CHAPTER 8

ANTI CANCER ACTIVITY

8.1 INTRODUCTION

Cancer is a disease in which there is an uncontrolled multiplication and spread, within the body, of abnormal form of the body's own cells. Uncontrolled replication of cells causes a tissue to forms, commonly known as tumour. It is a genetic term for a wide variety of malignant neoplasm that may result in deleterious effects on the host due to their invasive and metastasizing nature [127].

Mortality that results from the common forms of cancer is still unacceptably high. Despite many therapeutic advances in the understanding of the processes in carcinogenesis, overall mortality statistics are unlikely to change until there is a reorientation of the concepts for the use of natural products as new chemo preventive agents. Natural or semi synthetic compounds may be used to block, reverse or prevent the development of invasive cancers. Cellular carcinogenesis forms the biologic basis for the identification of preventive products, the assessment of their activity and ultimately the success or failure of a therapy.

As long ago as 480 BC, Hippocrates recognised that several aspects of what we now call ‘‘lifestyle’’ must come together to produce a healthy body. Cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition and via the environment. At least 35% of all cancers

worldwide are caused by an incorrect diet and in the case of colon cancer, diet may account for 80% of the cases. When one adds alcohol and cigarettes to their diet, the percentage may increase to 60%. Genetic predisposition to cancer lends itself to 20% of cancer cases, thus leaving the majority of cancers being associated with a host of environmental carcinogens [128].

Drug discovery from medicinal plants has played an important role in the treatment of cancer and most clinical applications of plant secondary metabolites and their derivatives over the half century have been applied towards combating cancer. Of all available anticancer drugs between 1940 and 2002, 40% were natural products or natural product derived, with another 8% considered natural product mimics [129]. An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds [130]. Plants have a long history of use in the treatment of cancer, though many of the claims for the efficacy of such treatment should be viewed with some scepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine. One of the best approaches in search for anticancer agents from plant resources is the selection of the plants based on ethno medical leads and testing the selected plants efficacy and safety in light of modern sciences[131].

Cancer is one of the most prominent diseases in humans and currently there is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from natural product sources [132]**.** The potential of using natural products as anticancer agents was recognized in 1950s by the U.S. National Cancer Institute (NCI) and has since made major contributions to the discovery of new naturally occurring anticancer agents [133].

8.1.2 Cancer

a) Carcinogens

The majority of human cancers result from exposure to environmental carcinogens; these include both natural and manmade chemicals, radiation, and viruses. Carcinogens may be divided into several classes.

(i) Genotoxic carcinogens, if they react with nucleic acids. These can be directly acting or primary carcinogens, if they are of such reactivity so as to directly affect cellular constituents.Primary, direct-acting alkylating agents- Dimethylsulfate, Ethylene imine, and B-propiolactone

(ii) Alternatively, they may be procarcinogens that require metabolic activation to induce carcinogenesis. Examplesare, Polycyclic aromatic hydrocarbons-eg. Benzo[a] pyrene, Nitrosamines – eg. Dimethyl nitrosamine. Hydrazine – eg.1, 2-Dimethyl hydrazine, Inorganic- eg. Cadmium, Plutonium.

(iii) Epigenetic carcinogens are those that are not genotoxic. Molecular diversity of the cancer-initiating compounds ranges from metals to complex organic chemicals and there is large variation in potency. The variation in structure and potency suggests that more than one mechanism is involved in carcinogenesis. Examples are: Promotors- Phorbol esters, saccharin, bile acids.Solid state-Asbestos, plastic, Hormones-Estrogens, Immunosuppressant- Purine analogues,Co-carcinogens- Catechol.

(iv) Unclassified - Peroxisome proliferators- Clofibrate, Phthalate esters.

Apart from exposure to carcinogens other factors such as the genetic predisposition have been documented. Thus, patients with genetic xero derma pigmentosum are more susceptible to skin cancer. Furthermore, incidence of bladder cancer is significantly higher in those individuals who have the slow acetylator phenotype, especially if they are exposed to aromatic amines.

Carcinogens in the diet that trigger the initial stage include moulds and aflatoxins (for example, in peanuts and maize), nitrosamines (in smoked meats and other charred products), rancid fats and cooking oils, alcohol, and additives and preservatives. A combination of foods may have a cumulative effect and when incorrect diet is added to a polluted environment, smoking, UV radiation, free radicals, lack of exercise and stress, the stage is set for DNA damage and cancer progression. On the protective side, we know that a diet rich in fruit, vegetables and fibre is associated with a reduced risk of cancer at most sites [134].

8.1.3 Global Cancer Incidence

Modern man is confronted with an increasing incidence of cancer and cancer deaths annually. Statistics indicate that men are largely plagued by lung, colon, rectum, and prostate cancer, whilst women increasingly suffer from breast, colon, rectum, and stomach cancer [135].

8.1.4 Natural Products and Defence against Carcinogenesis

The literature indicates that many natural products are available as chemoprotective agents against commonly occurring cancers occurring

worldwide. A major group of these products are the powerful antioxidants, others are phenolic in nature, and the remainder includes reactive groups that confer protective properties. These natural products are found in vegetables, fruits, plant extracts, and herbs. Some examples of natural products that shows anticancer activity are olives, apples, strawberries, melon, leafy greens, cauliflower, wheat germ, yellow-orange, carrots, tomatoes, grapes, citrus fruits, garlic, onions, common bean etc[136].

Although the mechanism of the protective effect is unclear, the fact that the consumption of fruit and vegetables lowers the incidence of carcinogenesis at a wide variety of sites is broadly supported. The epidemiological evidence suggests that protection against a wide array of cancers, particularly those of the respiratory and digestive tracts and to a lesser extent the hormone related cancers. A host of plant constituents could be responsible for the protective effects and it is likely that several of them play a role under some circumstances. Most of the non-nutrient antioxidants in these foods are phenolic or polyphenolic compounds, such as isoflavones in soybeans, catechins in tea, phenolic esters in coffee, phenolics acid in red wine, quercetin in onions, and rosmarinic acid in rosemary.

Of the many anti carcinogens already detected in plant foods, the antioxidants, vitamins C and E and the provitamin β-carotene have received the most attention [137]. Although there has been considered enthusiasm for the potential anticarcinogenic properties of β-carotene research findings suggest that several different carotenoids are likely to be associated with reduced cancer risks. In two intervention trials to investigate the potential protective effects of b-carotene against cancer, an unexpected significantly higher incidence of lung cancer was found in men taking supplements compared with those not taking additional β-carotene. These men were long-time heavy smokers and may represent a special case in that their lung cancer

may have been initiated many years before the study took place. These results cause concern and need serious consideration. They do not invalidate the concept of the importance of antioxidant nutrients but do underline the need to examine the relative influence of supplements of a single antioxidant nutrient (as distinct from complex mixtures of antioxidants in foods) as well as interactions between the effects of smoking, antioxidant nutrients, and disease progression. A number of naturally occurring compounds from vegetables and herbs exert chemo preventive properties against carcinogenesis. Most studies appear to test the natural products on human leukaemia cells. The Chinese medicinal herb *Rhizoma zedoariae*, for example, produces a compound called lemene, which has been shown to exhibit antitumor activity in human and murine tumour cells in *vitro* and in *vivo* [138].

The IC_{50} values of lemene indicated severe inhibition of promyelocytic HL-60 cells, erythroleukemia K562 cells and especially peripheral blood leukocytes. This was associated with cell arrest from S to G_2, M phase transition and with induction of apoptosis [139].Similar inhibitory effects were produced by allicin, a natural organosulfide from garlic. *In vitro* inhibition of proliferation of HL-60 cells or induction of apoptosis in promyelocytic leukemia was also demonstrated by Donget al (1997) [140] using other Chinese medicinal products, namely the bis benzyl iso quinoline alkaloids, tetrandrine and berberine, by Jing et al (1994) [141] using bufalin, by Sheng et al [142] using extracts of *Uncaria tomentosa, a*nd by Hirano et al [143] using cholestane glycosidase.

The successful identification of novel effective anticancer drugs is largely dependent on the use of appropriate preclinical experimental models that should possibly mimic the complexity of different cancer diseases. The huge number of targets suitable for the design of new anticancer drugs is

producing hundreds of novel molecules that require appropriate experimental models to investigate their mode of action and antitumor activity in order to select for clinical investigation the ones with higher chances of being clinically effective. There are no ideal models that can be used for any kind of drug, but depending on the drug. Genetic models are particularly relevant for the validation of the potential therapeutic value of new targets. The most suitable experimental models should be selected. Once a compound has demonstrated robust cytotoxic activity against a panel of human cancer cell lines and deserves further investigation in *in vivo* models, it is important to clarify its mechanism of action and to identify its exact molecular targets. An example of the importance of the *in vitro* studies aimed at clarifying the mechanism of action of a given drug.

8.1.5 Plants Used In the Treatment of Cancer

Some of the plants claimed for the treatment of cancer enumerated as follows [144], *Acacia arabica, Ageretum conzyoides, Allium sativum, Aloe vera, Bauhinia variegata, Cassia fistula, Calotropis gigantean, Berberis aristata, Boswellia serrata, Centella asiatica,Curcuma longa,Ficus glomerata,Hygrophila auriculata, Momordica charantia,Morinda citrifolia, Mucuna prurita,Solanum ndicum, Shorea robusta,Trianthema monogyna, Viola odorata, Vinca rosea, Woodfordia fructicosa, Xanthium strumarium, Tinospora cordifolia,Trigonella foenum-graecum, Viscum album, Withania somnifera.*

8.1.6 Herbal Formulations Used In Cancer

Immunotone, **Cancertame**, **Oncotame** are unique herbal formulations that helps to fight cancer more effectively by inhibiting mutations in the genes and enhancing immunity of the body. The herbal formulation contains active principles isolated from selected antimutagenic and immune enhancing herbs.

8.1.7 Experimental models for Anticancer Activity

Animal models of cancer, particularly mouse models of cancer, are commonly used to study tumour biology and develop new approaches to conquering human cancer. As the most commonly used systems in cancer drug development, mouse cancer models have helped us circumvent lots of ethical and economical problems for human cancer experiments. Because mice can develop human cancers. Furthermore, human tumours can be implanted in mice. In addition, mice have simpler genetics with humans. The above reasons proposed the utility of Dalton's lymphoma ascites (DLA) for the present study in mice.

Ascites is the presence of excess fluid in the peritoneal cavity. It is a common clinical finding with a wide range of causes, but develops most frequently as a part of the decompensation of previously asymptomatic chronic liver disease.

Rapidly developing (acute) ascites can occur as a complication of trauma, perforated ulcer, appendicitis or inflammation of the colon or other tube-shaped organ (diverticulitis). This condition can also develop when intestinal fluids, bile, pancreatic juices or bacteria invade or inflame the smooth, transparent membrane that lines the inside of the abdomen (peritoneum). However, ascites is more often associated with liver disease and other long-lasting (chronic) conditions.

8.2 MATERIALS AND METHODS

Dalton's Lymphoma Ascitic (DLA) cells, Mouse lung fibroblast, Minimum essential medium (MEM), Streptomycin, Camptothecin, Normalsaline, Tryphanblue, 10%formalin, Ethanol, Paraffin , Hematoxylin, Eosin, CO_2 water-jacketed incubator (Heraeus, Germany)

8.2.1 Cell Lines

Dalton's Lymphoma Ascites (DLA) cells was originally obtained from Amala Cancer Institute, Trissur, Kerala, and are being maintained in our laboratory as described below.DLA cells were maintained by weekly intraperitoneal (i.p.) inoculation of 2 x 10^6 cells/mouse.

Maintenance of DLA Cell Lines

The DLA cells were grown in Dulbecco's modified Eagle medium (DMEM) with10 % v/v foetal calf serum (FCS) in a CO_2 water-jacketed incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.The cells were propagated in the peritoneal cavity of the mice by injecting 10^6 cells. The cells were aspirated aseptically from developed tumour mice, during the log phase on the 15th day of tumour transplantation using 18 gauge needle by withdrawing the fluid from peritoneal cavity. The ascitic fluid was washed three times in PBS (Phosphate buffer saline) and the cell pellet was suspended in PBS. The tumour cell count was done using Tryphan blue dye exclusion method in a haemocytometer. The cell suspension was diluted to get $2x10^6$/0.1 ml [145]

8.2.2 *In Vitro* Cytotoxicity

Mouse lung fibroblast (L-929) cells were obtained through the courtesy of the National Institute of Virology, Pune, India.

Long term cytotoxicity of MELA to L-929 was determined by seeding 1 x 10^6 cells L-929) in a culture bottle containing 10 ml minimum essential medium (MEM) supplemented with 10% heat inactivated goat serum and 100 mg of Streptomycin. After 24 h incubation at 37°C, the cells were exposed to different (1–100µg) concentration of the extract or Camptothecin (a reference drug). Inhibition of the cell proliferation was assessed after 6 days by trypsinising and counting the cells with haemocytometer [146].

Tryphan Blue Dye Exclusion Technique

Tryphan Blue is a vital dye. The reactivity of Tryphan blue was based on the fact that the chromophore was negatively charged and does not interact with the cell unless the membrane was damaged. Therefore, all the cells which exclude the dye are viable.

Procedure

Place 0.5ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of 1×10^5 to 2×10^5 cells per ml) in a screw cap test tube. To this add 0.1ml of 0.4% Tryphan Blue Stain and mix thoroughly then allow to stand for 5 min at 15 to 30ºC (room temperature).The prepared solution was then observed in the haemocytometer for viable cell counting under a microscope and examined for the viability of the cells (non – viable cells are stained and viable cells excluded the stain).Cells were counted by the following formulae (8.1, 8.2)

Cell count = Number of cells ×Dilution (Area ×Thickness of fluid film) (8.1)

Percentage of cell viability = (Live cell count/Total cell count) ×100. (8.2)

8.2.3 *In vivo* Anti-tumour Activity in Mice

After acclimatization, mature male Swiss albino mice divided into four groups (n=10) and given food and water *ad libitum*. All the groups (Table 1) except group I were injected with DLA Cells (2×10^6 cells/mouse. i.p). This was taken as day 0.

Experimental Design

The animals were grouped as follows (n=10)

Group I : The animals of this group received normal saline (5 ml/kg, p.o)

Group II : The animals of this group was injected with DLA cells only without any drug treatment (2×10^6 cells/mouse. i.p)

Group III : Received a MELA at a dose of 200mg / Kg body weight dose administered orally for 14 consecutive days + DLA Cells (2×10^6 cells/mouse. i.p)

Group IV : Received a MELA at a dose of 400mg / Kg body weight dose administered orally for 14 consecutive days + DLA Cells (2×10^6 cells/mouse. i.p)

On day 1, MELA at a dose of 200 and 400 mg/kg body weight (Gr-III and IV) were administered orally for 14 consecutive days.

On day 15, five mice from each group were sacrificed 24 h after the last dose and the rest were kept with food and water *ad libitum* to check the life span of the tumour hosts.

The effect of MELA on tumour growth and host's survival time were examined by studying the parameters like tumour volume, tumour cell count, viable tumour cell counts, nonviable tumour cell count, mean survival time, Packed cell volume (PCV) and increase in life span [147,148].

8.2.4 Determination of PCV

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 g for 5 min.

8.2.5 Determination of RBC Count

The ascitic fluid was taken in a RBC pipette and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in 64 small squares was counted.

8.2.6 Estimation of Viable Tumour Cell Count

The cells were then stained with Tryphan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and non-viable cells were counted using the formula given below (8.3).

Cell count = (No. of cells x Dilution) / (Area x Thickness of liquid film) (8.3)

8.2.7 Percentage Increase Life Span

Recording the mortality monitored the effect of MELA on tumour growth and percentage increase in life span (ILS %) were calculated [186] by the following formulae (8.4 and 8.5)

$$\text{ILS}(\%) = \left[\frac{\text{Mean survival of treated group}}{\text{Mean survival of control group -1}} \right] \text{X } 100 \qquad (8.4)$$

$$\text{Mean survival time} = \frac{[1\text{st Death} + \text{Last Death}]}{2} \qquad (8.5)$$

8.2.8 Effect of MELA on Median Survival Time (MST)

Animal of group II, III, IV was inoculated with 2×10^6 / mice on day 0. The MELA 200mg/kg and 400mg/kg b.w was administered orally to group III and group IV respectively from the 1st day of cancer induction. The doses were given continuously for a period of 9 days. The MST of each group consisting of 5 mice was noted. The mortality of the group was calculated.

8.2.9 Hematological Parameters

The effect of MELA on peripheral blood was investigated. The drug treatment was started from 1st day of tumor transplantation for group III and group IV. Group II was injected with DLA cells ($2x10^6$i.p) the appropriate dose was given continuously for a period of 14 days. Blood was gently withdrawn from retro – orbital puncture on 15th day and used for hematological studies.

Enumeration of Erythrocytes (RBC)

Blood was collected from the animals through the tail vein. The blood was then drawn up to the mark 0.5 in the RBC pipette and diluting fluid was mixed by sucking up to mark 101.The content in the RBC pipette was mixed by rolling between the palms. The first few drops were discarded and the rest was gently dropped onto Neubauer chamber. The total RBC was calculated as number of cells per cubic millimeter in counting area.

Enumeration of Leukocytes (WBC)

The blood was collected from the animals through the tail vein. The blood was then drawn up to the mark 1 in the WBC pipette and diluting fluid was mixed by sucking up to mark 11. The content in the WBC pipette was mixed by rolling between the palms.The first few drops were discarded and the rest was gently dropped onto Neubauer chamber.The total WBC was calculated as number of cells per cubic millimeter in counting area.

Enumeration of Heamoglobin (Hb) content

The blood was collected from the animals through the tail vein. The diluting pipette of the hemoglobinometer was filled with N/10 HCl up to the mark of 2gm till the micropipette touches the level of acid in the tube. The blood was added to the mark 20µl and immediately deposited at the bottom of the graduated tube. The blood was mixed with N/10 HCl with the help of a stirrer and then allowed to stand for 15 min, so that hemoglobin was converted to "Acid hematin". It was diluted drop by drop with water and stirred well till it exactly matches with standard glass tube. The scale at the side of the diluting tube was then read.

Estimation of Total Serum Protein

a) Determination of Total Protein (Biuret's method)

In tubes marked as Blank add 1000µl of Alk. $CuSO_4$ reagent and 20 µl Serum respectively incubated for 10 min at room temperature. Add Lowry reagent 0.2 ml to each tubes Mix well, incubated for 30 min. Read the absorbance at 660 nm using colorimeter method and the results were calculated using the following formula (8.6)

$$\text{Total Protein} = \frac{\text{Abs of Sample}}{\text{Abs of Std}} \text{ X Conc. of Std} \qquad (8.6)$$

Biuret blank was prepared by adding 2ml of sulphate – sulphite solution to 5ml of Biuret reagent.0.4ml of standard protein solution was pipetted into 6ml of sulphate – sulphite solution as above and 2ml of the mixture was transformed into 5ml of Biuret reagent. The tube was heated in water bath at 37ºC for 10 min and the color developed was read at 570nm.

b) Albumin

To the rest of the plasma, sulphate – sulphite mixture provided in the diagnostic kits was added and from this 3ml of reagent mixture was added and shaken for about 40 times. The tubes were capped and centrifuged till a firm globulin layer was formed. After centrifugation, the tube was tilted and 1ml of clear solution below the globulin layer was pipetted out into a tube and 5ml of Biuret reagent was added.

Effect of MELA on Solid Tumor

Mice divided into four groups (n=10). Tumor cell (2×10^6 cells / mice) were injected into the right limb of all the animals intra muscularly. Mice of group I were tumor control group II and III received MELA orally for 5 alternative days. Tumor mass was measured from 11^{th} day of tumor induction and repeated every 5 days for a period of 30 days.

The volume of tumor was calculated by using the formula (8.7)

$$V = 4/3\pi r^3 \qquad (8.7)$$

where r was the mean of r_1 and r_2 which are two independent radii of the tumor mass.

8.3 STATISTICAL ANALYSIS

The experimental results were expressed as mean ± S.E.M. Data were assessed by the method of One-way ANOVA followed by Dunnet's post hoc test. *P* value of <0.05 was considered as statistically significant.

8.4 RESULTS

Invitro Cytotoxicity

In long term chemo sensitive cytotoxic assay, 65 µg/ml of the MELA extract produced 50% death of L-929 cells whereas 2 µg/ml camptothecin produced the same result.

Haematological Parameters

The RBC count in the cancer control group was decreased as compared to the normal group. Treatment with 200mg and 400mg/kg MELA increased the RBC count to normal levels. Treatment with standard drug was shown to increase the RBC count when compared with Control group 400mg/kg MELA treated group had shown significant increase in RBC count when compared with 200 mg/kg extract treated group.

The total WBC count was found to have increased significantly in the cancer control group when compared with normal group ($p < 0.001$). Administration of 200mg/kg and 400mg/kg MELA in DLA bearing mice reduced the WBC count as compared with cancer control group. Also administration of 200 mg MELA ($P < 0.01$) and 400mg MELA ($p < 0.001$) in DLA bearing mice significantly reduced the WBC count as compared with cancer control group. 400mg/kg MELA treated group had significantly reduced the WBC count when compared to the other dose of the extract treated group.

The haemoglobin content in cancer control group was significantly ($p<0.001$) decreased as compared to the normal group. Treatment with 200mg MELA ($p<0.05$) and 400mg MELA ($p<0.001$) treated group significantly increases the haemoglobin content to more or less equal to normal levels.400mg MELA treated group ($p<0.001$) had shown significant increase in haemoglobin content when compared to 200 mg/kg treated groups. The results are depicted in Table 8.1.

The total protein was found to be decreased significantly in the cancer control group when compared with the normal group ($p<0.001$). Administration of 200 and 400 mg/kg MELA treated groups had shown an increase in the total protein when compared to DLA treated group. The group treated with 400 mg/kg MELA group had shown significant increase in total protein when compared with 200mg/kg extract treated group. The results are compiled in Table 8.2.

Table 8.1 Effect of MELA on Life Span and Viability in DLA bearing mice

Treatment group	Survival time (Days)	Increase of life span (%)	Tumour volume (ml)	Viable cell count x 10^6 cells/ml	Non viable cell count x10^6 cells/ml	Body wt in gms
Normal saline (5 ml/kg p.o)	45.84±2.32	100	-	-	-	30.0 ± 0.90
DLA control (2 x 10^6 cell)	21.40±1.41	11.46	3.68±0.11	9.42±0.14	3.41±0.21	40.4± 0.74[a]***
DLA (2 x 10^6 cell) + MELA (200 mg/kg p.o)	30.81±1.02*	43.50	2.40±0.11*	3.89±0.04*	1.91±0.09*	37.8 ± 0.96
DLA (2 x 10^6 cell) + MELA (400 mg/kg p.o)	35.64±1.05*	66.35	1.01±0.04*	2.62±0.09*	2.14±0.19*	32.8 ± 1.02[b]***

Data were assessed by One-way ANOVA followed by Dunnet's t test. *P* value <0.05 considered significant

*p< 0.05, **p< 0.01, ^{ns}P > 0.05; ± SEM calculated by comparing treated groups with DLA control group n = 10

a *vs*. Normal group, b *vs*. DLA control. n = 10.

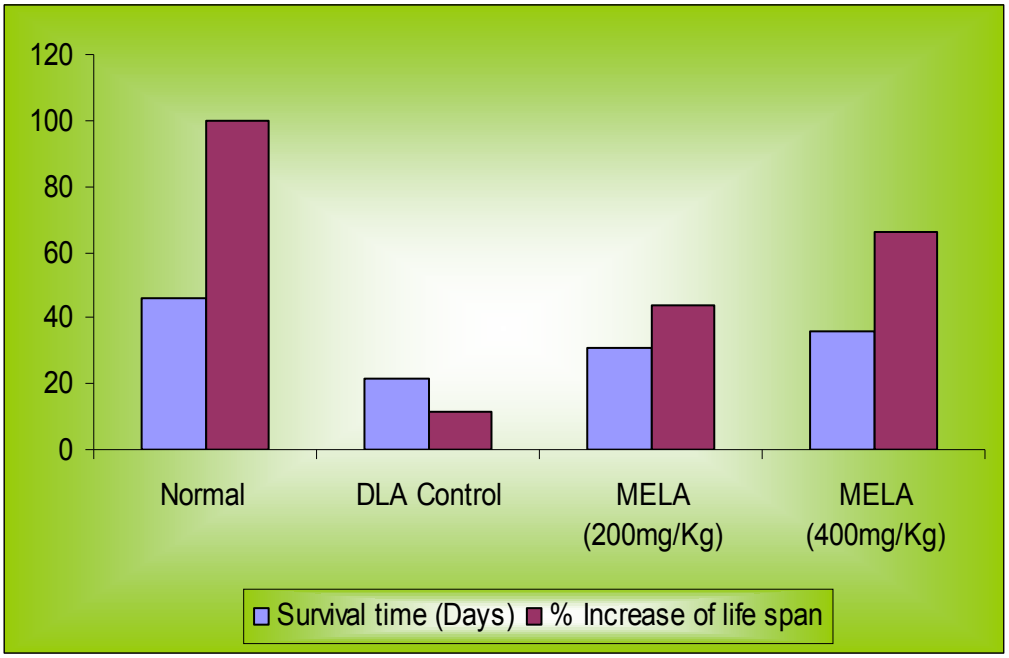

Figure 8.1 Effect of MELA on Life Span in DLA bearing mice

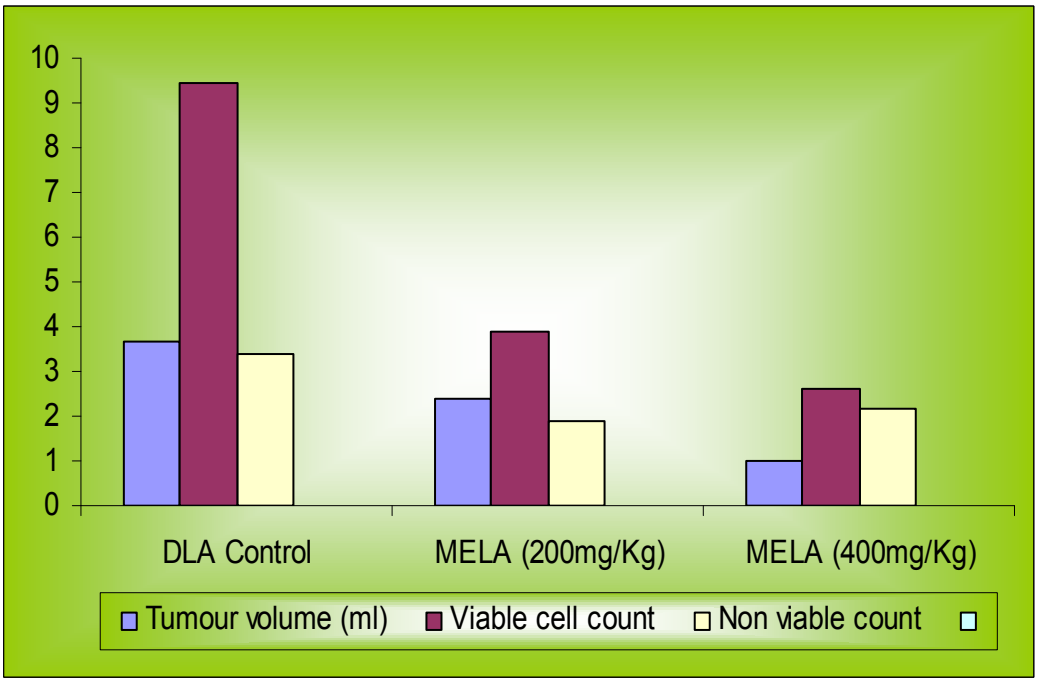

Figure 8.2 Effect of MELA on Viability in DLA bearing mice

Table 8.2 Effect of MELA on haematological parameters in DLA bearing mice

Treatment Group	Hb (g %)	RBC (million/mm^3)	WBC (10^3cells/ mm^3)	Total Protein (g %)	PCV (mm)	Differential count %		
						Lymphocytes	Neutrophil	Monocytes
Normal saline (5 ml/kg)	14.5±0.2	6.5±0.2	7.2±0.2	8.5±0.2	17.8±0.7	70.2±1.31	29.8±1.1	2.2±0.4
DLA control ($2x10^6$ cell)	7.8±0.6^{a}**	3.8±0.1 ^{a}**	15.2±1.3^{a}**	14.6±1.4 ^{a}**	27.5±0.4 ^{a}**	30.3±0.4 ^{a}**	68.6±1.6 ^{a}**	3.8±0.5^{a}*
DLA ($2 x 10^6$ cell) + MELA(200mg/kgp.o)	10.2±0.6^{b}**	5.1±0.5 ^{b}**	11.2±0.7 ^{b}*	11.8±0.1^{b}*	21.4±0.4^{b}**	55.8±1.1 ^{b}**	42.1±1.3 ^{b}**	2.9±0.4
DLA ($2 x 10^6$ cell) + MELA (400mg/kg p.o)	12.4±0.4 ^{b}**	5.8±0.3 ^{b}**	8.6±0.7 ^{b}**	9.2±0.1^{b}**	18.4±0.1^{b}**	67.3±2.1 ^{b}**	30.1±2.2^{b}**	2.8±0.3

Data were assessed by One-way ANOVA followed by Dunnet's t test. *P* value <0.05 considered significant

*p< 0.05, **p< 0.01, ^{ns}P > 0.05; calculated by comparing treated groups with DLA control group n = 10

a *vs*. Normal group, b *vs*. DLA control.

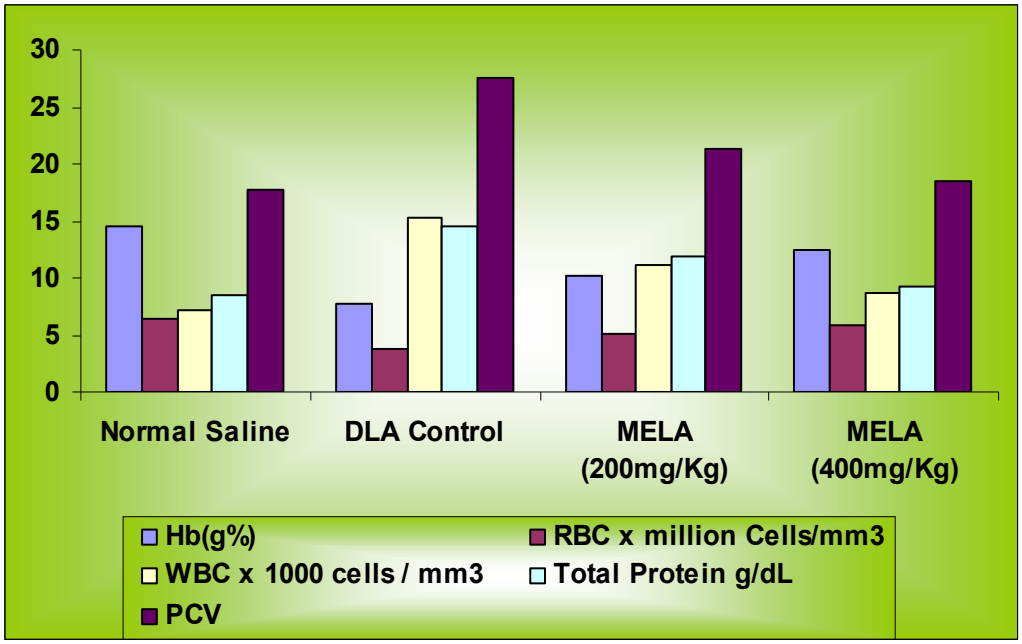

Figure 8.3 Effect of MELA on haematological parameters in DLA bearing mice.

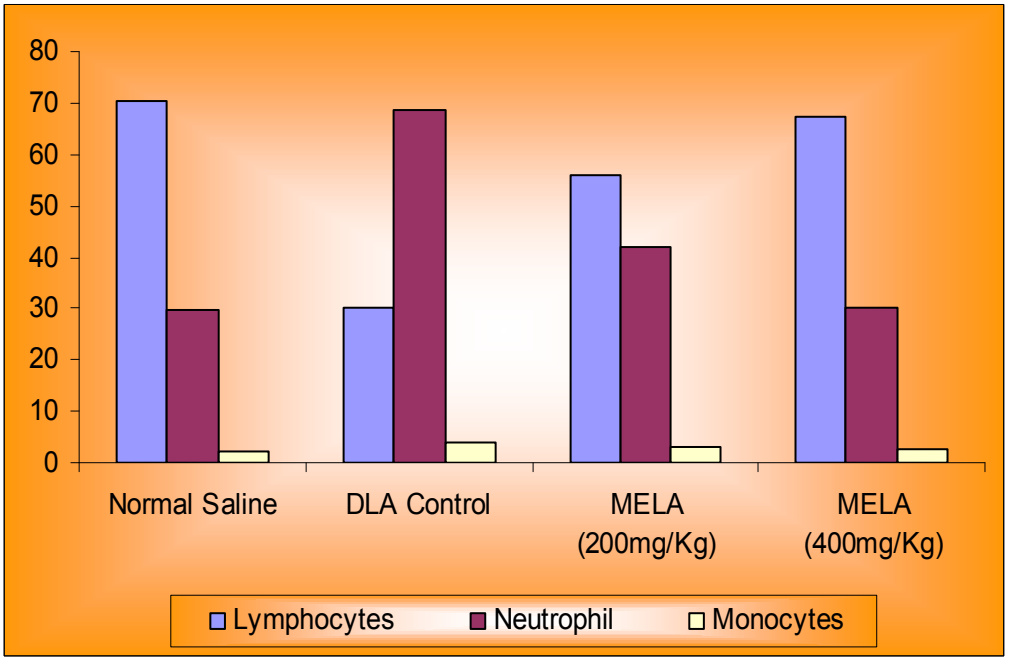

Figure 8.4 Effect of MELA on Differential count in DLA bearing mice.

The average tumour volumes for DLA treated animals were found to be 3.68±0.11. MELA treatment at both dose level significantly (P<0.05) reduced tumour volume which was found to be 2.40±0.11 and 1.01±0.04 respectively. Viable cell count of the tumour bearing mice was significantly decreased while non- viable cell count was increased in MELA treated groups in dose dependant manner when compared with DLA treated group.

The effect of MELA on the survival of tumour bearing mice on MST for DLA treated group to be 21.40±1.41 days, while it was 30.81±1.02 days (43.50%) and 35.64±1.05 days (66.35%) for the animals treated with MELA at the dose of 200 and 400 mg/kg respectively.

Moreover, haematological parameters of tumour bearing mice on day 15 were found to be significantly altered from normal group. The total WBC count, protein and PCV were found to be increased with a reduction of the haemoglobin and RBC. In differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased. At the same time interval, MELA treatment could change those altered parameters to near normal value.

8.4.3 Effect of MELA on Lipid per oxidation and Reduced Glutathione (LPO and GSH)

The levels of LPO in liver tissue were significantly increased by 48.9% in the DLA control group as compared to the normal group (P<0.001)as shown in Table 8.3. After administration of MELA at different doses (200 and 400 mg/kg) to DLA bearing mice, the level of LPO was reduced by 32 and 54%, respectively, in comparison to the DLA control group (P<0.05). Inoculation of DLA drastically decreased the GSH content to 28% in the DLA control group when compared with the normal group

(P<0.001). The administration of MELA at the dose of 200 and 400 mg/kg to the DLA bearing mice increased GSH levels by 32.5%, and 61.2%, respectively, as compared with the DLA control group (P<0.05).

Table 8.3 Effect of MELA on Different Antioxidant Parameters of DLA bearing mice

Treatment	LPO (nmoles MDA/mg protein)	GSH (mg/ g wet tissue)	SOD (U/mg protein)	CAT (U/mg protein)
Solvent control (5 ml /kg)	0.96 ± 0.04	2.35 ± 0.12	4.49± 0.18	2.04±0.05
DLA ($2x10^6$) cells	1.66 ± 0.01 a**	1.42±0.19 a**	2.63 ± 0.37 a**	1.02 ± 0.10 a**
MELA (200 mg/kg) + DLA ($2x\ 10^6$) cells	1.28 ± 0.01	2.39 ± 0.23	4.52 ± 0.27	1.99±0.13*
MELA 400mg/kg)+ DLA ($2x\ 10^6$) cells	1.02±0.020b**	2.47± .17b**	4.67± 0.42 b**	2.02±0.08 b**

Data were assessed by One-way ANOVA followed by Dunnet's t test.

*$P < 0.05$, **$P < 0.01$, $^{ns}P > 0.05$; a *vs*. Normal group, b *vs*. DLA control. n = 10.

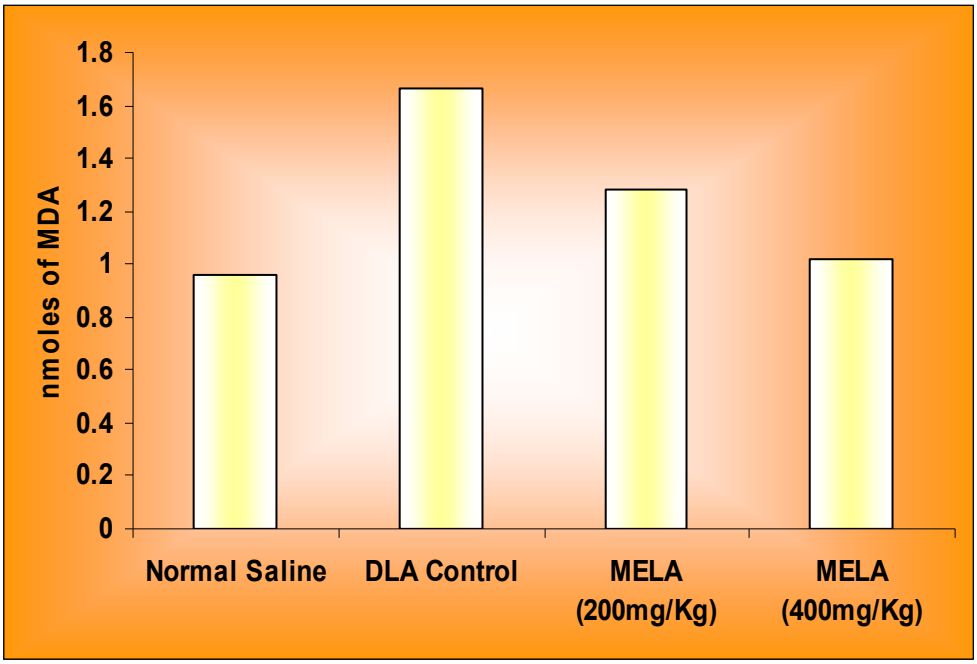

Figure 8.5 Effect of MELA on Lipid Peroxidation Levels of DLA bearing mice

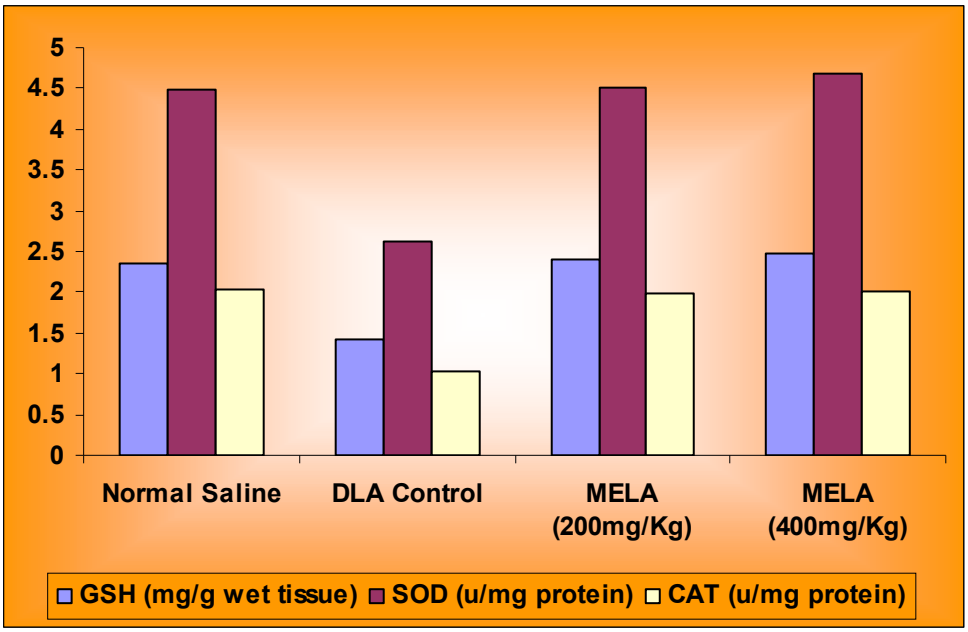

Figure 8.6 Effect of MELA on Different Antioxidant Parameters of DLA bearing mice

Effect of MELA on SOD and Catalase Levels

SOD level in the liver of DLA bearing mice was significantly decreased by 35.6% in comparison with the normal group ($P<0.001$) as shown in Table.12.Administration of MELA at the dose of 200, and 400 mg/kg increased levels of SOD by 21% and 53.2%,respectively, as compared to DLA control group ($P<0.05$). The CAT level in the DLA control group significantly decreased by 60% in comparison with the normal group ($P<0.001$). Treatment with MELA at the dose of 200 and 400 mg/kg increases CAT levels by 32% and 52.8%, respectively, when compared to that of the DLA control ($p<0.05$) group.

8.5 DISCUSSION

The result of the present study reveals that, the in *vitro* cytotoxic activity was more pronounced in long term exposure of fibroblasts (L-929) to MELA. Although the cytotoxicity of camptothecin to L-929 cell was more than that of the extract, it may be noted that camptothecin is a pure compound whereas the extract is a crude one containing numerous compounds.

The reliable criterion for judging the value of any anticancer drug is the prolongation of lifespan of the animal and decrease of WBC count from blood [149].The above results demonstrated the antitumor effect of MELA against DLA in Swiss albino mice. A significant ($P<0.05$) enhancement of MST and non-viable cell count in peritoneal exudates ($P<0.05$) was observed due to MELA treatment.

To evaluate whether MELA treatment indirectly inhibiting tumour cell growth, the effect of MELA treatment was examined on the viable and

non-viable cell counts against tumour bearing mice. Normally, each mouse contains about 2 x 10^6 intraperitoneal cells, 50% of which are macrophages. MELA treatment was found to enhance non-viable cell counts in peritoneal exudates and decrease the viable cell count. It might be due to the absorption of MELA by viable cells which leads to lysis of cell through to the activation of macrophages or some cytokine production in peritoneal cavity.

The Preliminary phytochemical study results indicated the presence of flavonoids and terpenoids in MELA. Flavonoids have been shown to possess antimutagenic and antimalignant effects [150]. Furthermore, Flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [151]. Thus, the antitumor effect produced by MELA may be due to the presence of flavonoids and terpenoids content as well as its antioxidant potential.

Many mechanisms are responsible for the anti-cancer activity, most important of these is that the genes that control apoptosis have a major effect on malignancy through the disruption of the apoptotic process that leads to tumour initiation, progression and metastasis. Therefore, one mechanism of tumour suppression by natural products may be to induce apoptosis, thereby providing a genetic basis for cancer therapy by natural products. Another mechanism for the anti-tumour action of MELA and its constituents is the inhibitory effect on free radical chain reactions, because most limonoids might act as membrane-associated high efficiency free radical scavengers, connects with their antioxidant properties [152].

Ascitic fluid is the direct nutritional source to tumour growth could possibly be a makes to meet more nutritional requirement of tumour cells. A regular rapid increase in ascites tumour volume was noted in tumour bearing mice. Linear progression in the body weight of tumour bearing mice with advancement of duration was observed. Such increase in body weight was

significantly retarded following the MELA treatment. Most anticancer drugs are antiproliferative and will also affect radically dividing normal cells. Bone marrow depression occurs within 10-14 days after a single dose of a cytotoxic drug indicated by a fall in circulating leukocytes and platelets. This may lead to bleeding disorders, increased susceptibility to infection and bone marrow aplasia. The elevated WBC count in tumour bearing mice was significantly reduced by MELA treatment. The MELA treatment inhibits tumour cell growth, enhances the survival of treated mice and restores the heamatological parameters.Thus our present study suggests that MELA possess potent anticancer activity and increases the life span of the treated animals

Usually, in cancer chemotherapy, the major problems that are being encountered are of myelosuppression and anemia [153], but the results have clearly shown that MELA has not only brought back hemoglobin content to normal but also the RBC count to normal. Analysis of the other hematological parameters showed minimum toxic effect in the mice which were treated with MELA. After 14 days of transplantation, MELA -treated groups were able to reverse the changes in the hematological parameters consequent to tumour inoculation.

Lipid per oxidation, an autocatalytic free radical chain propagating reaction, is known to be associated with pathological conditions of a cell. Malondialdehyde (MDA), the end product of lipid per oxidation, was reported to be higher in cancer tissues than in non diseased organ [154]. Glutathione, a potent inhibitor of the neoplastics process, plays an important role in the endogenous antioxidant system. It is found in high concentration in the liver and is known to have a key function in the protective process. Excessive production of free radicals resulted in oxidative stress, which leads to damage of macromolecules, for example, lipid per oxidation *in vivo* [155]. It was also reported that the presence of tumours in the human body or in experimental

animal is known to affect many functions of the vital organs, especially in the liver, even when the site of the tumour does not interfere directly with organ function. MELA significantly reduced the elevated levels of lipid per oxidation and increased the glutathione content in DLA-induced mice. The antitumorogenic effect of MELA may be due to the antioxidant and the free radical quenching property of the phytoconstituents present in the extract.

Cells are also equipped with enzymatic antioxidant mechanisms that play an important role in the elimination of free radicals. SOD, CAT, and Glutathione peroxides are involved in the clearance of superoxide and hydrogen peroxide.SOD catalyses the diminution of superoxide into H_2O_2, which has to be eliminated by glutathione peroxidase or catalase [156]. Consistent with this, it has been reported that a decrease in SOD activity in DLA-bearing mice may be due to loss of Mn^{2+} containing SOD activity in DLA cells and the loss of mitochondria, leading to a decrease in total SOD activity in the liver. The inhibitions of SOD and CAT activities as a result of tumour growth were also reported. Similar findings were observed in the present investigation with DLA-bearing mice.The administration of MELA at different doses significantly increased the SOD and CAT levels in a dose dependent manner.

It was reported that plant-derived extracts containing antioxidant principles showed cytotoxicity towards tumour cells [157] and antitumor activity in experimental animals [158]. Antitumor activity of these antioxidants is either through induction of apoptosis or by inhibition of neovascularisation [159].The implication of free radicals in tumours is well documented [160].In our earlier studies, we found that MELA possess hepatoprotective and antioxidant properties [161]. The free radical hypothesis supported the fact that the antioxidants effectively inhibit the tumour, and the observed properties may be attributed to the antioxidant and antitumor

principles present in the extract. It is known that antioxidants can inhibit proliferation of atleast certain types of cancers [162-165].

The phytochemical study indicated the presence of flavonoids and terpenoids in MELA. Flavonoids have been shown to possess antimutagenic and antimalignant effects [166]. Furthermore, flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation [167] and angiogenesis [168]. Thus, the antitumor effect produced by MELA may be due to its flavonoids and terpenoid content as well as its antioxidant potential. The methanolic extract of *Limonia acidissima* restored the mean survival time, decrease tumor volume, cancer cell count in treated mice. Thus our present study suggests that MELA possess potent anticancer and antioxidant activity and increases the life span of the treated animals.

8.6 CONCLUSION

All these data point to the possibility of developing methanolic extract of fruit pulp of *Limonia acidissima* as a novel, potential agent in the area of cancer chemotherapy. The present study highlighted the anti-tumor and antioxidant activities of MELA in experimental animal model against DLA cells induced tumor and thereby proves the supportive traditional claim of the plant part. Further works are being carried out to isolate and identify the active principle involved in the anti tumor and antioxidant activities of this plant extract.