Chapter V

ANTICANCER ACTIVITY
INTRODUCTION

Cancer is a major aliment that affects a several organs and chemotherapy is widely practiced to treat the disease (Rubin, 1985; Ganesan et al., 2010). Chemotherapy, with or without radiation, although effective for many cancers, is accompanied by severe side effects due to the toxicity of the anticancer drugs (Lasic, 1996; Partridge et al., 2001; Koopman et al., 2007).

All major anticancer drugs such as Cyclophosphamide (CP), doxorubicin, cisplatin, bleomycin etc. are associated with toxic side effects of varying degree affecting various vital systems (Hassan et al., 2011; Christie et al., 2012; Pabla and Dong, 2012). CP is an oxazaphosphorine class of alkylating agent widely used in cancer treatment (Schmidt and Koelbl, 2012). CP frequently causes hematopoetic depression, nausea, vomiting, alopecia, hemorrhagic cystitis, water retention, cardiac damage, gonadotrophy and carcinogenicity (Bhatia et al., 2008; Panahi et al., 2012). CP also induces mutagenicity in mice (Sharma et al., 2001). The suppressive effects of CP on lymphoid organs, WBC counts and other immune functions are well documented (Zaidi et al., 1990; Bin-Hafeez et al., 2001; Jena et al., 2003; Ramadan et al., 2011; Shreder et al., 2012). Several anticancer drugs are derived from plants such as taxol, vincristine, vinbalstine and etc. (Kametani et al., 2007; Pandey and Madhuri, 2009; Kaur et al., 2011).

Recently there is increased interest in the search for therapeutic agents of plant origin that are capable of minimizing the toxicity induced by anticancer drugs to normal cells, without compromising the efficacy.
A large number of single natural products and herbal extracts have shown protective effects against CP-induced toxicity (Kaneko et al., 1999; Sharma et al., 2001; Haque et al., 2003; Bhatia et al., 2006, 2008). However, there is limited knowledge about the efficacy of natural products.

Traditional system of Indian medicine extensively uses the plant derived compounds and formulations to modulate the immune system of the host. These herbal formulations are believed to be relatively less toxic or non-toxic (Kumar and kuttan, 2005; Hussain et al., 2013; Mishra et al., 2013).

*Decalepis hamiltonii* (Wight and Arn.) (family: Asclepiadaceae), a climbing shrub, grows in the forests of peninsular India. Its tuberous roots are consumed as pickles and juice for their health promoting properties in southern India. The roots are also used in folk medicine and ayurvedic (the ancient Indian traditional system of medicine) preparations as a general vitaliser (Nayar et al., 1978). The roots of *D. hamiltonii* possess strong antioxidant properties and several novel antioxidant compounds have been isolated and characterized (Srivastava et al., 2006a, 2006b, 2007). Further, the root extract shows hepatoprotective and neuroprotective potential against toxicity induced by xenobiotics in the laboratory rat (Srivastava and Shivanandappa, 2006a, 2009, 2010a, 2010b).

In view of the chemoprevention potential of *D. hamiltonii* investigations were carried out to study the protective effect against CP toxicity in mice (Chapter III and IV). Our studies show that the aqueous extract of *D. hamiltonii* exhibits amelioration potential against the oxidative stress and toxicity of CP in the liver and brain (Zarei and Shivanandappa, 2013a) (please see chapter III and IV). Therefore, it was postulated that *D. hamiltonii* root extract and its antioxidant compounds could be used
as adjunct to cancer chemotherapy in order to reduce the toxicity of CP. However, this raises important question regarding its therapeutic application: a) whether efficacy of the anti-cancer drug (CP) is affected, b) whether the root extracts and molecules, per se, have any anticancer activity that could potentiate the efficacy of CP.

Therefore, in this chapter, we have investigated whether *D. hamiltonii* affects the anticancer activity of CP and whether the extract and compounds have anticancer potential by using the *in vivo* model of Ehrlich Ascites Tumor in mice. Further, the mode of action of *D. hamiltonii* extracts and its bioactive molecules on cancer cells *in vitro* has been studied using different cell lines, on induction of apoptosis cell death.
MATERIALS AND METHODS

In vivo studies

Chemicals: Trypan blue and Leishman’s stain were purchased from Sigma chemical Co. (St. Louis, MO, USA). Cyclophosphamide (Ledoxan) was obtained from Dabur Pharma Limited, New Delhi, India. All other chemicals were purchased from Sisco Research Laboratories, Mumbai, India, and were of highest purity grade available.

Preparation of the root extract and isolation of antioxidant compounds:

Preparation of the root extract and isolation of antioxidant compounds was done as described previously (see Chapter II).

Animals:

Swiss albino mice were procured from the animal colony maintained at the Department of Zoology, University of Mysore, Mysore, India. The animals were kept at room temperature (25 ± 2°C), with 70 ±10% humidity and day light cycle of 12 h light and 12 h dark, and standard laboratory animal feed and water were provided ad libitum. Animals were acclimatized to the experimental conditions for a period of one week before the initiation of the experiment. Appropriate guidelines of the local animal ethics committee were followed for the animal experiments (UOM/1AEC/33/2011).

Experimental Protocol:

Eight weeks old adult male mice (30-32 g) were divided into groups of 12 each. Dosage of DHA, DHA-I and DHA-II was selected based on our preliminary experiments and earlier reports (Srivastava and Shivanandappa, 2010b, Zarei and shivanandappa, 2013b).
Anticancer Activity

Group I  -  Control
Group II  -  EAT-bearing mice + Saline (1 ml/kg b.w.)
Group III  -  EAT- bearing mice + CP (25 mg/kg b.w.) (diluted in saline)
Group IV  -  EAT- bearing mice + DHA (100 mg/kg b.w.) + CP (25 mg/kg b.w.)
Group V  -  EAT- bearing mice + DHA (100 mg/kg b.w.)
Group VI  -  EAT- bearing mice + DHA-I (100 mg/kg b.w.) + CP (25 mg/kg b.w.)
Group VII  -  EAT- bearing mice + DHA-I (100 mg/kg b.w.)
Group VIII  -  EAT- bearing mice + DHA-II (100 mg/kg b.w.) + CP (25 mg/kg b.w.)
Group IX  -  EAT- bearing mice + DHA-II (100 mg/kg b.w.)

EAT cells were originally obtained from National Center for Cell Science, Pune, India and maintained by in vivo propagation in mice. EAT cells were grown in the peritoneal cavity of mice by transplantation of 0.5 ml of cell suspension (10^6 cells/ml) in sterile citrate saline (0.9%). 24 hours after the inoculation of EAT cells, DHA, DHA-I and DHAII (100mg/kg b.w) were administered orally and cyclophosphamide (25 mg/kg.b.w) was given daily by intraperitoneal injection for the next ten days. Six mice from each group were scarified 24h after the last administration, by ether anesthesia for the study of antitumor activity, hematological analysis, and biochemical assays. The remaining six animals in each of the group were maintained without any further treatment to determine the median survival time (MST) of the tumor bearing mice.

**Antitumor activity:**

Body weights of the animals were recorded from the day of transplantation to sacrifice. The ascites tumor volume was determined by collecting the ascites fluid from the peritoneal cavity of mice by using syringe. Packed cell volume was
determined by centrifuging the ascites fluid at 1000 rpm for 5 min. An aliquot of the ascites fluid (10µl) was used for counting the number of viable cells stained with 0.4% trypan blue by using haemocytometer. Mean survival time (MST) of animals was monitored by recording the mortality daily for six weeks and percent increase in life span (ILS) was calculated by the following formula:

\[
\% \text{ ILS} = \{ (\text{MST of treated group} / \text{MST of control group}) - 1 \} \times 1000
\]

\[
\text{MST} = (\text{Day of 1}\text{st death} + \text{Day of last death}) / 2
\]

**Hematological profile:**

On the 15\textsuperscript{th} day post-inoculation of EAT cells, blood was collected from the animal by retro-orbital puncture and the hematological parameters such as red blood cells (RBC), white blood cells (WBC), differential count and hemoglobin content were determined by conventional laboratory method (Ramnik, 1999).

Hemoglobin was estimated by adding 20 µl of blood to 5 ml Drabkin’s solution (1 g sodium bicarbonate + 0.05 g potassium cyanide + 0.2 g ferricyanide/L H\textsubscript{2}O). The change in the absorbance was recorded at 340 nm.

RBC was measured by using RBC pipette of a hemocytometer, blood was drawn to the 0.5 mark in the RBC pipette following diluting fluid (3 g sodium citrate + 1 ml formalin + 100 ml distilled H\textsubscript{2}O) to the 101 mark. Shake for 3 min and charged the chamber. Counted the RBC using 40X objective in smaller square of the chamber.

WBC was estimated by drawing the blood to the 0.5 ml mark in the WBC pipette followed by diluting fluid (Turke’s fluid: 1.5 ml glacial acetic acid + 1 ml of
aqueous solution of gentian violet + 98 ml of distilled water) till the mark. Mix the fluid and blood gently. The WBC’s were counted uniformly in four corner squares.

Differential count was determined by smearing a thin layer of blood on a dry slide and stained by using the Leishman’s stain. The cells were counted using 100X objective.

**Biochemical assays:**

Blood was collected in polypropylene tubes, allowed to clot and the serum was separated by centrifugation at 2000g for 10 min and stored at 4 ºC for biochemical analysis. Lactate dehydrogenase (LDH) activity was assayed by Kornberg’s method (Kornberg, 1955). The reaction mixture consisted of NADH (0.02 M), sodium pyruvate (0.01 M) in sodium phosphate buffer (0.1 M, pH 7.4). The change in the absorbance was recorded at 340 nm at 30 s intervals for 3 min. Serum ALT (Alanine transaminases), AST (Aspartate transaminases) and ALP (Alkaline phosphatase) levels were measured spectrophotometrically by the standard enzymatic methods using commercial kits (Span Diagnostics Ltd, Surat, India).

**Statistical analysis:**

The data are expressed as Mean ± SEM, were analysed statistically by using one way ANOVA followed by multiple-comparison test and significance at $p < 0.05$ were considered as statistically significant.
**In vitro studies**

**Materials:** PC-12 (pheochromocytoma of the rat adrenal medulla) and HEK 293 (Untransformed Human embryonic kidney) cell lines were procured from Cambrex Biosciences, Walkersville, USA. DMEM, FBS, penicillin-streptomycin and trypsin-EDTA were from Invitrogen, USA. Giemsa's stain solution and Acridine orange were from Sigma Aldrich, USA. A MTT assay kit was from BD Biosciences, USA. All other reagents were of the highest analytical grade.

**Preparation of the root extract and isolation of antioxidant compounds:**

Preparation of the root extract and isolation of the antioxidant compounds (DHA-I and DHA-II) was done as described previously (see Chapter II).

**In Vitro cell culture:**

EAT (mouse mammary carcinoma) cells which are routinely maintained in our laboratory in Swiss albino mice were drawn in aseptic condition and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. PC-12 and HEK 293 cells were also maintained in DMEM with 10% FBS and 1% Penicillin-Streptomycin and Gentamycin. All the cells were incubated in a humidified atmosphere of 37°C and 5% CO₂. When cells reached confluency, they were passage by trypsinizing with 0.025% trypsin/0.01% EDTA and were used for experiments or frozen using freezing mixture (90% FBS and 10% DMSO).

**MTT assay:**

Cells (5 × 10³ cells/well) were plated in 96-well flat bottomed titer plate and incubated for 24 hours at 37°C in 5% CO₂ atmosphere. The following day, cells were
then treated with various concentrations of DHA, DHA-I, DHA-II (0-100 µg/mL) with or without CP (0-20 µg/ml) and incubated for 72 hours. At the end of incubation, 20 µl of MTT (2 mg/ml in PBS) was added to each well and incubated for another 4 hours at 37°C. The formazan formed was solubilised in 100 µl dimethylsulphoxide (DMSO) and the absorbance was measured at 540 nm using a multi-plate reader (BIO-RAD model 680). The absorbance value that was determined for cells cultured in complete media (control) was based on 100% viable cells.

**Giemsa staining:**

EAT Cells (5×10³ cells/well) were seeded in 6-well plates (BD Labware, England), and the plates were then incubated overnight at 37°C in 5% CO₂ atmosphere. DHA, DHA-I and DHA-II and CP (LC₅₀) were added to the cells and incubated for an additional 24 hours. After harvesting by trypsinisation, cells were washed twice with 1× phosphate buffer saline (pH 7.4), 25 µl of the cell suspension was smeared on clean glass slides and then fixed in methanol/acetic acid (3:1) and stained with freshly prepared Wright’s Giemsa’s stain solution for 6 minutes, rinsed again three times with sterile water and mounted with a cover slip. Morphological changes were examined using an inverted microscope (Nikon, TMS) with 100X actual magnification.

**Acridine orange and ethidium bromide staining (AO/EB staining):**

Nuclear staining was performed according to the Srinivas method (Srinivas et al., 2003). PC-12 Cells (5×10³ cells/well) were seeded in 6-well plates (BD Labware, England), and the plates were then incubated overnight at 37°C in 5% CO₂ atmosphere. DHA, DHA-I and DHA-II and CP (LC₅₀) were added to the cells and incubated for an additional 24 hours. After harvesting by trypsinisation, cells were
washed twice with 1× phosphate buffer saline (pH 7.4), 25 µl of the cell suspension was smeared on clean glass slides and then fixed in methanol/acetic acid (3:1) and air dried on a humidified chamber. The cells were hydrated with PBS and stained with 1 µl of the dye mixture, containing 100 mg/ml of acridine orange and 100 mg/ml of ethidium bromide. After staining, cells were washed with PBS and visualized immediately under a fluorescence microscope (Leitz-DIAPLAN).

**DNA fragmentation:**

Cells (1 × 10^5 cells/well) were treated with LC₅₀ concentration of DHA, DHA-I and DHA-II and CP and incubated for 24 hours at 37°C in 5% CO₂ atmosphere. After incubation, the cells were lysed in lysis buffer (10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 200 mM NaCl, 0.2% SDS) and incubated for 20 minutes at 37°C. The cell lysate was subjected to 8 M potassium acetate and left for 1h at 4°C. After spinning at 7,000 rpm, an equal volume of distilled phenol, chloroform and isoamyl alcohol (25:24:1) mixture was added and subjected to centrifugation (3,000 rpm, 30 min, and 37°C). The above step was repeated twice using the supernatant obtained, and to the final supernatant, an equal volume of chloroform was added. After centrifugation (2,000 rpm, 30 min and 37°C), the DNA was digested with 20 µg/ml RNase at 37°C for 30 min. Later, DNA was participated by adding 1:2 volumes of ice-cold ethanol. The solution was centrifuged (10,000 rpm, 10 min and 4°C) and the pellet obtained was dissolved in Tris/EDTA buffer (pH 8.0), and resolved on 1.5% agarose gel. DNA was stained with ethidium bromide and viewed under UV-light and documented using UVP-Bio Doc It TM system.
Single cell gel electrophoresis (Comet assay):

Comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly, after treatment, the cells were centrifuged (1,000 rpm, 5 min and 23°C) and the pellet resuspended with 200 µl PBS. A fully frosted microscopic glass slide was coated with 100 µl of a solution of 1% (w/v) normal melting-point agarose. Subsequently, about 50 µl of the cells mixed with 50 µl of a solution of 0.5% (w/v) low, melting-point agarose was overlaid to solidify at 4°C for 10 min. The cells were lysed by dipping in a freshly prepared lysis solution (100 mM Na<sub>4</sub>EDTA, 10 mM Tris, 2.5 M NaCl, 1% Triton X-100, and 10% DMSO, pH 10). The lysis was done for 2 h at 4°C in the dark. After lysis, slides were kept at 4°C for 40 min in an electrophoresis buffer (0.3 M NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13.0), and then electrophoresized at 18 V/300 mA at 4°C in the dark for 30 min. The glass slide was neutralized with 0.4 M Tris–HCl buffers (pH 7.4) three times and stained with ethidium bromide and mounted with a cover slip. The slides were randomly examined and photographed using a fluorescence microscope (Leitz-DIAPLAN). The images captured were analyzed using the software ‘CASP’ which gives %DNA in tail, tail length, tail moment and olive tail moment directly. The parameter, tail moment, is the product of tail length and %DNA in tail and olive tail moment is the product of distance between the center of the head and the center of the tail and % DNA in tail (Konca et al., 2003).

RT-PCR assay:

Oligonucleotide primer sequences of the selected genes for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized by Maxim Biotech Inc. (San Francisco, California, USA). The following primers of the mouse genes were used:
Bcl2 (Forward 5′- CCGGAGAACAGGGTATGATAA -3′ and Reverse 3′- CCCACTCGTAGCCCTCTCTTG -5′; 81bp),

Bax (Forward 5′- GGAAGGCCTCCTCCTCTACCTTC -3′ and Reverse 3′- GAGGACTCCAGGCCACAAAGATG -5′; 71bp),

Caspase-3 (Forward 5′- CAGAGCTGGACTGCGGTATTGA -3′ and Reverse 3′- AGCATGGCGCAAAGTGACTG -5′; 116 bp),

Caspase-9 (Forward 5′- AGCCAGATGCTGTTCCCATAC -3′ and Reverse 3′- CAGGAGACAAACCTGGGAA -5′; 102 bp) genes were amplified against GAPDH (Forward 5′- CATGGCCTTGCTGCTCCATAC -3′ and Reverse 3′- GCGGCACGTCAGATCCA -5′; 127bp) standard. RNeasy mini kit and QuantiTect Reverse transcription kit were obtained from Qiagen, Germany.

Cells (5×10^3 cells/well) were seeded in 96-well flat-bottom titer plate (BD Labware, England), and the plates were then incubated overnight at 37°C in 5% CO₂ atmosphere. Experimental treatment of cell with CP, DHA, DHA-I and DHA-II were as discussed previously.

Total RNA was isolated from cells using RNeasy mini kit (Qiagen, Germany) according to the protocol of the manufacturer. The quality and concentration of total RNA were checked at 260 nm using Pico-drop Spectrophotometer (Picodrop, Germany). Equal concentrations of RNA from each sample were prepared for the subsequent reverse transcription and polymerase chain reaction technique (RT-PCR). Reverse transcription was carried out using QuantiTect Reverse transcription kit (Qiagen, Germany) at 37°C for 1 h to generate the first strand cDNA. The cDNA samples were subjected to PCR amplification using the automated thermal cycler (Eppendorf AG / 22331, Hamburg, Germany) under optimum conditions for each pair of primers of the investigated genes. Polymerase chain reactions for each gene were
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performed in 25 µl reaction mixture consisting of 0.5 µl of Taq DNA polymerase (Sigma- Aldrich, Germany), 2 µl of 10X buffer, 4 µl of 2.5 mM dNTP mix, 1 µl of 50 mM MgCl$_2$, 1 µl each oligonucleotide primer (sense and antisense), and 1µl of template cDNA. Gene expression studies were performed by PCR analysis, for the Bcl2, Bax, Casapse-3 and Casapse-9 genes, and amplified against GAPDH standard. The PCR program was as follows: pre denaturation for 5 min at 95°C, followed by 50 cycles of: 20 s at 95°C, 15 s at 60°C, 15 s at 72°C, and finished with 1 min at 72°C. Amplified PCR products were separated on 1.8% agarose gel electrophoresis and stained with 10 µg/ml ethidium bromide (EB). The ethidium bromide-stained gel bands were documented using gel documentation system (UVP-Bio Doc It TM system). The signal intensities of the gel bands were semi-quantified using the Gel-Pro image software analyzer (Version 3.1 for Windows).

Statistical analysis:

The data are expressed as Mean ± SEM and were evaluated statistically by using one way ANOVA followed by multiple-comparison test. $p < 0.05$ were considered as statistically significant.
RESULTS

In vivo antitumor activity

Ehrlich ascites tumor bearing mice showed increased body weight gain due to ascites tumor volume whereas a significant reduction in the body weight was seen in the DHA and DHA-I administered groups. CP treated EAT bearing mice also showed decrease in body weight gain. In a groups treated with D. hamiltonii root extract and molecules along with CP treatment a further significant decrease was seen (Table 5.1). DHA-I treatment along with CP was shown the highest reduction in body weight compared to that of control.

There was a marked increase in the ascites fluid volume and packed cell volume in EAT bearing animals, whereas in DHA and DHA-I treated groups the ascites fluid volume, packed cell volume and number of cancer cells was significantly reduced. Reduction in cancer cell count was also seen in CP treated EAT bearing mice. Furthermore, The combination treatment of DHA and molecules treatment + CP on tumor bearing mice resulted in an significant decreases in the ascites fluid volume, packed cell volume and number of cancer cells compared to the CP treated EAT bearing mice (Fig. 5.1.).

D. hamiltonii and the antioxidant compounds significantly increased the survival time of tumor bearing mice. The Mean survival time of EAT bearing mice was 18.86 ± 0.24 days, whereas, administration of the DHA, DHA-I and DHA-II significantly prolonged the MST to 31.24 ± 0.54, 33.18 ± 0.61 and 26.69 ± 0.22 days, respectively. Cyclophosphamide (25 mg/kg) treated groups improved MST to of 37.24 ±1.16 days. The increase in the life span of tumor bearing mice treated with cyclophosphamide were 88.14 as compared to the EAT bearing mice, but these levels
were improved significantly in tumour-bearing mice treated with DHA, DHA-I, DHA-II along with CP to 94.27, 93.56 and 92.01% respectively (Fig. 5.2.).

**Hematological profile**

Hematological profile of EAT bearing mice was significantly altered compared to normal mice. There was a significant decrease in Hb content and RBC count, whereas, WBC count, was markedly higher in EAT bearing mice. Administration of DHA, DHA-I and DHA-II to EAT bearing mice significantly restored the RBC count, Hb content and WBC count as compared to that of untreated EAT mice. In a differential count, neutrophils were increased while lymphocytes were decreased in *D. hamiltonii* and its molecules treated mice. DHA and DHA-I treatment restored hematological parameters comparable to that of control group. The combination therapy of CP with *D. hamiltonii* root extract and molecules reduced the RBC count, Hb content and WBC count significantly compared to CP treated EAT bearing mice (Table 5.2).

**Biochemical assays**

Activity of the serum marker enzymes, AST, ALT, LDH, and ALP, were elevated in the EAT bearing mice as compared to that of normal control group. Treatment of EAT bearing mice with DHA, DHA-I and cyclophosphamide showed a significant restoration of the enzyme activities. The administration of DHA and its antioxidant molecules along with CP resulted in a drastic decline in the serum marker enzymes activities considerably when compared with either drug alone (Table 5.3).
In vitro studies

Cytotoxicity

CP inhibited the growth of cultured cells in a dose-dependant fashion as evident from MTT assay. IC\textsubscript{50} value for CP in EAT, PC-12, HEK-293 cells were found to be 2.18, 1.9 and 17.78 (µg/ml), respectively. Therefore, CP was cytotoxic to both carcinoma (EAT, PC-12) and non-carcinoma (HEK-293) cells (Fig. 5.3).

Aqueous extract of D. hamiltonii roots and the antioxidant compounds significantly affected the growth of EAT, PC-12 cells at 24 h. These results suggest that DHA and its bioactive molecules inhibited the growth of cancer cells in a concentration-dependent manner. The IC\textsubscript{50} values for DHA, DHA-I and DHA-II were, 29.30, 27.16, 37.1 in PC-12 cells and 24.83, 30.17, 34.2 (µg/ml) in EAT cells, respectively. The aqueous extract and antioxidant compounds from D. hamiltonii were not cytotoxic to HEK-293 cells at the highest concentration used (Fig. 5.4).

The combination treatment of CP + DHA/ DHA-I/ DHA-II on PC-12 and EAT cell lines resulted in a significant growth inhibition of cancer cells in a time-dependent manner as compared to the CP treated cells. However, cytotoxicity of CP to HEK-293 cells was reduced in the presence of DHA and the antioxidant compounds as evident from MTT assay (Fig. 5.5).

Apoptosis:

a) Giemsa staining

To determine whether apoptosis was involved in D.hamiltonii induced growth inhibition in carcinoma cells, we first examined the cell morphological changes. D.hamiltonii can induce apoptosis in EAT cells, as proven using Giemsa's staining. The observation showed that EAT cells displayed characteristically morphological
changes after *D. hamiltonii* roots extract and its antioxidant compounds treatment, including cell shrinkage, volume reduction, chromatin condensation, cell blebbing and formation of membrane embedded apoptotic bodies compared to that of control cells. The cells in CP group, due to the increase in cell membrane permeability, showed exhibited nucleus fragmented or apoptotic bodies. The combination therapy of CP with *D. hamiltonii* root extract and molecules induced marked apoptosis compared to CP treated as evident from morphological changes (Fig. 5.6).

b) Acridine Orange and Ethidium bromide staining

Staining cells with the fluorescent dyes, acridine orange and ethidium bromide (AO/EB staining), is used in evaluating the nuclear morphology of apoptotic cells. To see whether apoptosis is induced by the root extract of *D. hamiltonii* and its antioxidant compounds, PC-12 cells treated with DHA, DHA-I and DHA-II were analysed for apoptotic morphology. The observations showed that DHA and the antioxidant compounds induced apoptosis in cells at 24 h exposure. Further, in combination treatment of CP + DHA/ DHA-I/ DHA-II, most cells underwent apoptosis and showed typical apoptotic morphology, including chromatin condensation, membrane blebbing, formation of apoptotic bodies and condensed nuclei as compared to the CP treated cells.

c) Apoptotic Index (AI)

Analysis of the apoptotic index (AI), a measure of cell death by apoptosis showed that in control cells, only 3% exhibited apoptotic features. The AI percentage of PC-12 and EAT cells increased significantly in cells exposed to DHA and the antioxidant compounds compared to the control cells. Furthermore, the AI percentage
of cancer cells treated with CP in combination with DHA, DHA-I and DHA-II was higher than that of CP treated cells (Fig. 5.8).

**DNA fragmentation**

DNA fragmentation, the hallmark of cell apoptosis, was analyzed in PC-12 and EAT cells. Genomic DNA showed the typical formation of DNA fragments as a ladder in *D. hamiltonii* treated cells on agarose gel, whereas untreated cells produced intact genomes. Cells treated with CP as well as DHA and DHA-I showed DNA fragmentation.

Further, combined treatment of CP + DHA/ DHA-I/ DHA-II, in cancer cells was higher than that of CP alone indicating potentiation effect of DHA and the antioxidant compounds on the CP-induced DNA fragmentation in cancer cells (Fig. 5.9).

**Comet assay**

The extent of DNA damage in PC-12 and EAT cells treated with DHA, DHA- I and DHA-II was significantly increased as compared to the control cells. Further, in cancer cells treated with DHA, DHA-I and DHA-II in combination with CP, the olive moment were significantly increased than that of cells treated with CP only (Fig. 5.10-5.11).

DHA and the antioxidant compounds did not induce any DNA breakage in HEK-293 cells. Interestingly, co-treatment of CP with *D. hamiltonii* roots extract and the antioxidant molecules attenuated DNA damage induced by CP in HEK-293 which are non-carcinoma cells (Fig. 5.12).
Gene Expression

RT-PCR analysis revealed a significant down regulation in the expression of anti-apoptotic gene Bcl-2 in cells treated with DHA, DHA-I and CP treated groups. At the same time, expression of pro-apoptotic genes such as Bax and Caspase-3, 9, were significantly up-regulated in PC-12 and EAT cells exposed to *D. hamiltonii* roots extract and the bioactive compounds (Fig. 5.13-14).

Increased transcription levels of Bax and Caspase-3, 9 mRNA was observed in carcinoma cells treated with combination of CP and DHA/ DHA-I/ DHA-II as compared to the CP treated cells (Fig. 5.15-5.16).
Table 5.1.: Effect of *D. hamiltonii* root extract and its bioactive molecules on EAT bearing mice: body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight(g)</th>
<th>Weight Gain</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>I</td>
<td>30.4 ± 2.42</td>
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<tr>
<td>II</td>
<td>29.8 ± 2.17</td>
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<tr>
<td>IX</td>
<td>30.2 ± 2.2</td>
<td>39.8 ± 1.14</td>
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</tbody>
</table>

Groups: I – control; II– EAT bearing mice; III–EAT + CP (25mg / kg b.w.); IV–EAT + DHA (100mg / kg b.w.)+ CP; V– EAT+DHA (100mg / kg b.w.); VI–EAT+DHA-I (100mg / kg b.w.)+ CP; VII– EAT + DHA-I (100mg / kg b.w.); VIII–EAT+DHA-II (100mg / kg b.w.)+ CP; IX– EAT + DHA-II (100 mg / kg b.w.) Values are mean of 6 mice ± SEM. ** p < 0.01, and * p < 0.01. ** Values are significantly different from control, * Values are significantly different from EAT bearing mice.
Figure 5.1.: Antitumor activity of *D. hamiltonii* root extract and its bioactive molecules on EAT carcinoma in mice

Treatment: **I**– EAT bearing mice; **II**– EAT + CP (25mg / kg.b.w.); **III**– EAT + DHA (100mg / kg.b.w.)+ CP; **IV**– EAT + DHA (100mg / kg. b.w.); **V**– EAT + DHA-I (100mg / kg. b.w.)+ CP; **VI**– EAT + DHA-I (100mg / kg.b.w.); **VII**– EAT + DHA-II (100mg / kg. b.w.)+ CP; **VIII**– EAT + DHA-II (100mg / kg.b.w.). Values are mean of 6 mice ± SEM. *a p < 0.05 and * p < 0.01. * Values are significantly different from EAT bearing mice.
Figure 5.2.: Effect of *D. hamiltonii* root extract and its bioactive molecules on mean of survival time in EAT bearing mice

Treatment: **I**– EAT bearing mice; **II**– EAT + CP (25mg / kg.b.w.); **III**– EAT + DHA (100mg / kg.b.w.)+ CP; **IV**– EAT+DHA (100mg / kg. b.w.); **V**– EAT+DHA-I (100mg / kg. b.w.)+ CP; **VI**– EAT + DHA-I (100mg / kg.b.w.); **VII**– EAT+DHA-II (100mg / kg. b.w.)+ CP; **VIII**– EAT + DHA-II (100mg / kg.b.w.). Values are mean of 6 mice ± SEM. *a* p < 0.05 and *p* < 0.01. * Values are significantly different from EAT bearing mice.
Table 5.2.: Effect of *D. hamiltonii* root extract and its bioactive molecules on hematological profile of mice with Ehrlich Ascites tumor

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hb  (g %)</th>
<th>RBC (10^6 million / mm³)</th>
<th>WBC (10^3 million cells / mm³)</th>
<th>Lymphocyte (%)</th>
<th>Neutrophils (%)</th>
<th>Monocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>13.76±0.17</td>
<td>5.23±0.07</td>
<td>8.051±0.17</td>
<td>75.10±0.46</td>
<td>21.20±0.51</td>
<td>2.32±0.16</td>
</tr>
<tr>
<td>II</td>
<td>9.18±0.4**</td>
<td>3.55±0.12**</td>
<td>21.92±0.78**</td>
<td>34.20±1.7**</td>
<td>62.30±1.63**</td>
<td>1.51±0.1**</td>
</tr>
<tr>
<td>III</td>
<td>12.63±0.45*</td>
<td>4.92 ± 0.14 *</td>
<td>10.56 ± 0.23 *</td>
<td>68.46 ± 3.04 *</td>
<td>23.24 ± 1.24 *</td>
<td>2.02 ± 0.32 *</td>
</tr>
<tr>
<td>IV</td>
<td>13.04±0.12*</td>
<td>5.17±0.08*</td>
<td>9.83±0.18*</td>
<td>72.14±0.62*</td>
<td>22.80±0.32*</td>
<td>2.17±0.14*</td>
</tr>
<tr>
<td>V</td>
<td>11.08±0.17*</td>
<td>4.41±0.05*</td>
<td>14.18±0.82*</td>
<td>59.30±1.44</td>
<td>29.56±1.86</td>
<td>1.93±0.26*</td>
</tr>
<tr>
<td>VI</td>
<td>12.77±0.31*</td>
<td>5.08±0.11*</td>
<td>9.50±0.53*</td>
<td>71.65±2.16*</td>
<td>21.72±1.31*</td>
<td>2.09±0.20*</td>
</tr>
<tr>
<td>VII</td>
<td>11.42±0.08*</td>
<td>4.64±0.04*</td>
<td>13.47±0.21*</td>
<td>62.70±2.80*</td>
<td>28.40±1.74*</td>
<td>1.87±0.15*</td>
</tr>
<tr>
<td>VIII</td>
<td>12.57±0.22*</td>
<td>5.00±0.06</td>
<td>10.23±0.25*</td>
<td>70.45±2.16*</td>
<td>23.01±0.68*</td>
<td>2.05±0.36*</td>
</tr>
<tr>
<td>IX</td>
<td>10.02±0.06</td>
<td>4.12±0.10</td>
<td>17.10±0.16*</td>
<td>49.83±1.20</td>
<td>39.04±1.62</td>
<td>1.71±0.11*</td>
</tr>
</tbody>
</table>

Groups: I – control; II– EAT bearing mice; III–EAT + CP (25mg / kg.b.w.); IV– EAT + DHA (100mg / kg.b.w.)+ CP; V– EAT+DHA (100mg / kg. b.w.); VI– EAT+DHA-I (100mg / kg. b.w.)+ CP; VII– EAT + DHA-I (100mg / kg.b.w.); VIII– EAT+DHA-II (100mg / kg. b.w.)+ CP; IX– EAT + DHA-II (100mg / kg.b.w.) Values are mean of 6 mice ± SEM. ** p < 0.01, and * p < 0.01. ** Values are significantly different from control, * Values are significantly different from EAT bearing mice.
Table 5.3.: Effect of *D. hamiltonii* root extract and its antioxidant compounds on serum enzymes profile in mice with Ehrlich Ascites tumor

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>32.58±1.08</td>
<td>24.28±2.07</td>
<td>78.64±2.23</td>
<td>873.10±26.24</td>
</tr>
<tr>
<td>II</td>
<td>62.27±3.19**</td>
<td>58.02±3.08**</td>
<td>121.03±4.12**</td>
<td>1420.60±102.10**</td>
</tr>
<tr>
<td>III</td>
<td>39.16±1.12*</td>
<td>32.15±1.12</td>
<td>84.24±1.42*</td>
<td>924.12±38.14*</td>
</tr>
<tr>
<td>IV</td>
<td>36.82±2.10*</td>
<td>30.40±0.44*</td>
<td>81.33±1.16*</td>
<td>907.00±33.05*</td>
</tr>
<tr>
<td>V</td>
<td>41.03±2.32*</td>
<td>36.71±0.62*</td>
<td>89.20±2.15*</td>
<td>960.00±41.34*</td>
</tr>
<tr>
<td>VI</td>
<td>34.22±2.30*</td>
<td>28.62±1.23*</td>
<td>82.09±1.74*</td>
<td>918.25±36.46*</td>
</tr>
<tr>
<td>VII</td>
<td>40.61±1.19*</td>
<td>34.20±1.61*</td>
<td>88.45±2.02*</td>
<td>973.04±45.51*</td>
</tr>
<tr>
<td>VIII</td>
<td>38.84±1.46*</td>
<td>32.07±1.34*</td>
<td>84.00±1.22*</td>
<td>922.69±38.02*</td>
</tr>
<tr>
<td>IX</td>
<td>45.11±2.59*</td>
<td>40.60±2.78</td>
<td>95.21±3.07*</td>
<td>1104.02±80.77</td>
</tr>
</tbody>
</table>

Groups: **I** – control; **II**– EAT bearing mice; **III**–EAT + CP (25mg / kg.b.w.); **IV**–EAT + DHA (100mg / kg.b.w.)+ CP; **V**– EAT+DHA (100mg / kg. b.w.); **VI**–EAT+DHA-I (100mg / kg. b.w.)+ CP; **VII**– EAT + DHA-I (100mg / kg.b.w.); **VIII**–EAT+DHA-II (100mg / kg. b.w.)+ CP; **IX**– EAT + DHA-II (100mg / kg.b.w.). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase. Values are mean of 6 mice ± SEM. **p < 0.01, and * p < 0.01. ** Values are significantly different from control, * Values are significantly different from EAT bearing mice.
HEK-293, PC-12, EAT cells were seeded in 96-well plates and treated with various concentrations of CP for 24 h. Cell viability was assayed by MTT method for 4 h at 37°C. The LC\textsubscript{50} values (µg/ml) were: HEK-293 (17.78), PC-12 (1.9) and EAT cells (2.18). Data are mean ± SD and representative of three experiments. *p<0.05, **p<0.01 versus the control group.
HEK-293, PC-12, EAT cells were seeded in 96-well plates and treated with various concentrations of *D. hamiltonii* root extract and its bioactive molecules [A: DHA, B: DHA-I, C: DHA-II] for 24 h. Cell viability was assayed by MTT method for 4 h at 37°C. The LC\textsubscript{50} values (µg/ml) were: PC-12 [A: 29.30, B: 27.16, C: 37.1] and EAT cells [A: 24.83, B: 30.17, C: 34.2]. Data are mean ± SD and representative of three independent experiments. *p<0.05, **p<0.01 versus the control group.
Figure 5.5.: Potentiation of CP action on cell viability in the presence of *D. hamiltonii* root extract and its bioactive molecules

HEK-293, PC-12, EAT cells were seeded in 96-well plates and treated with aqueous extract of the roots of *D. hamiltonii* and its bioactive molecules + CP for different time intervals. [A: DHA + CP, B: DHA-I+ CP, C: DHA-II+ CP] Cell viability was assayed by MTT method for 4 h at 37°C. (For respective control group please see Fig. 5.3, 5.4). Data are mean ± SD and representative of three independent experiments. *p<0.05, **p<0.01 versus the control group.
Figure 5.6.: Morphology of apoptotic cell death in EAT cells exposed to CP and *D. hamiltonii* (Giemsa staining)

Cells were treated with LC\textsubscript{50} concentration of CP and *D. hamiltonii* for 24 h and stained with Wright’s Giemsa. Arrows indicate the plasma membrane blebbing and formation of apoptotic bodies. Groups: I- Control; II- CP (2.18µg/ml); III- DHA (24.83µg/ml) + CP; IV- DHA (24.83µg/ml); V- DHA-I (30.17µg/ml) + CP; VI- DHA-I (30.17µg/ml); VII- DHA-II (34.2µg/ml) + CP; VIII- DHA-II (34.2µg/ml). All data shown are representative of three independent experiments [magnification, x 100].
Anticancer Activity

Figure 5.7.: Morphology of apoptotic cell death in PC-12 cells exposed to CP and *D. hamiltonii* (Acridine orange/Ethidium bromide staining)

Cells were treated with LC₅₀ concentration of CP and *D. hamiltonii* for 24 h and stained with acridine orange/ethidium bromide. Viewed under fluorescence microscope. Arrows indicates the the formation of apoptotic bodies, condensed nucleus and membrane blebbing as evidence of apoptosis. Groups: I- Control; II- CP (1.9µg/ml); III- DHA (29.30µg/ml) + CP; IV-DHA (29.30µg/ml); V- DHA-I (27.16µg/ml) + CP; VI- DHA-I (27.16µg/ml); VII- DHA-II (37.1µg/ml) + CP; VIII- DHA-II (37.1µg/ml). All data shown are representative of three independent experiments [magnification, x 400].
Figure 5.8: Induction of apoptosis by CP, *D.hamiltonii* root extract and its bioactive molecules in cancer cells

A: EAT Cells, B: PC-12 Cells; Groups: I- Control; II- CP; III- DHA + CP; IV- DHA; V- DHA-I + CP; VI- DHA-I; VII- DHA-II + CP; VIII- DHA-II. All data shown are representative of three independent experiments. Data are mean ± SD and representative of three independent experiments. *p<0.05, **p<0.01 versus the control group.
Figure 5.9.: DNA fragmentation in carcinoma cells exposed to CP, *D. hamiltonii* root extract and its bioactive molecules

Cells were treated with LC$_{50}$ concentration of CP, *D. hamiltonii* root extract and its bioactive molecules for 24 h. The genomic DNA was isolated and electrophoresed on 1.5 % agarose gel and stained with ethidium bromide, **A)** EAT cells: Lane I- control; Lane II- CP (2.18µg/ml); Lane III- DHA (24.83µg/ml) + CP; Lane IV-DHA (24.83µg/ml); Lane V- DHA-I (30.17µg/ml) + CP; Lane VI- DHA-I (30.17µg/ml); Lane VII- DHA-II (34.2µg/ml) + CP; Lane VIII- DHA-II (34.2µg/ml); **B)** PC-12 cells: Lane I- control; Lane II- CP (1.9µg/ml); Lane III- DHA (29.30µg/ml) + CP; Lane IV-DHA (29.30µg/ml); Lane V- DHA-I (27.16µg/ml) + CP; Lane VI- DHA-I (27.16µg/ml); Lane VII- DHA-II (37.1µg/ml) + CP; Lane VIII- DHA-II (37.1µg/ml).
Figure 5.10.: Induction DNA damage in EAT cells by CP and *D. hamiltonii* (Comet assay)

A: Photomicrographs showing the DNA migration pattern in EAT cells exposed to Cp and *D. hamiltonii* by comet assay [magnification, x 100].

B: Histogram derived from comet assay in EAT cells exposed to Cp and *D. hamiltonii*: quantification of DNA breakage and damage.

Cells were treated with LC$_{50}$ concentration of CP, *D. hamiltonii* root extract and its bioactive molecules for 24 h. The comet assay was performed. Groups: I- Control; II- CP (2.18µg/ml); III- DHA (24.83µg/ml) + CP; IV- DHA (24.83µg/ml); V- DHA-I (30.17µg/ml) + CP; VI- DHA-I (30.17µg/ml); VII- DHA-II (34.2µg/ml) + CP; VIII- DHA-II (34.2µg/ml). Data are mean ± SD and representative of three independent experiments. * p<0.05, ** p<0.01 versus the control group.
Anticancer Activity

A:

Figure 5.11: Induction DNA damage in PC-12 cells by CP and D. hamiltonii (Comet assay)

A: Photomicrographs showing the DNA migration pattern in PC-12 cells exposed to Cp and D. hamiltonii by comet assay [magnification, x 100].

B: Histogram derived from comet assay in PC-12 cells exposed to Cp and D. hamiltonii: quantification of DNA breakage and damage.

Cells were treated with LC50 concentration of CP, D. hamiltonii root extract and its bioactive molecules for 24 h. The comet assay was performed. Groups: I- Control; II- CP (1.9µg/ml); III- DHA (29.30µg/ml) + CP; IV- DHA (29.30µg/ml) + CP; V- DHA-I (27.16µg/ml) + CP; VI- DHA-I (27.16µg/ml); VII- DHA-II (37.1µg/ml) + CP; VIII- DHA-II (37.1µg/ml). Data are mean ± SD and representative of three independent experiments. * p<0.05, ** p<0.01 versus the control group.
A:

Photomicrographs showing the DNA migration pattern in HEK-293 cells exposed to Cp and *D. hamiltonii* by comet assay [magnification, x 100].

B:

Histogram derived from comet assay in HEK-293 cells exposed to Cp and *D. hamiltonii*: quantification of DNA breakage and damage.

Cells were treated with LC$_{50}$ concentration of CP (17.78µg/ml) and 100µg/ml of DHA, DHA-I and DHA-II for 24 h. The comet assay was performed. Groups: I- Control; II- CP; III- DHA+ CP; IV- DHA; V- DHA-I + CP; VI- DHA-I; VII- DHA-II + CP; VIII- DHA-II (37.1µg/ml). Data are mean ± SD and representative of three independent experiments. * p<0.05, ** p<0.01 versus the control group.
Figure 5.13: RT-PCR profile showing expression of apoptotic genes in EAT cells exposed to CP, \textit{D. hamiltonii} root extract and its bioactive molecules.

EAT cells were exposed to LC$_{50}$ concentration of CP, \textit{D. hamiltonii} root extract and its bioactive molecules for 24 h, cDNA was synthesized and amplified with appropriate primers using PCR. The Bax, Bcl-2 and caspase3, 9 genes were amplified against GAPDH standard. Amplified PCR products were subjected to electrophoresis on a 1.8% agarose gel and stained with ethidium bromide and photographed under UV light. Lane \textbf{I} - control; Lane \textbf{II} - CP (2.18µg/ml); Lane \textbf{III} - DHA (24.83µg/ml) + CP; Lane \textbf{IV} - DHA (24.83µg/ml); Lane \textbf{V} - DHA-I (30.17µg/ml) + CP; Lane \textbf{VI} - DHA-I (30.17µg/ml); Lane \textbf{VII} - DHA-II (34.2µg/ml) + CP; Lane \textbf{VIII} - DHA-II (34.2µg/ml).
Anticancer Activity

Figure 5.14: RT-PCR profile showing expression of apoptotic genes in PC-12 cells exposed to CP, D. hamiltonii root extract and its bioactive molecules.

PC-12 cells were exposed to LC$_{50}$ concentration of CP, D. hamiltonii root extract and its bioactive molecules for 24 h, cDNA was synthesized and amplified with appropriate primers using PCR. The Bax, Bcl-2 and caspase3, 9 genes were amplified against GAPDH standard. Amplified PCR products were subjected to electrophoresis on a 1.8% agarose gel and stained with ethidium bromide and photographed under UV light. Lane I- control; Lane II- CP (1.9µg/ml); Lane III- DHA (29.30µg/ml) + CP; Lane IV- DHA (29.30µg/ml); Lane V- DHA-I (27.16µg/ml) + CP; Lane VI- DHA-I (27.16µg/ml); Lane VII- DHA-II (37.1µg/ml) + CP; Lane VIII- DHA-II (37.1µg/ml).
Anticancer Activity

Figure 5.15: Semi-quantitative RT-PCR analysis of Bax, Bcl-2 and caspase3, 9 mRNA levels in EAT cells.

EAT cells were exposed to LC₅₀ concentration of CP, *D. hamiltonii* root extract and its antioxidants for 24 h. cDNA was synthesized and amplified with appropriate primers using PCR. The mRNA expression levels of apoptotic genes were normalized with GAPDH (housekeeping gene). Groups: I- Control; II- CP (2.18µg/ml); III- DHA (24.83µg/ml) + CP; IV- DHA (24.83µg/ml); V- DHA-I (30.17µg/ml) + CP; VI- DHA-I (30.17µg/ml); VII- DHA-II (34.2µg/ml) + CP; VIII- DHA-II (34.2µg/ml).

Data are mean ± SD and representative of three independent experiments. * p<0.05, ** p<0.01 versus the control group.
Figure 5.16: Semi-quantitative RT-PCR analysis of Bax, Bcl-2 and caspase3, 9 mRNA levels in PC-12 cells.

PC-12 cells were exposed to LC$_{50}$ concentration of CP, D. hamiltonii root extract and its bioactive molecules for 24 h, cDNA was synthesized and amplified with appropriate primers using PCR. The mRNA expression levels of apoptotic genes were normalized with GAPDH (house-keeping gene). Groups: 

- **I**- Control;
- **II**- CP (1.9µg/ml);
- **III**- DHA (29.30µg/ml) + CP;
- **IV**- DHA (29.30µg/ml);
- **V**- DHA-I (27.16µg/ml) + CP;
- **VI**- DHA-I (27.16µg/ml);
- **VII**- DHA-II (37.1µg/ml) + CP;
- **VIII**- DHA-II (37.1µg/ml). Data are mean ± SD and representative of three independent experiments. * p<0.05, ** p<0.01 versus the control group.
DISCUSSION

Cancer chemotherapy by anti-cancer drugs targets inhibition of malignant cell transformation, prevention of invasion and metastasis (Miyoshi et al., 2003; Ramawat and Goyal, 2009; Demain and Vaishnav, 2011; Wu et al., 2011). There is evidence that antioxidants when properly combined with chemotherapy drugs, have been shown to reduce the toxicity and side effects of drugs (Wang and Wieder, 2004; Li et al., 2006; Beer et al., 2007). The use of herbal medicine or dietary supplements are promising approaches in the management of cancer treatment (Rates, 2001; Jin and Russell, 2010; Raihan et al., 2012). Many of the plant derived antioxidants have greatly contributed to the efficacy of cancer chemotherapy (Srivasthava and Gupta, 2006; Desai et al., 2008; Bachrach, 2012). Hence, there is a great potential for the discovery of newer bioactive compounds including antioxidants from the untapped reservoir of the plant kingdom with therapeutic potential in cancer chemotherapy.

The edible roots *Decalepis hamiltonii* have been shown to possess health promoting potential and contain several many bioactive compounds that show chemopreventive properties (Srivastava et al., 2006b; Srivastava and Shivanandappa, 2006a; 2010a; 2010b; 2012 a). We have shown that the root extract of *D. hamiltonii* and its antioxidant compounds show ameliorating potential against CP-induced hepatotoxicity (Zarei and Shivanandappa, 2013a) (please see chapter III). In this study, we have demonstrated the modulation of antitumor activity of CP by *D. hamiltonii* aqueous extract and its bioactive molecules in EAT tumor bearing mice. EAT cell transplanted mice showed weight gain and increase in tumor volume due to growth of tumor cells (Ashok et al., 2009, Zarei and Javarappa, 2012). Administrations of DHA and DHA-I suppressed EAT tumor growth significantly as shown by the decreased cell number and enhanced survival time. Our results indicate
that combination treatment of CP with DHA, DHA-I and DHA-II shows potentiation of anti-cancer effect of CP in EAT bearing mice. As expected, administration of the antitumor drug, CP to EAT bearing mice significantly prolonged the MST. Prolongation of lifespan of the tumor bearing animal is considered as a significant measure for evaluating efficacy of an anticancer drug (Choudhury et al., 2010; Das et al., 2010; Dashora et al., 2011). *D. hamiltonii* roots extract treatment alone prolonged the lifespan of tumour bearing mice and the effect was comparable to that of CP treated group, which is indication of the antitumor activity of the DHA and its bioactive constituents (Zarei and Shivanandapa, 2013b).

A rapid increase in ascetic fluid volume is seen in EAT bearing mice and the tumor cells use ascetic fluid as a nutritional source (Feng et al., 2001; Rammath et al., 2002; Anbu et al., 2011). Treatment with DHA and its bioactive molecules showed a significant decrease in the tumor volume and cell count which suggests that *D. hamiltonii* was effective in arresting the tumor growth. Co treatment of *D. hamiltonii* root extract and its antioxidant compound with CP showed a significant reduction in the tumor volume and cell count suggesting their potentiating influence on the efficacy of CP.

In cancer chemotherapy, major problems encountered are myelosuppression and anemia (Price and Greenfield, 1958; Gordon, 2002; Govindan et al., 2012). The anemic changes seen in tumor bearing mice as evident from reduction in RBC or Hb content which could be due to hemolytic or myelopathic conditions (Hoagland, 1982; Svetislav, 2004; Upadhyay et al., 2012; Islam et al., 2012). Treatment with *D. hamiltonii* extract and DHA-I to EAT bearing mice restored the Hb content, RBC and WBC count and was comparable to that of normal mice which indicates the protective action of *D. hamiltonii* against tumor-induced changes in the hemopoietic system.
Combination therapy of CP with *D. hamiltonii* in EAT bearing mice restored the Hb content, RBC and WBC count and was comparable to that of CP treated mice.

Elevation in the activities of serum enzymes AST and ALT is associated with hepatic dysfunction in tumor bearing mice (Stefanini, 1985; Joy and Kuttan, 1998; Özkol et al., 2012; Kumar et al., 2012). In tumor bearing mice treated with the aqueous extract of *D. hamiltonii* and the antioxidant compound, serum levels of ALT and AST were not elevated which is consistent with reduced tumor growth. Altered serum enzyme levels in EAT bearing mice are indicative of changed metabolism including hepatic dysfunction due to cancerous growth, which was restored to normal levels by *D. hamiltonii* treatment indicating the therapeutic effect and physiological consequences towards normalcy. Further, *D. hamiltonii* and its bioactive molecules in combination with CP significantly improved the serum enzyme profile which could be due to the scavenging activity of *D. hamiltonii* against the toxic metabolite produced during the activation of the CP by liver microsomal enzymes.

Apoptosis is an important physiological mechanism between cell division and cell death (Osborne, 1993; Green and Reed, 1998; Günther et al., 2012). It is thought that cell death by apoptosis is physiologically advantageous because apoptotic cells are cleared by phagocytosis and subsequent intracellular degradation without pathological consequences (Roos and Kaina, 2006; Lee et al., 2012). Anticancer drugs, such as CP kill cancer cells by apoptosis (Hannun, 1997). Drug screening that kill cancer cells by apoptosis is often used to identify therapeutic compounds from plants. (Shao et al., 1997; Zhang et al., 2008; Mohan et al., 2010; Shawi et al., 2011; Ahmad et al. 2012).
In the present study, we shown that *Decalepis hamiltonii* root extract and its bioactive molecules exhibited significant anticancer activity and potentiated the ability of CP to induce apoptosis in cancer cells as revealed from the enhancement of apoptotic index in cells treated with DHA, DHA-I and DHA-II alone and in combination with CP. The apoptotic cellular morphology in treated cancer cells showing nuclear condensation, fragmentation of DNA, as well as comet assay, confirm that the mode of action involving induction of apoptosis. *In vitro* results support the *in vivo* observations that *D. hamiltonii* and its antioxidant compounds exhibit anticancer activity.

It is known that in cancer cells CP binds to DNA and induces DNA damage in the form of strand breaks, DNA–DNA cross-links, both interstrand and intrastrand, as well as DNA-protein cross-links (Crook et al., 1986; Colvin, 1999). The present studies using comet assay provided further evidence that CP caused a significant elevation of DNA single strand breakage in HEK-293 cells a non-cancer cell line. The important finding in our results is that *D. hamiltonii* and its bioactive molecules significantly inhibited CP-induced DNA damage in HEK-293 cell, and decreased the number of apoptotic cells, a desirable feature that protects normal dividing cells. Interestingly, we have shown that *D. hamiltonii* and its antioxidant molecules can induce apoptosis and DNA damage in cancer cells such as EAT and PC-12 cells in combination with CP, which shows potentiation effect.

There are at least two major apoptotic pathways, extrinsic pathways (death receptors) and intrinsic pathways (mitochondria) (Fulda and Debatin, 2006; Seitz et al., 2010). The mitochondrial death pathway is controlled by members of the Bcl-2 family, including the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins (Willis et al., 2003; Brunelle and Letai, 2009; Estaquier et al., 2012). The balance between
protein levels of these members is crucial for the cellular decision of starting the apoptotic process. The ratio of a Bcl-2 and Bax is proportional to the relative sensitivity or resistance to a wide variety of apoptotic stimuli (Li et al., 1997; Adams and Cory, 2007). In contrast, the mitochondrial pathway requires the release of mitochondrial cytochrome C and the formation of a large multiprotein complex comprising cytochrome c, and procaspase-9. caspase-9 will then proteolytically activate downstream caspases, in particular caspases-3 (Jänicke et al., 1998; Riedl and Shi, 2004; Wang et al., 2013). The activation of caspase-3, a key and irreversible point in the progression of apoptosis, is essential for DNA fragmentation and the morphological changes associated with apoptosis (Jänicke et al., 1998; Fan et al., 2005; Logue and Martin, 2008).

In our study we have analyzed the expression of apoptotic genes in cancer cells treated with CP and D. hamiltonii. Expression of pro-apoptotic genes such as Bax, Caspase-9 and 3 was significantly induced, suggesting that DHA and DHA-I was an initiator or inducer of the apoptotic mechanism. D. hamiltonii also enhanced the activation of Bcl-2 family of pro-apoptotic proteins such as Bax while it down-regulated the expressions of Bcl-2 in carcinoma cells.

In conclusion, our study has demonstrated the action of CP on normal cells causing oxidative stress-mediated cellular injury is responsible for its toxicity. Whereas, the anti-cancer activity of CP is attributed to its ability to kill cancer cells by DNA damage and mitochondrial pathway of apoptosis induction. D. hamiltonii and its antioxidant compounds protect normal cells against CP cytotoxicity by suppressing oxidative stress. D. hamiltonii promotes the anti-cancer effect via induction of apoptosis. Therefore, the roots of D. hamiltonii possess a safe therapeutic potential in cancer chemotherapy.