

4.2 EFFECT OF THE METHANOLIC EXTRACT OF *Coleus forskohlii* ON HEMATOLOGICAL PARAMETERS IN THE DLA TUMOR INDUCED MICE

Tumor growth normally affects various hematological parameters and the anticancer activity is generally assessed by the restoration of the changes in these parameters to normal, particularly in respect of White blood cells (WBC), Red blood cells (RBC), lymphocytes and hemoglobin. The acceptance criteria for determining the antitumor activity of a compound are the circulating WBC and the life span prolongation (Radha and Sivakumar, 2008).

The hematological parameters of the tumor bearing mice and the methanolic extract treated mice were studied for a period of 21 days with observations on 0^{th},10^{th} and 20^{th} day of the changes in hemoglobin level, WBC, lymphocyte , monocyte and differential cell count due to tumor induction and administration of methanolic extract.

4.2.1 Hemoglobin

The effect of the methanolic extract of *Coleus forskohlii* root on the blood hemoglobin content in the mice bearing Daltons lymphoma on 0^{th} ,10^{th} , and 20^{th} days is depicted in Table 14 and figure 3.

TABLE 14

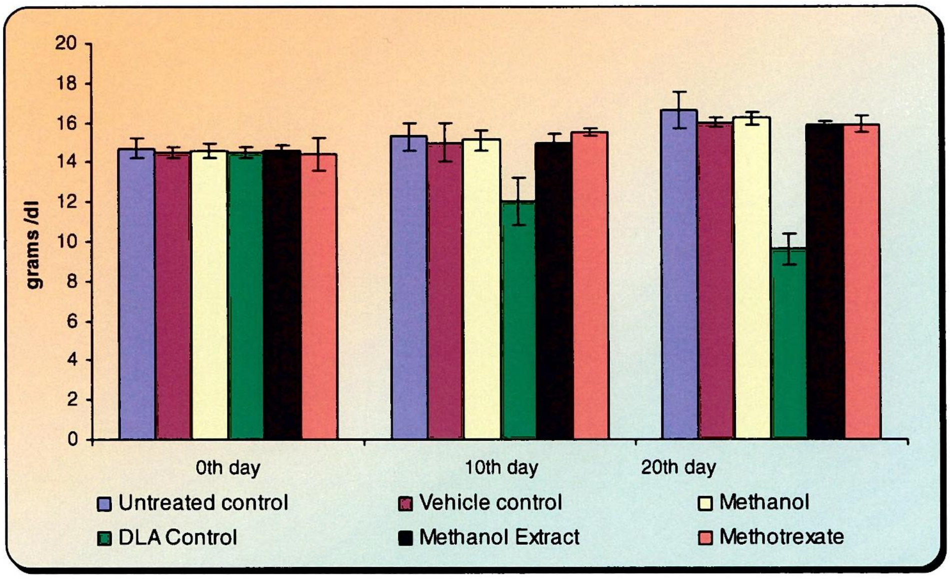
BLOOD HEMOGLOBIN CONTENT IN THE MICE BEARING DALTONS LYMOPHOMA AT DIFFERENT TIME INTERVALS

Groups	Groups	Blood hemoglobin (g/dl)		
		0^{th} day	10^{th} day	20^{th} day
1	Untreated control	14.7±0.5	15.3±0.7	16.6±0.9
2	Vehicle control	14.5±0.3	15.0±1.0	16.0±0.2
3	Methanol	14.6±0.1	15.1±0.5	16.2±0.3
4	DLA Control	14.5±0.3	12.0±1.2	9.6±0.8
5	Methanol Extract	14.6±0.1	15.0±0.4	15.9±0.2
6	Methotrexate	14.4±0.8	15.5±0.2	15.9±0.4
	CD (5%)	**1.065**		

Values are mean of six mice

FIGURE 3

BLOOD HAEMOGLOBIN CONTENT IN THE MICE BEARING DALTONS LYMOPHOMA AT DIFFERENT TIME INTERVALS



Fall in the hemoglobin level in the DLA control group were observed on the 10^{th} and on the 20^{th} days. As shown in the Table 17 the hemoglobin content in the DLA control mice has decreased significantly ($P<0.05$) from 14.5g% on the 0^{th} day to 12.0g% on the 10^{th} day and 9.6g% on the 20^{th} day. Methanolic extract treated animals showed a significant increase in the hemoglobin content of 15g% and 15.9g% on the 10^{th} and on the 20^{th} day respectively compared to the tumor control mice.

Usually in cancer chemotherapy, the major problems that are being encountered are myelosuppression and anemia. The anemia encountered in the tumor bearing mice is mainly due to the reduction in the RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic condition. Radha and Sivakumar (2008) observed that the methanolic extract of *Plumeria alba* brought back the hemoglobin and the RBC count to normal in the tumor bearing mice.

Sivakumar *et al.* (2008) also showed that the methanolic extract of *Triumfera rhomboidea* treated mice had a higher hemoglobin content compared to the DLA tumor induced mice.The haemoglobin content and RBC count in the EAC induced mice were found to have increased when they were treated with different concentrations of the methanolic extract of *Caesalpinia bonducella* leaves (Gupta *et al.*, 2004).

4.2.2. White blood corpuscles (WBC)

The effect of methanolic extract of *Coleus forskohlii* root on the white blood cell count in the mice bearing Daltons Lymphoma on the 0th, the 10th and the 20th days is shown in Table 15 and Figure 4.

TABLE 15

WHITE BLOOD CELLS CONTENT IN THE MICE BEARING DALTONS LYMPHOMA AT DIFFERENT TIME INTERVALS

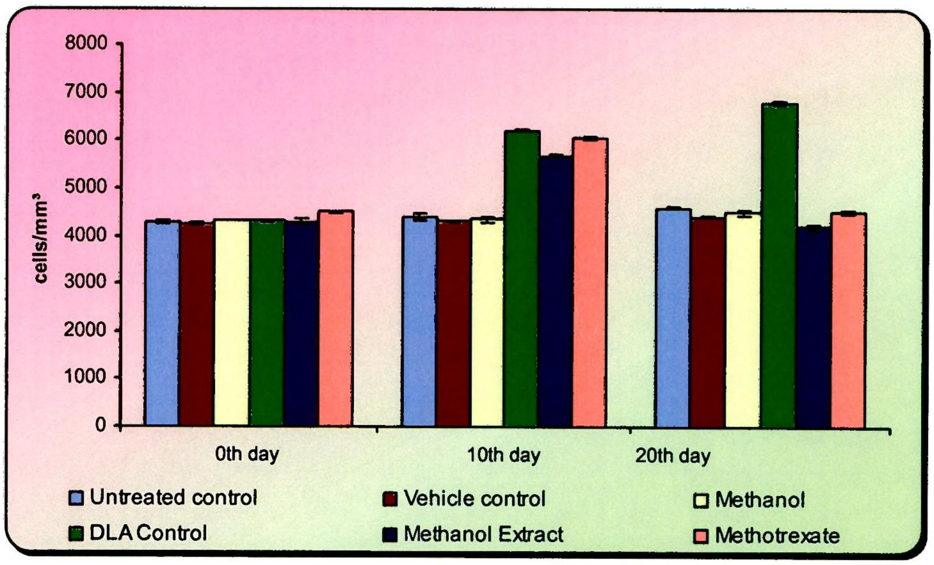
Groups	Treatments	White blood cells$^{\$}$		
		0th day	10th day	20th day
1	Untreated control	4266±40	4397±75	4600±22
2	Vehicle control	4250±43	4304±20	4400±20
3	Methanol	4312±10	4350±50	4500±55
4	DLA Control	4306±25	6214±23	6800±34
5	Methanol Extract	4300±55	5689±20	4200±55
6	Methotrexate	4300±47	5570±80	4140±05
	CD (5%)	**73.38**		

Values are mean of six mice

$^{\$}$ values expressed as cells/mm³

FIGURE 4

WHITE BLOOD CELLS CONTENT IN THE MICE BEARING DALTONS LYMPHOMA AT DIFFERENT TIME INTERVALS



White blood cell count has significantly increased ($P<0.05$) in the DLA control mice from 4306±25 cells / mm^3 to 6214 ±23 and 6800±34 cells / mm^3 respectively on the 10th and on the 20th days of treatment. Treatment with the methanolic extract of *Coleus forskohlii* has reduced the WBC count significantly ($P<0.05$) on the 10th day (5689±20 cells/ mm^3) as well as on the 20th day (4200±55 cells/ mm^3) of treatment. Treatment with the methanolic extract has normalized the WBC count. Radha *et al.* (2008) had observed an improvement in hematological parameters such as RBC and WBC cell count following the treatment of tumor bearing mice with methanolic extract of *Plumeria alba.*

4.2.3 Lymphocyte count

The effect of the methanolic extract of *Coleus forskohlii* root on the total lymphocyte count in the mice bearing Daltons lymphoma on the 0th, the 10th and the 20th day is presented in Table 16 and Figure 5.

TABLE 16

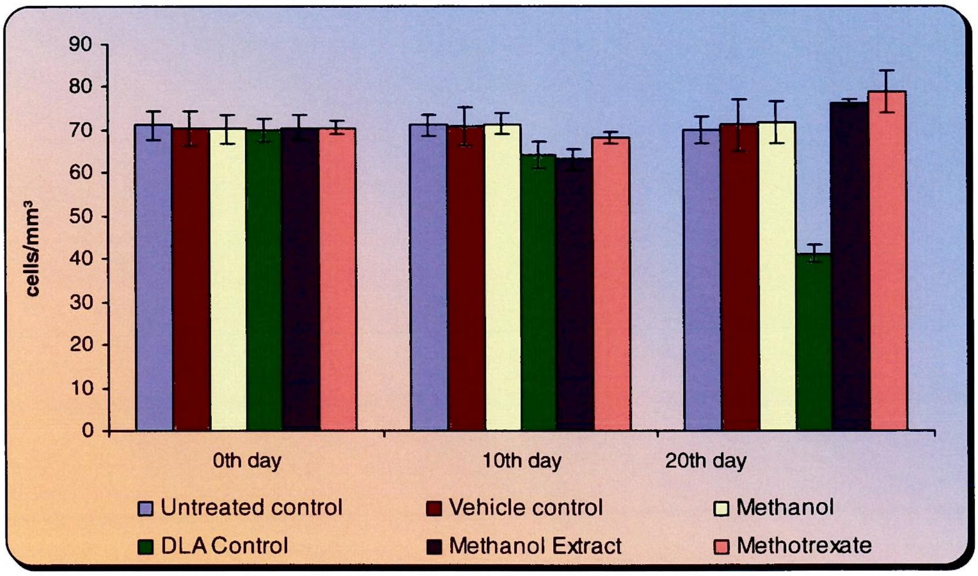
LYMPHOCYTE COUNT IN THE MICE BEARING DALTONS LYMPHOMA AT DIFFERENT TIME INTERVALS

Groups	Lymphocytes (Cells/mm^3)		
	0^{th} day	10^{th} day	20^{th} day
Untreated control	71.2±3.3	71.1±2.5	70.0±3.0
Vehicle control	70.4±4.0	70.8±4.5	71.2±6.0
Methanol	70.2±3.5	71.4±2.5	71.7±5.0
DLA Control	70.0±2.5	64.3±3.1	41.1±2.0
Methanol Extract	70.6±3.0	63.2±2.4	76.2±1.0
Methotrexate	70.5±1.5	68.2±1.2	78.8±5.0
CD (5%)	**4.538**		

Values are mean of six mice

FIGURE 5

LYMPHOCYTE COUNT IN THE MICE BEARING DALTONS LYMPHOMA AT DIFFERENT TIME INTERVALS



As shown in Table 16 and Figure 5 the lymphocyte count has significantly decreased in the DLA control from 70 cells/mm^3 on the 0th day to 64.3 cells/mm^3 on the 10th day and 41.1 cells/mm^3 on the 20th day. Treatment with methanolic extract of the root of *Coleus forskohlii* showed a moderate decrease in lymphocyte on the 10th day (63.2±2.4) and a significant increase ($P<0.05$) to 76.2 cells/mm^3 on the 20th day indicating the antitumor effect of *Coleus forskohlii* extract.

4.2.4 Monocyte count

The effect of the methanolic extract of the root of *Coleus forskohlii* on monocyte count of the mice bearing Dalton's lymphoma is given in Table 17 and Figure 6.

TABLE 17

MONOCYTE COUNT IN THE MICE BEARING DALTONS LYMPHOMA AT DIFFERENT TIME INTERVALS

Groups	Monocytes (cells/mm^3)		
	0th day	10th	20th day
Untreated control	2.1±0.05	2.0±0.30	2.0±0.02
Vehicle control	2.0±0.01	1.9±0.70	2.1±0.20
Methanol extract	1.8±0.30	2.0±0.01	2.1±0.10
DLA Control	1.5±0.01	2.0±0.05	3.4±0.3
Methanol Extract	1.6±0.04	2.0±0.01	1.9±0.2
Methotrexate	2.0±0.01	2.0±0.30	1.9±0.10
CD (5%)		**0.275**	

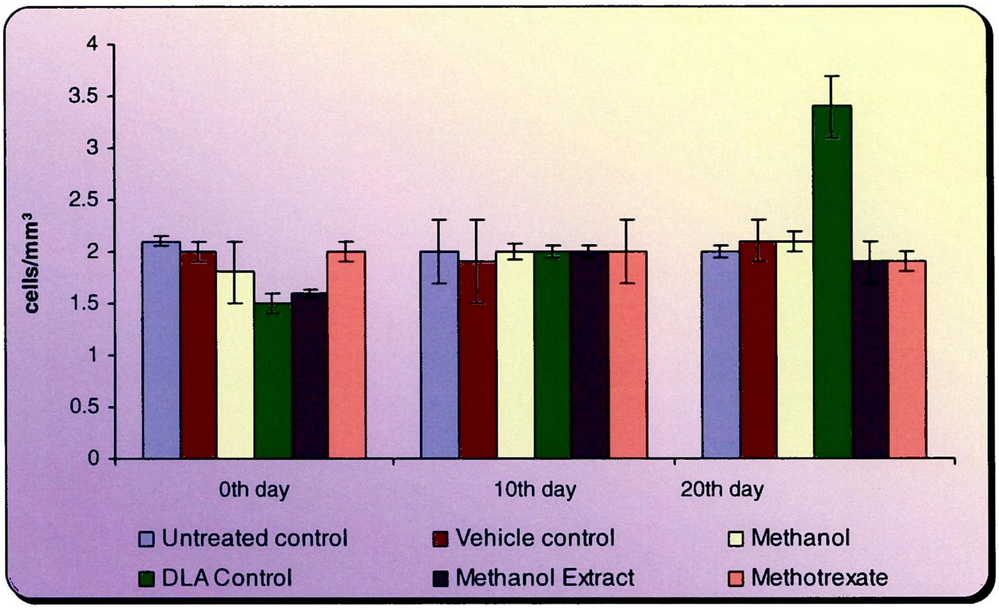
Values are mean of six mice

As shown in the Table 17 and Figure 6, the monocyte count has significantly increased in the DLA control from 1.5 cells/mm^3 on 0th day to 2.0 cells/mm^3 on the 10th day and 3.4 cells/mm^3 on the 20th day . Treatment with the methanolic extract of the root of *Coleus forskohlii* showed a significant

(P<0.05) decrease in the lymphocyte to 1.9 on the 20th day indicating the antitumor effect of *Coleus forskohlii* extract.

FIGURE 6

MONOCYTE COUNT IN THE MICE BEARING DALTONS LYMPHOMA AT DIFFERENT TIME INTERVALS



Immunoregualtion is a complex balance between the regulator and the effector cell and any imbalance in the immunological mechanism may lead to pathogenesis. Immunity is suppressed in cancer. An earlier study had shown that the extract from the plant *Viscum album,* could stimulate immunity in normal and tumor bearing mice. Similar results have also been reported using herbal immunostimulatory preparations and rasayanas which were used in the indigenous system of medicine. Guruvayoorappan and Kuttan (2007) also showed the immunomodulatory and antitumor activity of *B.sensitivum,* an important plant in the indigenous medicinal practices.

Treatment with the methanolic extract of *Coleus forskohlii* restored the hemoglobin content, RBC and WBC cell count to near normal values. This indicates that the extract possesses protective action on the haematpoietic system. Further analysis of haematological parameters showed minimum toxic effects in the mice treated with the *Coleus forskohlii* extract.

Immunomodulators are substances which can modify the activity of the immune system. Plant and plant products have been the basis of treatment for human diseases since time immemorial. There are several medicinal plants that are considered to possess immunomodulataory properties. They have shown to augment specific cellular and humoral immune response (Guruvayoorappan and Kuttan, 2007).

4.2.5 Differential count

Table 18 and Figure 7 and 8 represents the effect of methanolic extract of root of *Coleus forskohlii* on differential cell count of the mice bearing Dalton's lymphoma.

As seen from Table 18 and Figure 7, the Neutrophil count has significantly ($P<0.05$) increased in the DLA control from 41.1 cells/mm^3 on 0th day to 64.3 cells/mm^3 on the 10th day and 86.0 cells/mm^3 on the 20th day. Treatment with the methanolic extract of the root of *Coleus forskohlii* showed a significant decrease on the 10th day (59.1 cells/mm^3) and on the 20th day (57.0 cells/mm^3) compared to the control DLA mice indicating the antiinfectious action of the extract of *Coleus forskohlii.*

Neutrophils constitute the first line of defence against infectious agents that penetrate the body's physical barriers. This is the most abundant leukocyte present in the blood with mean concentration of 4.4 x 10^6 cells/mm^3. Neutrophils are derived from the common hematopoietic stem cell. Their development in the bone marrow takes approximately 10-14 days.

TABLE 18

EFFECT OF *Coleus forskohlii* ON DIFFERENTIAL COUNT OF THE DLA TRANSPLANTED MICE AT DIFFERENT TIME INTERVALS

Groups	**Neutrophils** (cells/mm^3)			**Eosinophils** (cells/mm^3)		
	0^{th} day	**10^{th} day**	**20^{th} day**	**0^{th} day**	**10^{th} day**	**20^{th} day**
Untreated control	56.0±4.0	54.2±5.5	56.6±2.0	1.5±0.05	1.5±0.01	1.7±0.10
Vehicle control	55.3±0.8	55.9±0.9	55.9±0.10	1.6±0.80	1.50±0.70	2.4±0.30
Methanol	55.4±2.5	56.8±1.0	57.0±0.8	1.5±0.04	1.50±0.02	1.6±0.01
DLA Control	41.1±2.0	64.3±3.1	86.0±2.0	1.5±0.01	2.0±0.05	2.4±0.30
Methanol extract	56.2±2.5	59.1±8.0	57.0±6.5	1.6±0.03	2.0±0.02	1.9±0.01
Methotrexate	56.0±0.30	70.1±0.80	59.0±0.20	1.5±0.03	1.9±0.07	1.9±0.01
CD (5%)	**17.88**			**0.18**		

Values are mean of six mice

FIGURE 7

FIGURE 7. EFFECT OF *Coleus forskohlii* ON NEUTROPHIL COUNT OF THE DLA TRANSPLANTED MICE AT DIFFERENT TIME INTERVALS

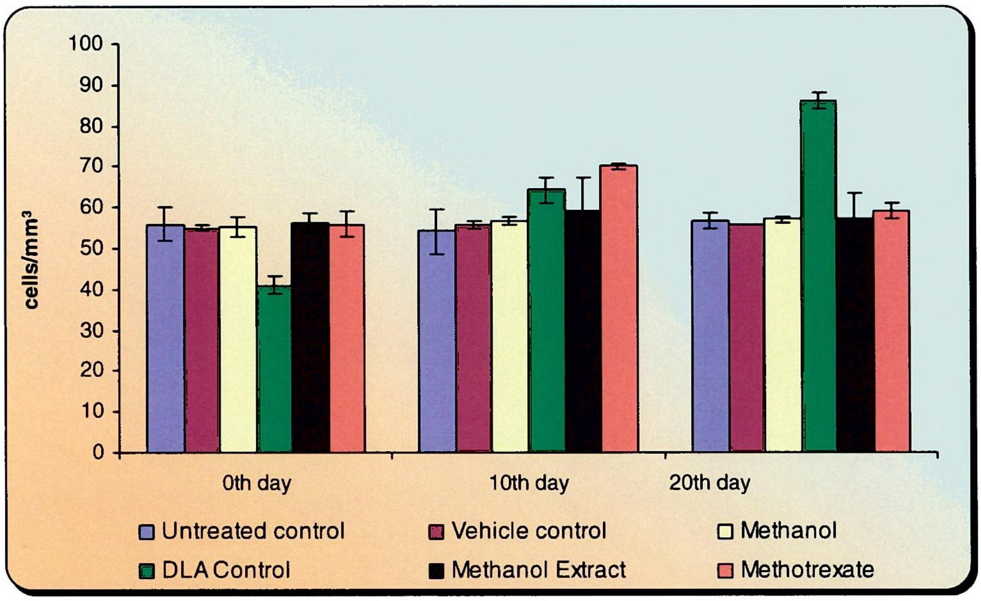
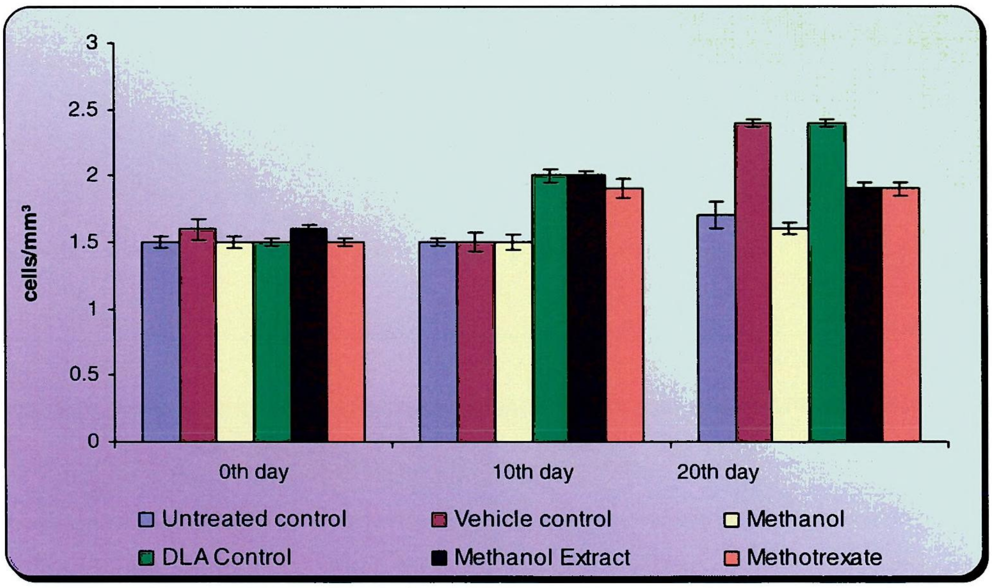


FIGURE 8

EFFECT OF *Coleus forskohlii* ON EOSHINOPHIL COUNT OF THE DLA TRANSPLANTED MICE AT DIFFERENT TIME INTERVALS



The bone marrow produces approximately $1x10^9$ neutrophils/kg/day. As the neutrophils mature, they develop the capacity to enter the blood through increasing formability and through changes in the adhesion protein in their surface membranes. Agents such as G-CSF, CM-CSF, corticosteroids or endotoxins can stimulate the release of neutrophils from the bone marrow. Neutrophils phagocytose the invading infectious agents and neutralize them (Vijayalakshmi, 2008). Shanta *et al.* (2000) observed a significant decrease in neutrophils count in cervical cancer patients compared to the healthy controls.

Table 18 and figure 8 show that the eosinophil count has significantly ($p<0.05$) increased in the DLA control from 1.5 cells/mm^3 on the 0^{th} day to 2.0 cells / mm^3 on the 10^{th} day and 2.4 cells / mm^3 on the 20^{th} day. Treatment with the methanolic extract of the root of *Coleus forskohlii* showed significant decrease on the 20^{th} day (1.9 cells/ mm^3) compared to the DLA control mice.

The reliable criteria for evaluating an anticancer drug are prolongation of lifespan of the animal and decrease in WBC count of blood (Radha *et al.*, 2008). The present results have showed a reduction in WBC count in the plant extract treated mice. The results clearly demonstrate the antitumor activity of the extract against the DLA.

The improvement in hematological profile of the tumor bearing mice following the treatment with the mrthanolic extract could be due to the action of the different phytoconstituents present in the extract of *Coleus forskohlii.*

4.2.6 Survival rate

The reliable criteria for judging the value of any anticancer drug are the prolongation of lifespan of the animals . The effect of *Coleus forskohlii* extract on the survival of the ascitis tumor bearing mice is presented in Table 19.

TABLE 19

EFFECT OF *Coleus forskohlii* EXTRACT ON THE SURVIVAL OF THE ASCITIS TUMOR BEARING MICE

Treatment	Mean survival days	Percentage increase in life span (%ILS)
Untreated Control	22.3±0.2	23
Vehicle control	22.1±0.7	22
Methanol	21.9±0.3	20
DLA control	18.1 ±0.4	--
Methanolic extract	22.8±0.7	25
Methotrexate	23.1±0.4	27.6

Values are mean of six mice

As shown in Table 19, the lifespan of the ascitis tumor bearing mice treated with methanolic extract of *Coleus forskohlii* was found to have increased. The control animals survived only 18.1 days after the tumor induction while the *Coleus forskohlii* treated animals survived up to 22.8 days.The percentage increase in the life span is 22.5% in the methanolic extract treated animals.

Dongre *et al.* (2008) also noticed a significant increase in the life span of the ascitis tumor bearing mice treated with methanolic extract of *Hypericum hookerianum.* A similar study by Rajkapoor *et al* .(2004) had shown an increased lifespan of the ascitis tumor bearing mice treated with *Indigofera asphalthoide.*

Sivakumar *et al.* (2008) had observed an increase in the mean survival time of 6 days when the DLA induced mice was treated with the methanolic extract of *Triumfera rhomboidea.*

4.2.7. Body weight

The effect of *Coleus forskohlii* methanolic extract on the body weight of the DLA induced mice is shown in Table 20.

TABLE 20

EFFECT OF *Coleus forskohlii* EXTRACT ON THE BODY WEIGHT OF ASCITIS TUMOR BEARING MICE

Treatment	Weight in g	Percentage increase/decrease
Untreated control	21.5±1.5	22
Vehicle control	22.0±1.9	20
Methanol	23.3±1.8	16
DLA Control	27.8± 2.2	--
Methanolic extract	21.0±0.8	24.4
Methotrexate	20.8±1.8	25

Values are mean of six mice

The body weight of the control animals was found to have increased to 22% indicating a mean increase in weight. The weight of the DLA control mice has increased significantly owing to an increase in the tumor volume. The methanolic extract treated mice showed a reduction in weight, compared to DLA control mice.

The ascitic tumor implantation induces *per se* local inflammatory reactions with an increase in vascular permeability, which results in intense edema formation, cellular migration and a progressive ascitic fluid formation.

4.2.8 Histopathology of liver

The presence of tumors in the human body or in experimental animals is known to affect many functions of the vital organs, especially the liver, even when the site of the tumor does not interfere directly with the organ function (Gupta *et al.*, 2004).

Histopathological changes like vacuolization, necrosis and apoptosis in cells are indicative of anticancer activity (Radha and Sivakumar, 2008). The results observed in the histopathological study of the liver of the Daltons lymphoma bearing mice are presented in Table 21.

TABLE 21

HISTOPATHOLOGY OF THE LIVER OF MICE

Groups	Treatments	Liver	Impressions
Untreated control	No treatment	Normal Histology	Healthy liver tissue
DLA Control	DLA induced	Section of liver tissue shows perivenular and periportal mixed inflammatory infiltrate composed of lymphocytes within the hepatocytes.	Lymphoid collections show pathology
Methanolic extract	DLA+Methanolic extract of CF	Shows liver tissue with a few collections of lymphocytes in between the hepatocytes. No perivenular and periportal collections seen.	Reductions in Lymphoid cells show no Specific pathology.

Plates 2, 3, 4 show the liver sections of the normal, the DLA induced and the methanolic extract treated mice. The histopathology results of normal mice show a healthy liver tissue whereas in the DLA induced mice liver sections show a perivenular and periportal mixed inflammatory infiltrate composed of lymphocytes within the hepatocytes. This indicates the clear pathology of the liver. The liver sections of methanolic extract treated mice showed liver tissue with a few collections of lymphocytes in between the hepatocytes. No perivenular and periportal collections were seen. Reductions in lymphoid cells show no specific pathology. It is likely that the administration of the extracts of *Coleus forskohlii* would have reverted the histological changes that had occurred due to cancer.

PLATE 2

HISTOLOGY OF THE UNTREATED MICE LIVER

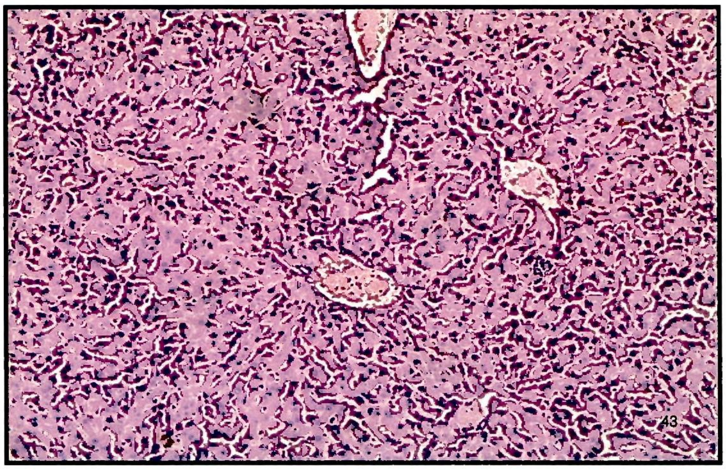


PLATE 3

HISTOPATHOLOGY OF THE LIVER OF THE DLA TREATED MICE
(Periportal inflammation)

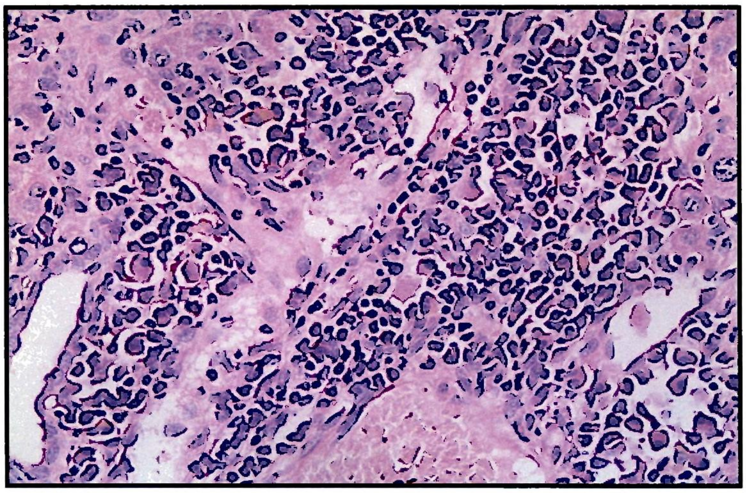


PLATE 4

HISTOPATHOLOGY OF THE LIVER OF THE DLA TREATED MICE
(Perivenular inflammation)

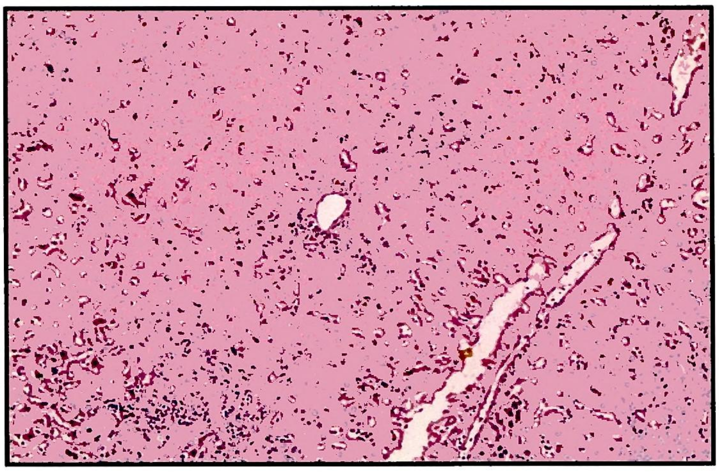
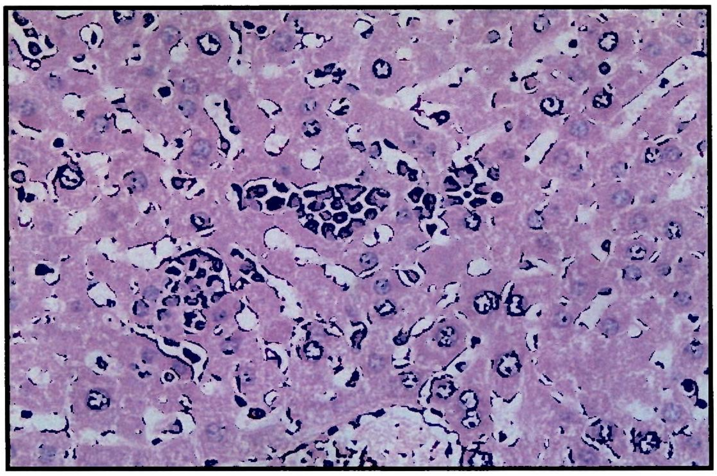


PLATE 5

HISTOPATHOLOGY OF THE LIVER OF THE METHANOLIC EXTRACT TREATED MICE



PHASE III

4.3 IDENTIFICATION OF THE PHYTOCHEMICALS IN THE ROOT OF *Coleus forskohlii*

Several medicinal plants are traditionally noted for their bio-medicinal properties, often exhibiting a wide range of biological and pharmacological activities, such as anti-inflammatory, antibacterial and antifungal properties. The extracts from these plant tissues, especially from the leaves, the roots, the barks, the seeds and the fruits have been used in traditional medicinal systems. The active constituents contributing to these protective effects are the naturally occurring phytochemicals, vitamins and minerals which give the plants their color and flavor. The alkaloids, the tannins, the flavonoids and the phenolic compounds play a major role in preventing a number of chronic diseases by a definite physiological action on the human body like anti-inflammatory, antithrombotic, antioxidant and anticarcinogenic activities (Craigg, 1999).

Plants have almost limitless ability to synthesize aromatic substances mainly secondary metabolites, of which, at least 12,000 have been isolated , a number estimated to be less than 10% of the total. In many cases, these substances serve as the molecules of plant defence against predation by microorganisms, insects and herbivores. Further, some of then involve in plant odour (terpenoids), pigmentation (tannins and quinines) and flavor (Capsaicin). However, several of these molecules possess medicinal properties (Mallikharjuna *et al.*, 2007).

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

Considerable attention has recently been focused on the interrelationship between the free radical related reactions and the development of a variety of pathological events. It is well established that lipid peroxidation is the deleterious result of free radical reaction, leading to disruption of biomembranes and dysfunction of cells and tissues (Cho *et al.*, 2003).

In recent years, there has been a global trend towards focusing on the area of natural phytochemicals present in herbs and functional foods as antioxidants. Since ancient times, human beings have derived many benefits from natural plants and compounds. It has been recognized that traditional medicines and the polyphenols isolated from them have potential therapeutic roles in the prevention and the treatment of many human diseases related to excessive oxidative stress (Harnafi and Amrani, 2008).

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 (Ali *et al.,* 2008).

Reactive oxygen species (ROS) such as superoxide anions ($O2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing injury through covalent binding and lipid peroxidation. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent disease (Youdim and Joseph, 2001). The free radical scavenging activities (inhibition of invitro SO, NO generation, DPPH radial scavenging and hydroxyl radical scavenging) of *Coleus forskohlii* were therefore assessed and the same are shown in Table 22 and Figure 9 and 10 respectively.

TABLE 22

DPPH AND HYDROXYL RADICAL SCAVENGING ACTIVITY OF MEHANOLIC EXTRACT OF ROOTS OF *Coleus forskohlii*

Concentration in mg/ml	Percent inhibition	
	DPPH radical	Hydroxy radical
10	27	5
20	39	15
30	43	12
40	59	44
50	65	60
IC_{50}	34	44

FIGURE 9

DPPH RADICAL SCAVENGING ACTIVITY OF THE METHANOLIC EXTRACT OF *Coleus forskohlii* ROOTS

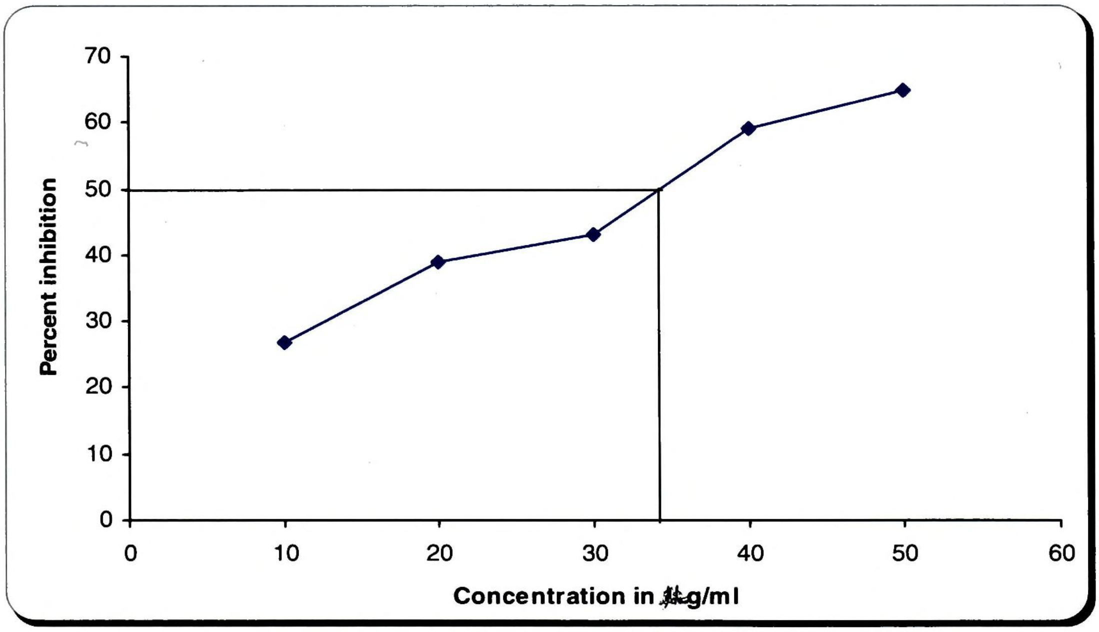


Table 22 and Figure 9 indicate DPPH radical scavenging activity of different concentrations of the methanolic extract. As the concentration increases from 10,20,30,40 to 50 mg/ml, the percent inhibition of DPPH radical also increases in a dose dependent manner from 27, 39, 43, 59, 65 respectively with a IC $_{50}$ of 34 percent.

1,1 diphenyl 2 picryl hydrazyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the methanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction which can be quantified by the decrease in absorbance at wavelength of 517nm. Radical scavenging activity increased with an increasing percentage of the free radical inhibition.

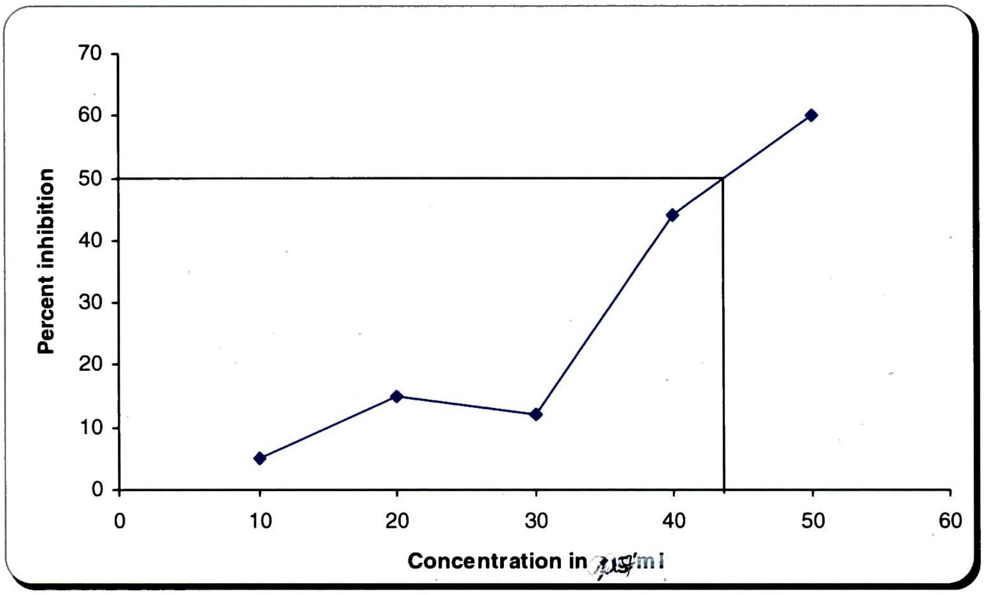
DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent. The electron becomes paired off and the solution loses color stochiometrically, depending on the number of electrons taken up. From the present results, it may be postulated that the root extract of *Coleus forskohlii* reduces the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principles (Sanchez-Moreno, 2002).

Puvanendran *et al* (2007) has also studied the DPPH radical scavenging activity of some *Annonaceae* plant extracts and showed the high radical scavenging activity of these plants. Zakaria *et al* (2008) had shown the DPPH radical scavenging activity of Lamiaceae family such as *Coleus blumei, Orthosiphon staminus, Ocimum basilicum* and *Mentha arvensis.*

The root extract of *Coleus forskohlii* is rich in antioxidants. These antioxidants might have contributed to the effective inhibition of *invitro* superoxide generation, nitric oxide generation, free radicals and hydroxyl radicals scavenging activity. These results are similar to that of Khalaf *et al* (2008) who have reported the antioxidant activity of some common plants. John *et al* (2008) also observed a significant free radical scavenging effect of alcoholic extract of *Cleorodendron paniculatum.*

FIGURE 10

HYDROXYL RADICAL SCAVENGING EFFECT OF THE METHANOLIC EXTRACT OF *Coleus forskohlii* ROOTS



As given in the Table 22 and Figure 10, the hydroxyl radical scavenging or inhibiting activity of the extract of *Coleus forskohlii* shows percent inhibition from 5 to 60 corresponding to 10 to 50mg/ml with the IC_{50} value of 44 mg/ml.

The hydroxyl radical scavenging activity is measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture by studying the competition between deoxy ribose and the extract for hydrogen radicals generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 systems. The hydroxyl radicals attack deoxy ribose which eventually results in TBARS formation. From the present results it is observed that the extract of *Coleus forskohlii* had better hydroxyl radical scavenging activity as reflected in terms of percentage inhibition.

Table 23 gives the superoxide and nitric oxide radical scavenging activities of the methanolic extract of *Coleus forskohlii* root.

TABLE 23

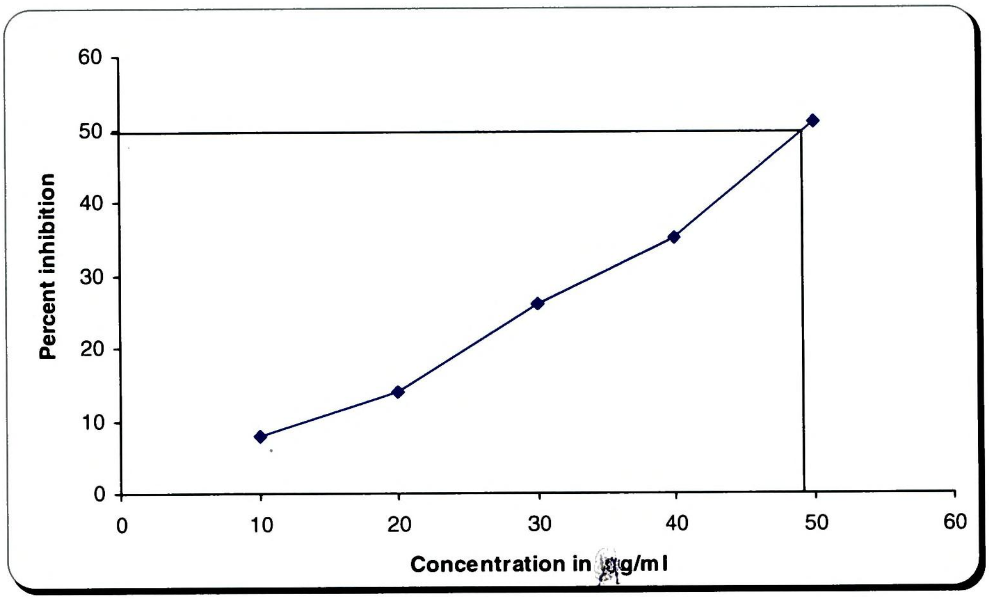
SUPEROXIDE AND NITRIC OXIDE RADICAL SCAVENGING ACTIVITIES OF METHANOLIC EXTRACT OF THE ROOTS OF *Coleus forskohlii*

Concentration in mg/ml	Percent inhibition	
	Superoxide	Nitric Oxide
10	8	9
20	14	18
30	26	23
40	35	20
50	51	55
IC_{50}	49	48

Table 23 indicates that the superoxide radicals are scavenged in a dose dependent manner from 8% to 51% with an IC_{50} value of 49 percent. Figure 11 represents the same.

FIGURE 11

SUPEROXIDE RADICAL SCAVENGING EFFECT OF THE METHANOLIC EXTRACT OF *Coleus forskohlii* ROOTS

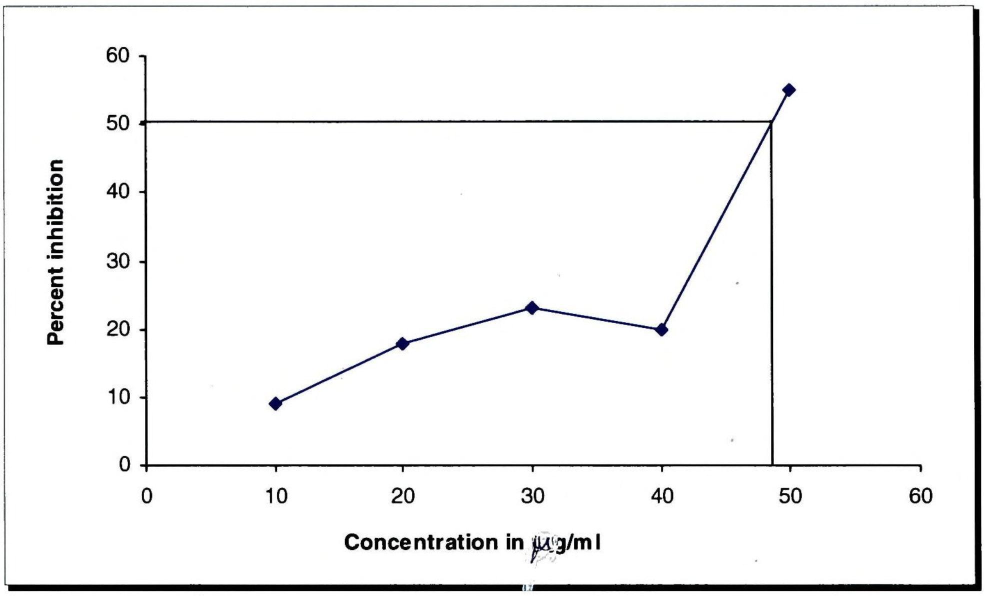


Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as *via* non enzymatic reaction such as auto oxidation by catelcholamines. The scavenging activity towards the superoxide radical is measured in terms of the inhibition of generation of $O_2^{\cdot-}$. In the present study, superoxide radicals reduce NBT to a blue colored formosan that is measured at 560nm (Khanam, 2004). The result shows that the extract of CF has a potent free radical scavenging activity increasing with increasing percentage inhibition. The probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of the extract towards generation of superoxides in the *in vitro* reaction mixture.

The percent inhibition of different concentrations of *Coleus forskohlii* extract on nitric oxide is given in Table 23 and Figure 12. The percent inhibition increases from 9 to 55 as the concentration increases from 10 to 50mg/ml with an IC_{50} of 48 percent.

FIGURE 12

NITRIC OXIDE RADICAL SCAVENGING EFFECT OF METHANOLIC EXTRACT OF THE *Coleus forskohlii* ROOTS



Nitric Oxide is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological process (Lata and Ahuja, 2000). Excess concentration of NO is associated with several diseases. Oxygen reacts with the nitrite anions, which act as free radicals (Baskar *et al.*, 2005). Peroxy nitrite ($ONOO^-$), the reaction product formed between nitric oxide ($NO^·$) and superoxide plays a critical role in the induction of inflammatory reaction and apoptosis. $O^·$ is also associated with tumor promotion and progression (Krishnamoorthy and Sangeetha, 2008). In the present study, the methanolic extract of *Coleus forskohlii* root showed better activity in competing with oxygen to react with nitric oxide and thus inhibit the generation of anions.

Rasineni *et al.* (2008) investigated the total antioxidant capacity of the three species of *Coleus* and found *Coleus forskohlii* having a high free radical scavenging activity among them.

The results of the present study indicate a strong *in vitro* antioxidant activity of the methanolic extract of the roots of *Coleus forskohlii*. It follows that it can protect the cells from oxidative damage and toxic effects of ROS and has potential role in the control of several diseases.

4.3.2 Qualitative detection of phytochemicals in the methanolic extract of *Coleus forskohlii*

Pharmaceutically significant secondary metabolites or phyto pharmaceuticals include terpenoids, alkaloids, glycosides, flavonoids, volatile oils, tannins, resins etc. A number of reports on flavanoids, triterpenoids, diterpenoids and steroids indicate that they exert multiple biological effects due to their antioxidant and free radical scavenging abilities. These phytoconstituents produce protective effects against tumors, heart disease and different pathologies (Gupta *et al.*, 2004). The root extract of *Coleus forskohlii* was screened qualitatively for the presence of phytochemicals such as alkaloids, flavonoids, phenols, terpenoids, tannins and saponins.

The results of the qualitative detection of phytochemicals in the extract of *Coleus forskohlii is* given in the Table 24.

The roots of *Coleus forskohlii* contain the phytochemicals tannins, saponins, flavanoids and terpenoids except steroids and cardiac glycosides.

Flavonoids are a large family consisting of more than 4000 ubiquitous secondary plant metabolites, which are further divided into five subclasses namely flavanoids, flavones, anthocyanins, catechins and flavanones (Merker, 2000). These flavonoid compounds have a common diphenylpropane structure with different degrees of hydroxylation, oxidation and substitution and these compounds commonly occur as glycosides in plants (Pietta, 2000).

TABLE 24

PHYTOCHEMICALS IN THE METHANOLIC EXTRACT OF *Coleus forskohlii* ROOTS

PHYTOCHEMICALS	OBSERVATION	INFERENCE
Tannins	Brownish green color was not obtained	+
Saponins	Emulsion was not observed	+
Flavonoids	Yellow color was not formed	+
Steroids	No color change was observed	–
Terpenoids	A reddish brown coloration at the interface was observed	+
Cardiac glycoside	No brown ring at the interface was observed	–

Note: - Absence + Presence

Terpenoids are known to have many important biological and physiological functions and are also known for their pharmaceutical significance. Terpenoids constitute the largest family of natural plant products comprising over 30,000 members. Terpenoids are classified, based on the homologous series of five carbon isoprene units in their structure.

The other important phytoconstituents are tannins, water soluble polyphenols which tend to reduce the mutagenic activity and also oxygen free radicals. The anticarcinogenic and the antimutagenic potentials of tannins may be related to their antioxidative capability like protecting against cellular oxidative damage, including lipid peroxidation and the inhibition of generation of superoxide radicals (King *et al.,* 1998).

Numerous types of bioactive compounds have been isolated from the plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation. Flavopiridol is a synthetic flavone derived from the plant alkaloid rohitukine, which has been isolated from *Dysoxylum binectariferum* Hook.f. (Kellard *et al.*, 2000). It has been currently used in clinical trials against a broad range of tumors including leukemia, lymphoma and solid tumors (Christian *et a*l.,1997). Synthetic agent roscovitine which is derived from the natural product olomucine originally isolated from *Raphanus sativus* L. is in clinical trials in Europe (Cragg and Newman, 2005; Meijer *et al.*, 2003). Combretastatins were isolated from the bark of the South African tree *Combretum caffrum.* Combretastatin A4 is active against colon, lung and leukemia cancers and it is expected that this molecule is the most cytotoxic phytomolecule isolated so far (Shoeb, 2006b).

Betulinic acid, a pentacyclic triterpene is a common secondary metabolite of plants primarily from Betula species (Cichewitz *et al.,* 2004). Betulinic acid was isolated from Zizyphus species like *Mauritiana rugosa* and oenoplia and displayed selective cytotoxicity against human melanoma cell lines (Balunas *et al.*, 2005). Pervilleine A was isolated from the roots of *rythroxylum pervillei* (Silva *et al.*, 2001). Pervilleine A was selectively cytotoxic against a multidrug resistant oral epidermal cancer cell line in the presence of the anticancer agent vinblastine (Mi *et al.*, 2001). Silvestrol was first isolated from the fruits of *Aglaila sylvestre* (Hwang *et al.*, 2004). Silvestrol exhibited cytotoxicity against the lung and the breast cancer cell lines (Cragg and Newman, 2005). Two novel alkaloids, schischkinnin and montamine have been isolated from the seeds of *Centaurea schischkinii* and *Centaurea Montana* (Shoeb *et al.*, 2005; 2006a). Both of the alkaloids exhibited significant cytotoxicity against human colon cancer cell lines. Hence the presence of alkaloids, flavonoids and terpenes might have contribuited to the antioxidant and the antitumor activities of *Coleus forskohlii.*

Among the fractionated methanolic extracts (Petroleum ether, chloroform, acetone and methanol), only chloroform fraction of methanolic extracts was cytotoxic against DLA cells.

4.3.3. Determination of DLA cell viability against chloroform fraction of the methanolic extract of *Coleus forskohlii* roots by cell viability and MTT assay

Gupta *et al.* (2004) reported that the plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells and antitumor activity in experimental animals. Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations involving *in vitro* and *in vivo* studies. The determination of cell number is useful in the determination of the cytostatic potential of anticancer compounds in toxicology testing. Also in such toxicological studies, *in vitro* testing techniques are very useful to evaluate the cytotoxic, the mutagenic, and the carcinogenic effects of the chemical compounds on human cells.

The cell viability of DLA cells against varying concentrations of chloroform fraction of the methanolic extract of *Coleus forskohlii* root is presented in Table 25 and Figure 13.

The chloroform fraction of the methanolic extract at a concentration of 0 to 15 µg does not have any effect on DLA cells, whereas there is a complete absence of tumor cells at concentration of 30µg and above, indicating the toxic effect of methanolic extract against DLA cells.

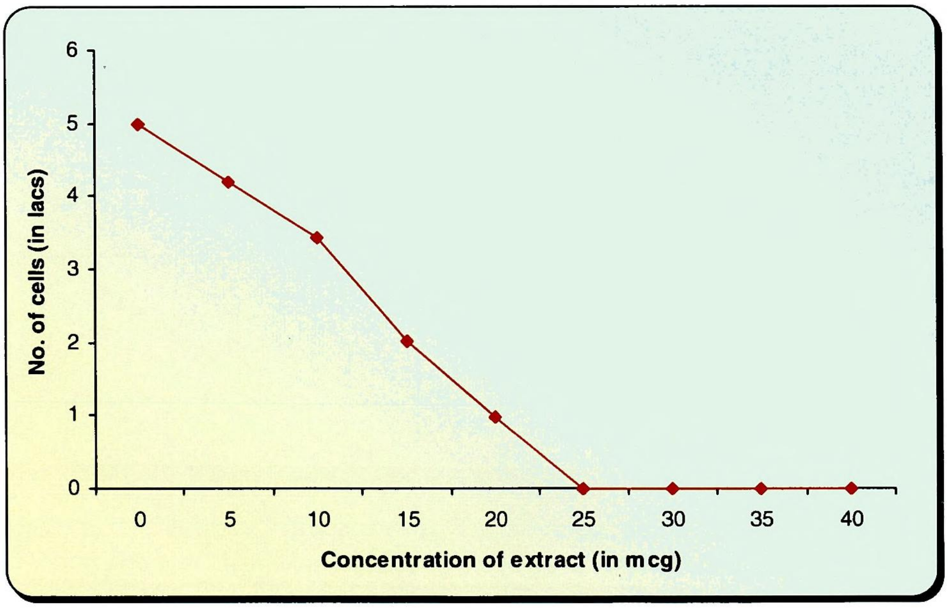
TABLE 25

DETERMINATION OF CELL VIABILITY OF DLA CELLS

S.No	Concentration of extract (in mcg)	Number of cells (in lacs)	Inference
1.	0	4.98	Healthy cells
2.	5	4.2	Healthy cells
3.	10	3.44	Healthy cells
4.	15	2.01	Healthy cells
5.	20	0.97	Unhealthy cells
6.	25	0.01	Unhealthy cells
7.	30	0	Nil
8.	35	0	Nil
9.	40	0	Nil

FIGURE 13

CELL VIABILITY OF DLA CELLS WITH VARYING CONCENTRATION OF CHLOROFORM FRACTION OF THE METHANOL EXTRACT OF THE ROOTS OF *Coleus forskohlii.*



The chloroform fraction of the methanolic extract inhibited the cell proliferation of DLA cell lines with IC_{50} of 13.5 μg/ml. Deregulation of apoptosis is the hall mark of all cancer cells and agents that activate programmed cell death in cancer cells could be a valuable anticancer therapeutics. Anticancer drugs act through different pathways converging ultimately into the activation of apoptosis in cancer cells, leading to cell cytotoxicity (Zalke *et al.,* 2010). According to the criteria of the American Cancer Institute, 30 μg/ml is the upper IC_{50} limit, considered promising for purification of a crude extract.

The anticancer potential of *P.langifolia* leaves extract and its chloroform fraction was observed when the extract and the chloroform fraction inhibited cell proliferation of various human cancer cell lines in which colon cancer cells SW-620 showed maximum inhibition with IC_{50} value 6.1 μg/ml (Verma *et al.*, 2008).

The quantification of cellular growth, including proliferation and viability has become an essential tool in any laboratory working on cell-based studies. Such techniques enable, not only the optimization of cell culture conditions, but also the determination of growth factor and cytokine activity. Even more importantly, the quantification of cell growth offers the opportunity to assess the efficacy of therapeutic agents in drug screening, the cytotoxic potential of anticancer compounds in toxicology testing and cell-mediated toxicity.

MTT ASSAY

Most viability assays are based on one of the two characteristic parameters, viz. metabolic activity and cell membrane integrity of healthy cells. Usually, the metabolic activity is measured in cell populations *via* incubation with a Tetrazolium salt (e.g., MTT, XTT and WST-1) that is cleaved into a colored formazan product by metabolically active cells.

The ATP status of cells can also be analyzed which will give an indication for cellular energy capacity and thus viability. The second type of assay also termed dye - exclusion assay takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue. The dead cells will be stained and the healthy cells can be counted directly. In cytotoxicity studies, this ability is also analyzed. However, in these assays, molecules are released from the dying cells with leaky cytoplasmic membranes and are measured in cell populations (e.g., lactate dehydrogenase). The MTT test offers a high degree of precision and is easy to do. It is suitable for the purpose of large scale assays (Romijn, 2004).

The MTT assay is based on the enzymatic reduction of the tetrazolium salt MTT (3-(4,5,dimethyl thiazole 2,5, diphenyl tetrazolium bromide) in living metabolically active cells but not in the dead cells. The MTT tetrazolium salt colorimetric assay previously described by Mosmann (1983) to measure cytotoxicity and cell proliferation was further explored to extend its application to the measurement of cell activation. The level of MTT cleavage by viable cells of various origins is directly proportional to the number of cells but increases as a non-linear function of time. This non-linear relationship is related to a time-linear cell death during MTT incubation.

The cleavage of MTT by viable cells was found to follow first order kinetics and could be fitted to Michaelis' kinetics. Different cell types exhibit similar apparent Km values (1949 microM) and different apparent maximal velocities (V). The apparent V values determined for a given cell type under different experimental conditions are rigorously similar. The analysis of MTT cleavage by viable cells suggests that the colorimetric MTT test can be useful to quantify the activation level of cells, independently of proliferation.

The results of the MTT test on cell viability of DLA cells against the methanolic extract of *Coleus forskohlii* roots is given in the Table 26 and Figure 14.

TABLE 26

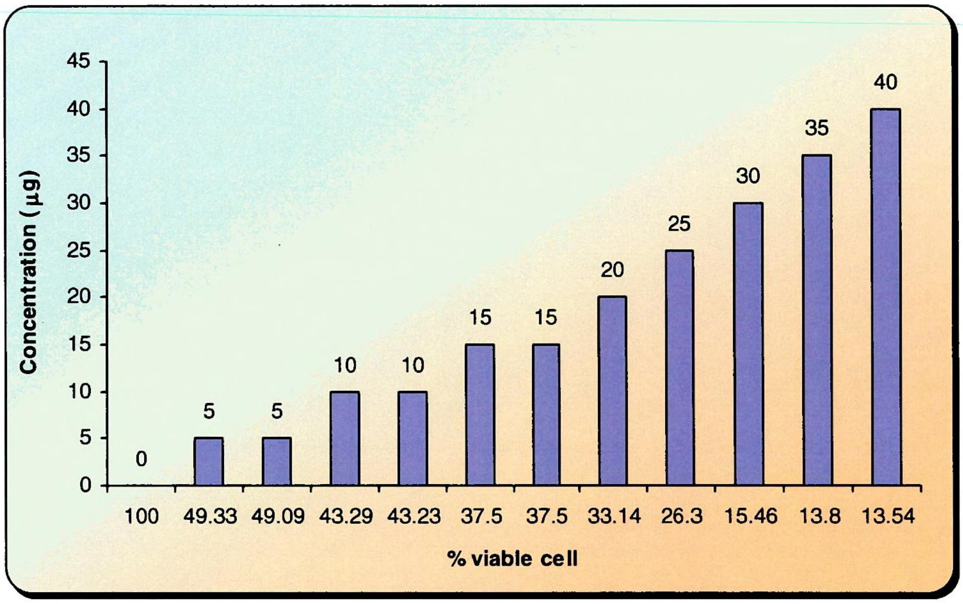
PERCENTAGE OF CELL VIABILITY WITH VARYING CONCENTRATION OF CHLOROFORM FRACTION OF THE METHANOLIC EXTRACT BY USING MTT ASSAY

No	Concentration of extract (mcg)	Category	Cell viability (%)
1.	0	Control (RPMI media)	100
2.	5	Treated	49.33
3.	5		49.09
4.	10		43.29
5.	10		43.23
6.	15		37.50
7.	15		37.50
8.	20		33.14
9.	25		26.30
10.	30		15.46
11.	35		13.80
12.	40		13.54

The percentage cell viability of DLA cells against the *Coleus forskohlii* extract showed that the percentage cell viability is reduced to 49.21% at 5 µg concentration and is further reduced as the concentration of the extract increases and the percentage of cells viable is only 13.54% at 40 µg concentrations, indicating the toxicity of the extract towards the DLA cells. Saeed *et al.* (2007) has determined the cytotoxic activity of quinolones against seven cancer cell lines using *invitro* cell culture by MTT assay system.

FIGURE 14

PERCENTAGE OF CELL VIABLE WITH VARYING CONCENTRATION OF CHLOROFORM FRACTION OF METHANOLIC EXTRACT



Bhagat *et al.* (2010) had studied the cytotoxic activity of different fractions of *Calotropis procera* roots and showed that the active constitutents are non polar and present in chloroform fraction which had exhibited cytotoxicity against the oral cancer cell line.

Mothana *et al.*, (2009) also reported that only the methanolic extracts of the plants exhibited cytotoxic activity.

4.3.4 Identification of the phytochemicals in *Coleus forskohlii* by high performance thin layer chromatography (HPTLC)

Phytochemical analysis is one of the tools for the quality assessment of plant products which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques. HPTLC is emerged as an important tool for the qualitative and quantitative phytochemical analysis of herbal drug and formulations (Kshirsagar *et al.*, 2008).

Plate 6 and Table 27 represents the high performance thin layer chromatogram of chloroform fraction of methanolic extract of roots of *Coleus forksohlii.*

PLATE 6

HIGH PERFORMANCE THINLAYER CHROMATOGRAM OF CHLOROFORM FRACTION OF THE METHANOLIC EXTRACT OF *Coleus forskohlii*

Before derivatization **After derivatization**

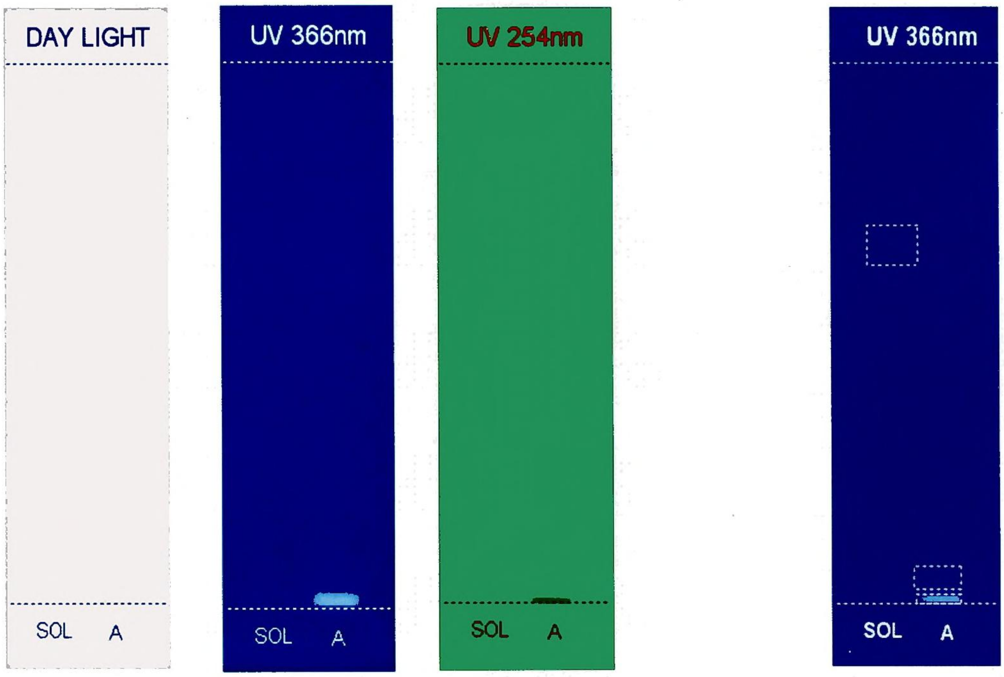


TABLE 27

HPTLC OF CHLOROFORM FRACTION OF THE METHANOLIC EXTRACT OF *Coleus forskohlii*

Track	Peak	Rf	Height	Area	Assigned substance
SOL	1	0.68	57.1	2183.8	Solanesol standard
A	1	0.04	214.7	7355.0	Terpenoid 1
A	2	0.08	149.4	3953.6	Terpenoid 2

It was observed from the chromatogram after derivatization that blue colored fluorescent zones at UV 366nm mode were present in the track which was compared with the standard diterpenoid solenesol. This confirmed the presence of terpenoids in the chloroform fraction of *Coleus forskohlii.*

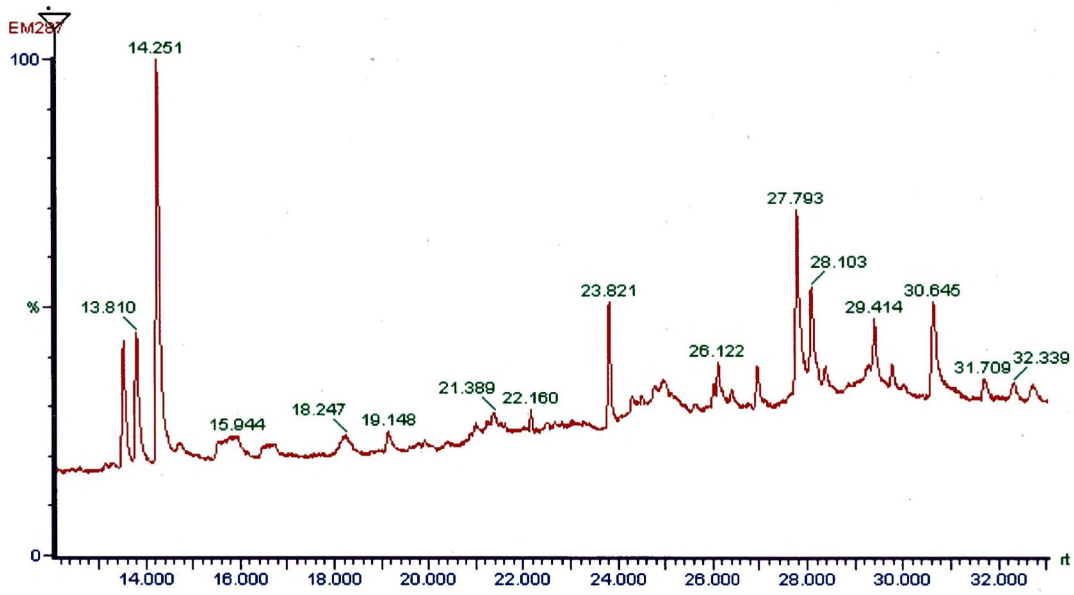
4.3.5 Identification of the anticancer constituents in the chloroform fraction of the methanolic extract of *Coleus forskohlii* by GC-MS

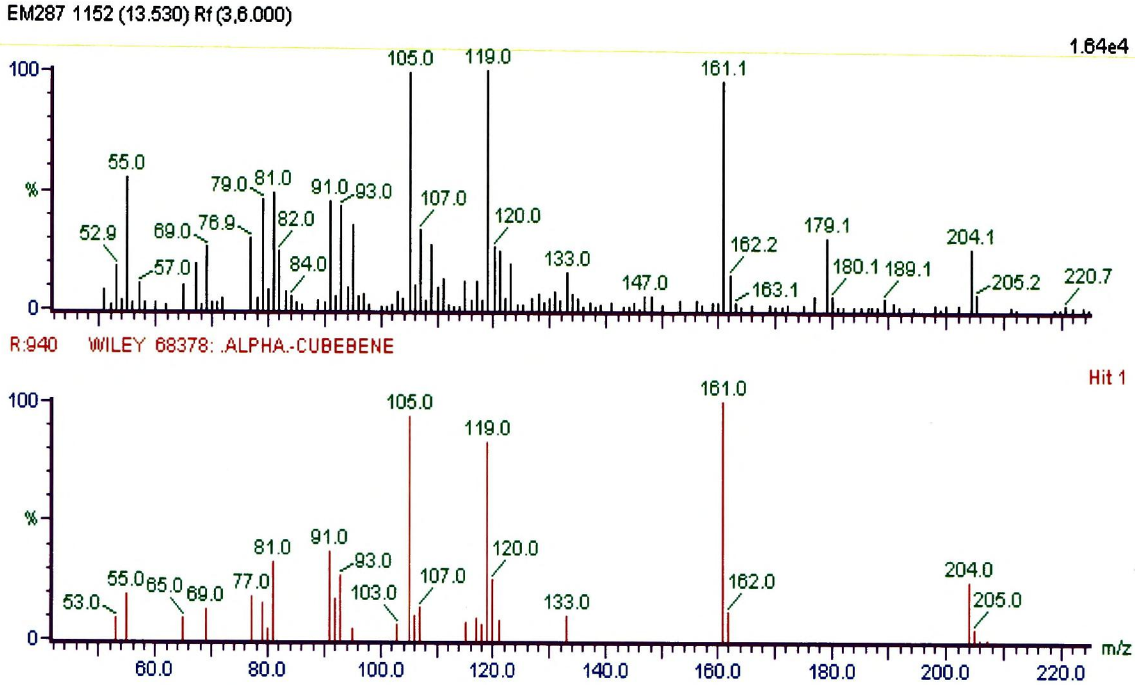
Over 100,000 metabolites are described in the plant kingdom and it can be assumed that the number of metabolites of an individual plant species may easily reach some 5,000 low molecular weight compounds. These compounds can be analyzed by a technique called Gas chromatography-Mass spectroscopy (GC-MS), which is the most common technique .

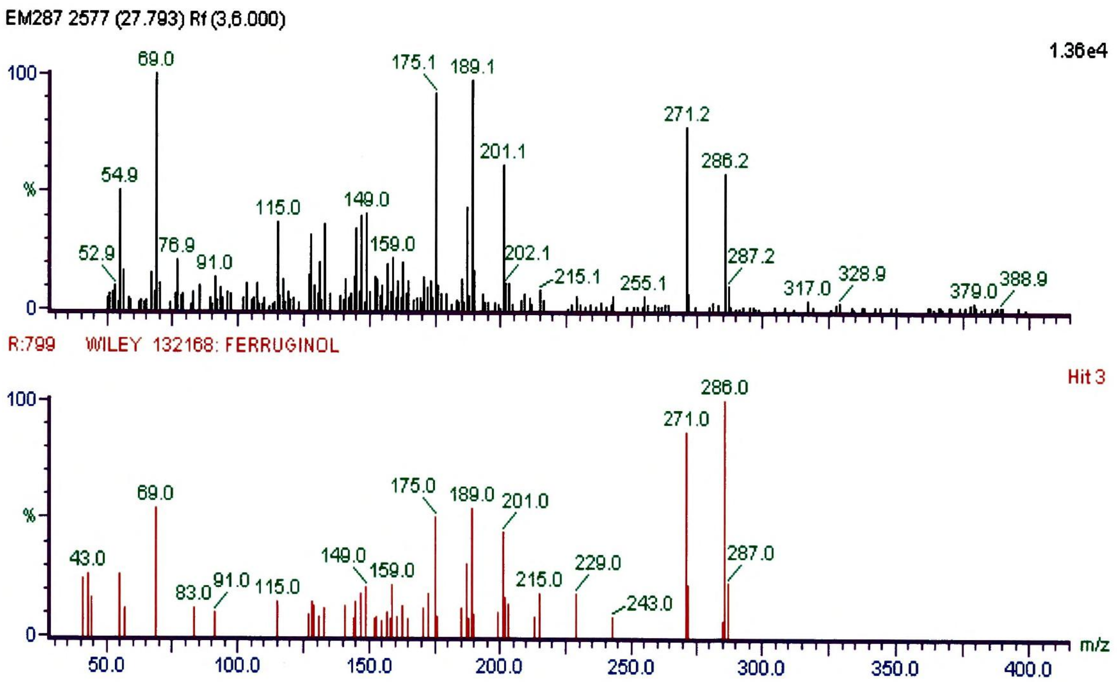
The GC-MS chromatogram of the *Coleus forskohlii* is shown in Figure 15 and the identified compounds are shown in Table 28.

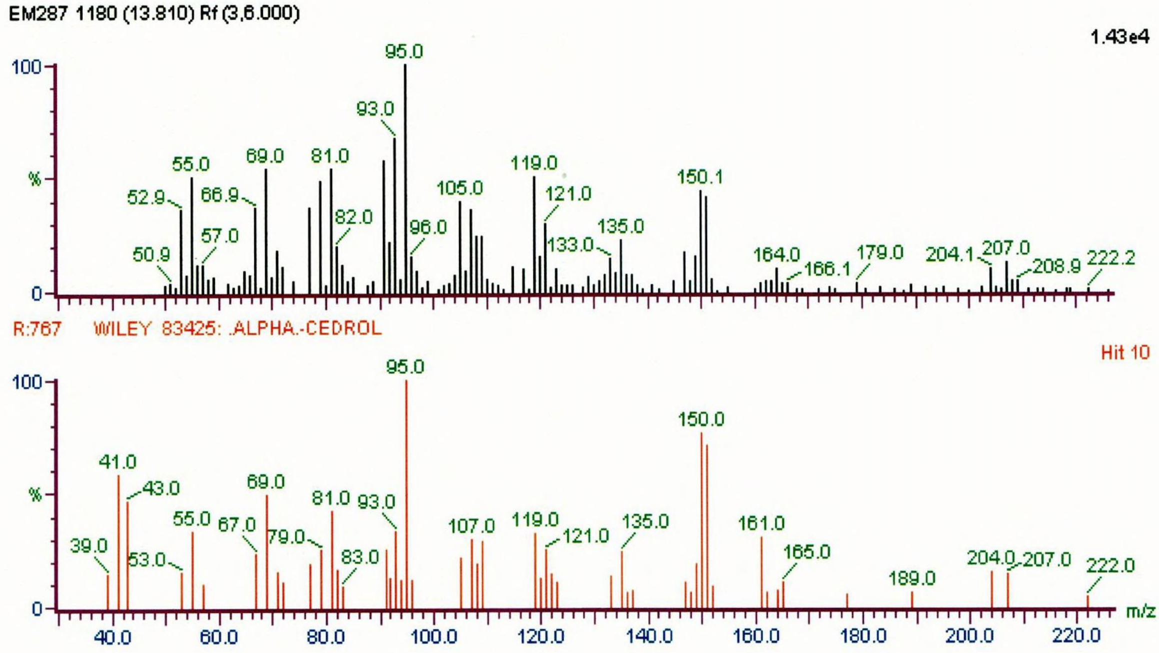
FIGURE 15

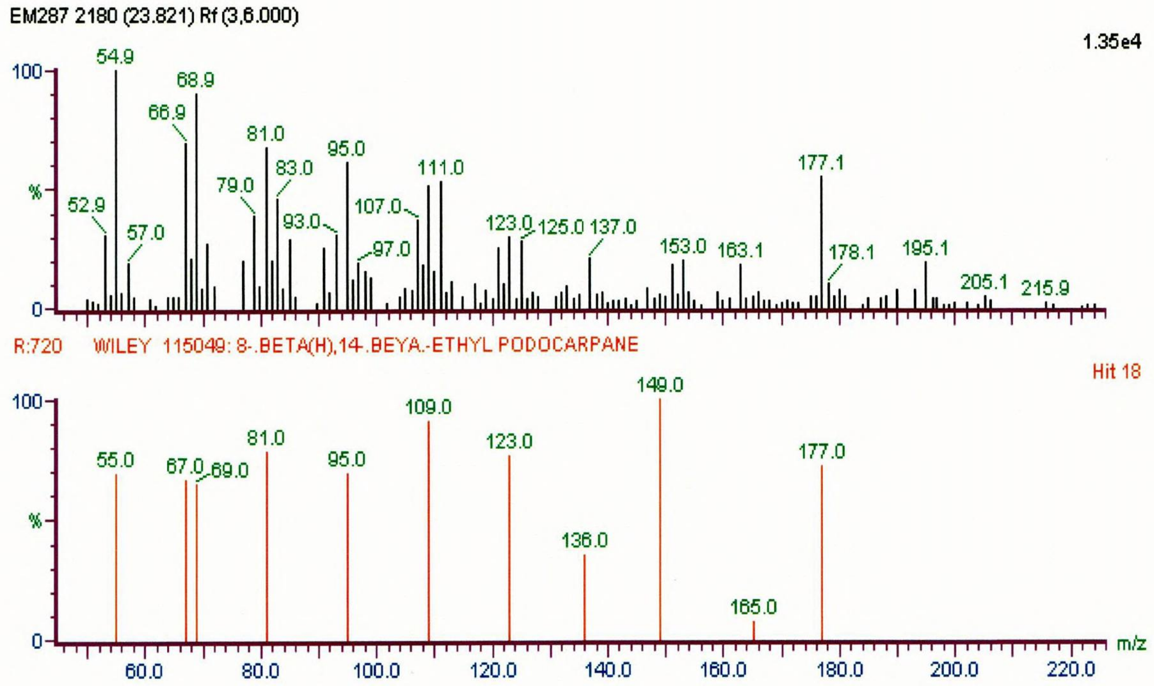
THE GC-MS CHROMATOGRAM OF CHLOROFORM FRACTION OF *Coleus forskohlii* ROOTS

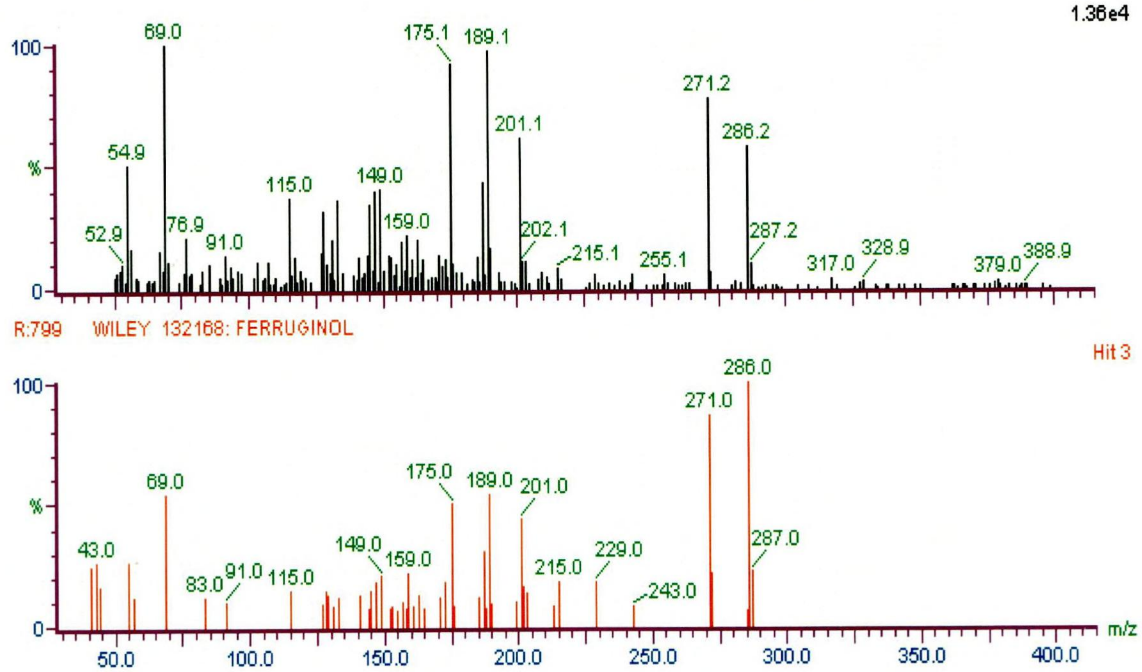



EM287 1152 (13.530) Rf (3,6.000)
1.64e4
100
%
0
105.0
119.0
161.1
55.0
79.0
81.0
91.0
93.0
107.0
52.9
69.0
76.9
82.0
120.0
179.1
204.1
57.0
84.0
133.0
162.2
180.1
189.1
147.0
163.1
205.2
220.7
R:940 WILEY 68378: .ALPHA.-CUBEBENE
Hit 1
105.0
161.0
119.0
81.0
91.0
93.0
120.0
55.0
65.0
69.0
77.0
53.0
103.0
107.0
133.0
162.0
204.0
205.0
m/z
60.0
80.0
100.0
120.0
140.0
160.0
180.0
200.0
220.0


EM287 2577 (27.793) Rf (3,6.000)
1.36e4
100
%
0
69.0
175.1
189.1
271.2
201.1
286.2
54.9
115.0
149.0
52.9
76.9
91.0
159.0
202.1
287.2
215.1
255.1
317.0
328.9
379.0
388.9
R:799 WILEY 132168: FERRUGINOL
Hit 3
286.0
271.0
69.0
175.0
189.0
201.0
43.0
149.0
159.0
229.0
83.0
91.0
115.0
215.0
243.0
287.0
m/z
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0


EM287 1180 (13.810) Rf (3,6.000)
1.43e4
R:767 WILEY 83425: .ALPHA.-CEDROL
Hit 10
100
%
0
m/z
40.0
60.0
80.0
100.0
120.0
140.0
160.0
180.0
200.0
220.0
95.0
93.0
55.0
69.0
81.0
119.0
150.1
52.9
66.9
105.0
121.0
82.0
57.0
96.0
135.0
133.0
50.9
164.0
166.1
179.0
204.1
207.0
208.9
222.2
150.0
41.0
43.0
55.0
67.0
79.0
81.0
93.0
107.0
121.0
161.0
39.0
53.0
83.0
165.0
189.0
204.0
207.0
222.0


EM287 2180 (23.821) Rf (3,6.000)
1.35e4
R:720 WILEY 115049: 8-.BETA(H),14-.BEYA.-ETHYL PODOCARPANE
Hit 18
100
%
0
m/z
60.0
80.0
100.0
120.0
140.0
160.0
180.0
200.0
220.0
54.9
68.9
66.9
81.0
95.0
83.0
111.0
177.1
79.0
52.9
107.0
57.0
93.0
97.0
123.0
125.0
137.0
153.0
163.1
178.1
195.1
205.1
215.9
149.0
109.0
81.0
123.0
55.0
67.0
69.0
95.0
177.0
136.0
165.0


1.36e4
100
%
0
69.0
175.1
189.1
271.2
201.1
286.2
54.9
149.0
115.0
159.0
52.9
76.9
91.0
202.1
287.2
215.1
255.1
317.0
328.9
379.0
388.9
R:799 WILEY 132168: FERRUGINOL
Hit 3
286.0
271.0
69.0
175.0
189.0
201.0
287.0
43.0
149.0
159.0
229.0
83.0
91.0
115.0
215.0
243.0
m/z
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0

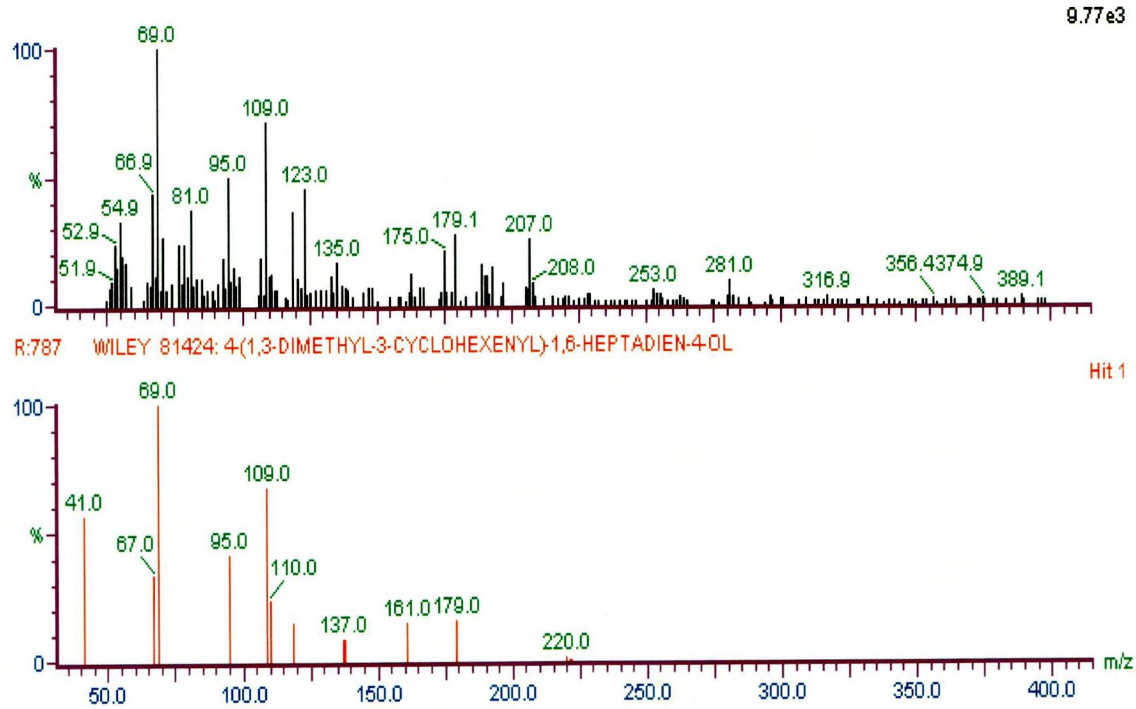

9.77e3
100
%
0
69.0
109.0
66.9
95.0
123.0
54.9
81.0
52.9
179.1
207.0
175.0
135.0
51.9
208.0
253.0
281.0
316.9
356.4374.9
389.1
R:787 WILEY 81424: 4-(1,3-DIMETHYL-3-CYCLOHEXENYL)-1,6-HEPTADIEN-4-OL
Hit 1
69.0
109.0
41.0
67.0
95.0
110.0
161.0
179.0
137.0
220.0
m/z
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0

TABLE 28

THE GC-MS PROFILE OF *Coleus forskohlii*

Sl.No	Name of the compound	RT	M/Z
1.	Alpha-Cubebene	13.53	204
2.	Alpha –Cedrol	13.81	222
3.	8ß(H),14-Beta.ethyl podocarpane	23.81	262
4.	Ferruginol	27.793	286

With the partition by different polarity ranges of solvents (petroleum ether, chloroform, acetone and methanol), open column chromatogram and GC-MS analysis were done to identify the constituents present in the extract of *Coleus forskohlii* root which had shown the presence of the diterpenoids such as α-cubebene, α-cedrol, 8ß Hydroxy 14 β-ethyl podocarpane and ferruginol. Of these, the compounds α-cubebene, podocarpane and ferruginol possess antitumor activity.

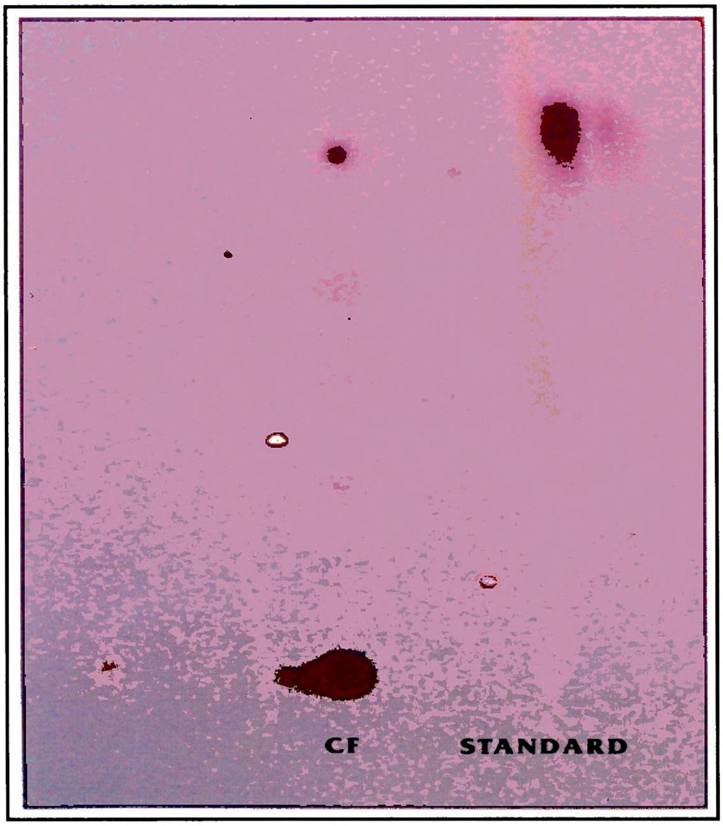
Diterpenes are a diverse group of compounds with four isoprene units, most of which have limited distribution in the living plants. Because of their high boiling points, they are not considered to be essential oils and are classically considered to be resins, the material that remains after steam distillation of a plant extract. Diterpenes constitute the second largest class of terpenes (Wang *et al.*, 2002).

4.3.6 Identification of the anticancer constituents in the chloroform fraction of the methanolic extract of *Coleus forskohlii* root by TLC and HPLC

The detection of active principles in medicinal plants plays a strategic role in the phytochemical investigation of crude plant extracts and is very important for potential pharmacological effects (Pascuel *et al.*, 2002). The TLC profile of the 70:30 fraction of chloroform methanolic extract of *Coleus forskohlii roots* is presented in plate 7.

PLATE 7

TLC PROFILE OF 70:30 FRACTION OF CHLOROFORM FRACTION OF THE METHANOLIC EXTRACT OF *Coleus forskohlii* ROOTS



The TLC profile of the chloroform fraction of the methanolic extract of *Coleus forskohlii* shows 2 spots of which one purple spot corresponds to forskolin

Figures 16 and 17 show the HPLC profile of standard forskolin and 70: 30 chloroform methanolic fraction of *Coleus forskohlii* respectively.

FIGURE 16

HPLC PROFILE OF THE STANDARD FORSKOLIN

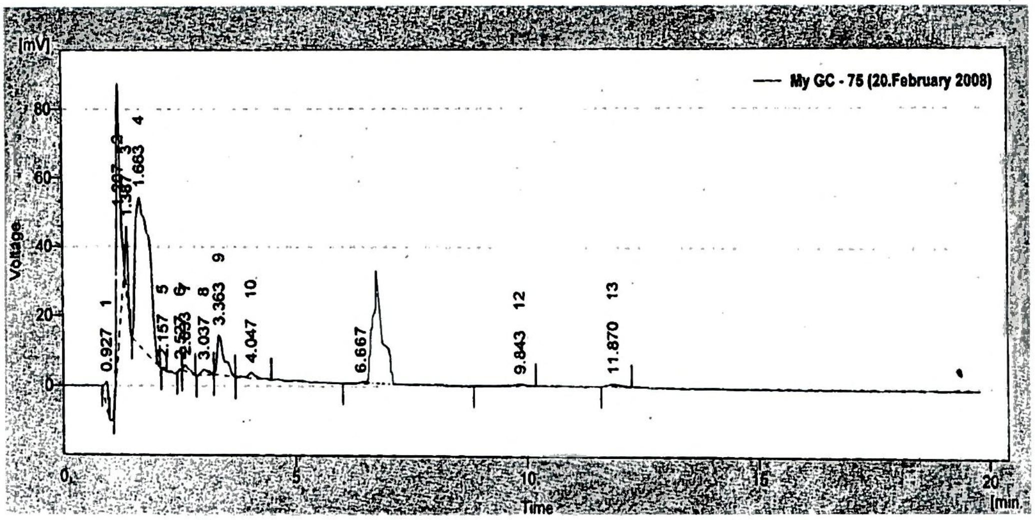
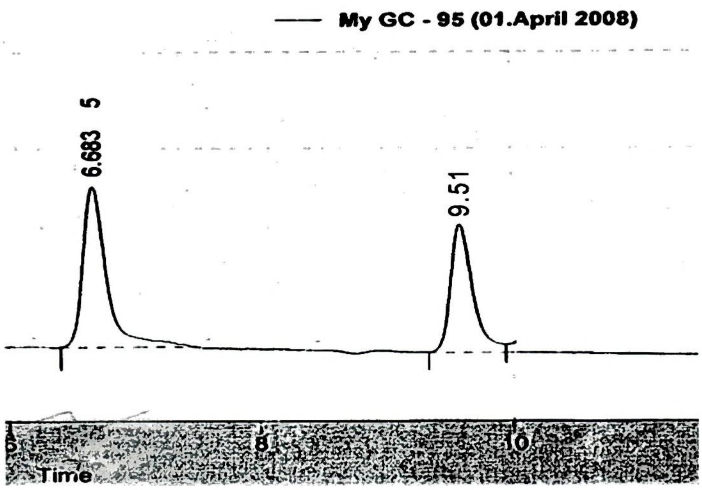


FIGURE 17

HPLC PROFILE OF THE 70:30 FRACTION OF CHLOROFORM METHANOL EXTRACT OF *Coleus forskohlii*



From the figure 16 and 17, the presence of forskolin is confirmed as it has the retention time of 6.6 min in the High performance liquid chromatogram. The diterpene forskolin, derived from the root of the plant is the primary constituent of clinical interest in *Coleus forskohlii.* Forskolin (7-beta acetoxy-8, 13 epoxy, 1 alpha, 6 beta 9 alpha trihydroxy labd14-ene 11- one) is the main active ingredient in the ayurvedic herb *Coleus forskohlii.* Forskolin has been extensively researched in the medical field for its use in the treatment of allergies, respiratory problems, cardiovascular diseases, glaucoma, psoriasis, hypo thyroidism and weight loss. Forskolin increases cyclic AMP and appears to have additional actions that are due to its ability to alter a number of membrane transport proteins. Many metastasizing tumor cell lines induce platelet aggregation both *in vitro* and *in vivo*. Upon aggregation, platelets release substances that promote tumor growth. Researchers have demonstrated forskolin's ability to block platelet aggregation *via* its stimulation of the activity of platelet adenylate cyclase and increase in intracellular cAMP. The isolation of Coleon S 33 from *Coleus forskohlii* has been examined by Yao *et al.*, 2002

The chromatogram also showed the presence of ferruginol which has the retention time of 9.51 min as shown by reference standard (Wei *et al.*, 2008). Ferruginol is an abietane diterpene phenol. It has been found recently to have many pharmacological properties, including anti-tumor (Son *et al.*, 2005; Jesus *et al.*, 2008), anti-fungal (Becerra *et al.*, 2002), antimalarial (Koch *et al.*, 2006), anti-bacterial (Kabouche *et al.*, 2005, Kuzma *et al.*, 2007;), cardio-protective (Ulubelen *et al.*, 2002), anti-plasmodial (Clarkson *et al.*, 2003) and gastroprotective effects (Rodriguez *et al.*, 2006, Espinoza *et al.*, 2008).

Thus, it could be concluded that the HPLC and the GC-MS peaks of the extract of the *Coleus forskohlii* root *sample* has shown the presence of diterpenoids like forskolin, ferruginol and it can be suggested to be the active principle which might be responsible for potential antioxidant and antitumor activity of roots of *Coleus forskohlii.*