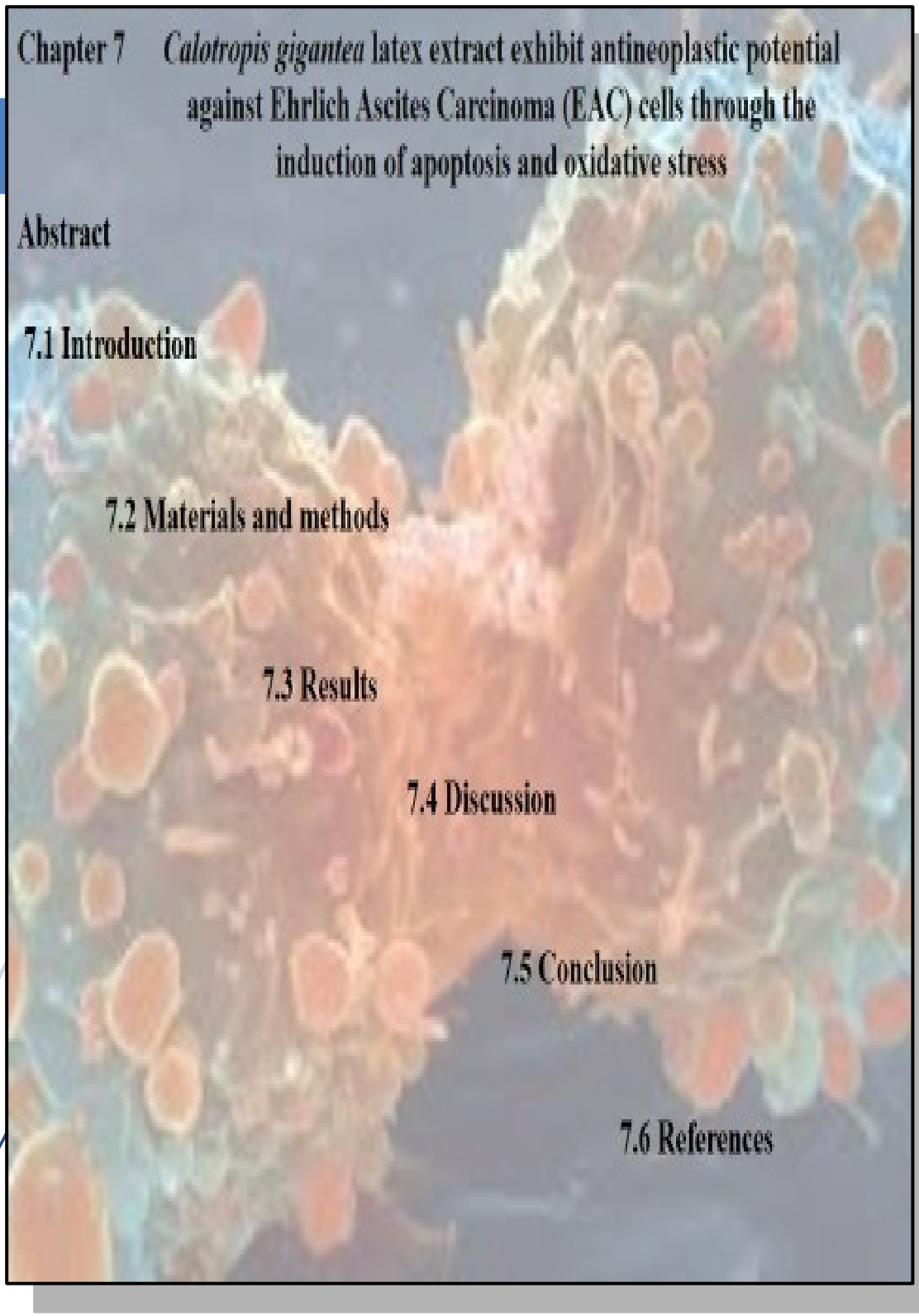
Chapter 7 *Calotropis gigantea* latex extract exhibit antineoplastic potential against Ehrlich Ascites Carcinoma (EAC) cells through the induction of apoptosis and oxidative stress
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Abstract

Cancer is the one of the deadly diseases and it is the third leading cause of death worldwide. The roots and leaves of *Calotropis gigantea* are used traditionally for the treatment of abdominal tumours. The present study designed to evaluate the efficacy of *Calotropis gigantea* latex extracts (EECGL and WECGL) from as anticancer agents against Ehrlich ascites carcinoma in comparison to EECGL. Study of cytotoxicity, cell viability, cell morphology, chromatin condensation, DNA fragmentation, nitric oxide (NO) generation and release level revealed that EECGL and WECGL possess antiproliferative and apoptotic effects on EAC cell line at IC_{50} of 25 and 40.98 $\mu g\, mL^{-1}$.EECGL and WECGL were administered intraperitoneally at the dose level of 100- and 200 mg/kg body weight/day respectively for 14consecutive days after 24 hour of EAC cell inoculation (1×10^6 cell) to mice using 5-fluorouracil as standard drug. Mean survival time, tumor volume, haematological and oxidative stress related parameters were studied. Decrease in tumor volume, and body weight of the EAC-bearing mice were observed in EECGL and WECGL-treated mice group compared to EAC-control mice. Treatments with EECGL and WECGL were associated to decrease in the levels of lipid peroxidation (MDA), and increase in the levels of reduced glutathione (GSH), and antioxidant enzymes activities. Up regulation of pro-apoptotic proteins as well as down regulation of anti-apoptotic proteins involving intrinsic apoptotic pathway were detected. Immuno-histochemical observations from treated solid tumors also established the anti-proliferative and anti-angiogenic capabilities of *Calotropis gigantea* latex extracts. From the present investigation, it is established that EECGL and WECGL possess *in vitro*cytotoxic, apoptotic, antioxidant and *in vivo* anti-proliferative and anti-angiogenetic activities against EAC cells but the efficacy ofEECGL is more than WECGL.

7.1 Introduction

Despite the therapeutic advances created in understanding the processes concerned in carcinogenesis, cancer is a major public health problem worldwide.Cancer constitute the second cause of mortality behind cardiovascular diseases in developed countries and the third after infectious and cardiovascular diseases in developing countries (Bieche, 2004; Mbaveng et al., 2011). The number of people affected with cancer is steadily increasing every day (Engel et al., 2011).). An estimate of 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer in 2012 worldwide was observed. 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions (Globocan, 2012).For this reason, cancer therapy has become a serious focus of current research.

Cancer is a complex disease that is normally associated with a wide range of escalating effects both at the molecular and cellular levels. Cancer is fundamentally a disease of regulation of tissue growth. In order to transform from a normal cell into a cancer cell, genes which regulate cell growth and differentiation, must be altered (Croce, 2008). Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide. There are two broad categories of genes which are affected by these changes. Oncogenes may be normal genes which are expressed in inappropriately high levels, or altered genes which have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Tumor suppressor genes are genes which inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer-promoting genetic changes (Knudson, 2001).

The serious side effects of the FDA approved chemo preventive drugs is an issue of particular concern when considering long-term administration of a drug to healthy people who may or may not develop cancer. This clearly indicates the need for agents, which are safe and efficacious in preventing cancer. Plant derived natural products will be potential candidates for this purpose (Anand et al., 2008). Over 60% of currently used anticancer agents are derived in one-way or another from natural sources, including plants, marine organisms and microorganisms. It is estimated that more than 50% of all the patients diagnosed with cancer explorecomplementary and alternative medicine, especially herbal medicine (Newman et al.,2003).

7.1.1 Etiology of Cancer

Cancers are primarily an environmental disease with 90-95% of cases due to lifestyle, environmental factors and 5-10% due to genetics. Common environmental factors that lead to cancer death include: obesity (30-35%), tobacco (25-30%), diet, infections (15-20%), radiation, stress, lack of physical activity and pollutants.

Diet

High-fat, high cholesterol diets are proven risk factors for several types of cancer such as colon, uterus and prostate. Obesity may be linked to breast cancer among older women as well as to cancers of the prostate, pancreas, uterus, colon and ovary.

Tobacco, smoking and alcohol

Tobacco is a known cause of cancer of the lung, bladder, mouth, pharynx, pancreas, kidney, stomach, larynx, oesophagus and possibly colon (Giovannucci and Wllett, 1994).

Smokers are seemingly to develop many types of cancer like those of the mouth, larynx, esophagus, pancreas, bladder, excretory organ and cervix. Smoking may increase

the probability of developing cancers of the abdomen, liver, prostate, colon and body part (Peto et al., 1992).Alcoholic beverages cause inflammation, cirrhosis of the liver, and liver cancer (International Agency for Research on Cancer, 1988). Alcohol is an important cause of oral and oesophageal cancer and possibly contributes to colorectal cancer (Glynn and Albanes, 1994; Giovannucci et al., 1995).

Sun Exposure

UV radiations from the sun are directly linked to melanoma and other forms of skin cancer. Artificial sources of UV radiation, such as sun lamps also increase the risk of skin cancer. Many of the 1.3 million skin cancers diagnosed in the year 2000 could have been prevented by protection from the sun rays.

Chemicals

Long term exposure to chemicals such as pesticides, uranium, nickel, asbestos, radon and benzene can increase the risk of cancer.

Medical Interventions

Some formerly used drugs, such as phenacetin and diethylstilbesteroland immunosuppressive agents such as cyclosporinwere associated with increased cancer risk (Ryffel et al.,1992).

Hereditary Factors

The abnormal gene responsible for causing cancer is passed from parent to child, posing a greater risk for that type of cancer in all descendants of the family, 20% of cancers are hereditary.

Oncogenes and tumour suppressor genes

Cancer is caused by the accumulation of genetic mutations in genes that normally play a role in the regulation of cell proliferation, thus leading to uncontrolled cell growth. Cells acquire mutations in these genes as a result of spontaneous and environmentally-induced DNA

damage. Genes involved in tumorigenesis include those whose products: 1) directly regulate cell proliferation, 2) control programmed cell death or apoptosis, and 3) are involved in the repair of damaged DNA. Depending on how they affect each process, these genes can be grouped into two general categories: tumor suppressor genes and proto-oncogenes. Mutant alleles of proto-oncogenes are called oncogenes.

Tumor suppressor genes can be defined as genes which encode proteins that normally inhibit the formation of tumors. Their normal function is to inhibit cell proliferation, or act as the "brakes" for the cell cycle. Mutations in tumor suppressor genes contribute to the development of cancer by inactivating the inhibitory function.

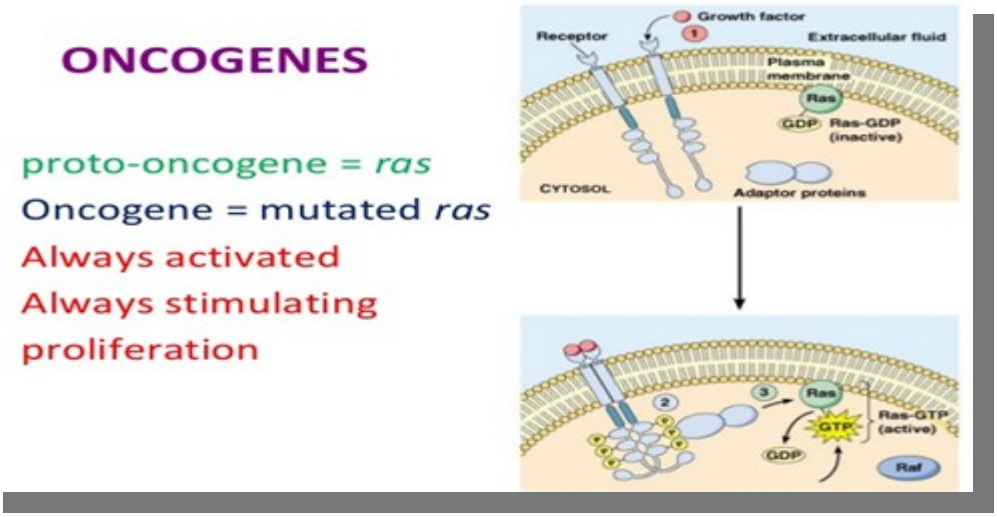


Figure 7.1Role of oncogenes in cancer development

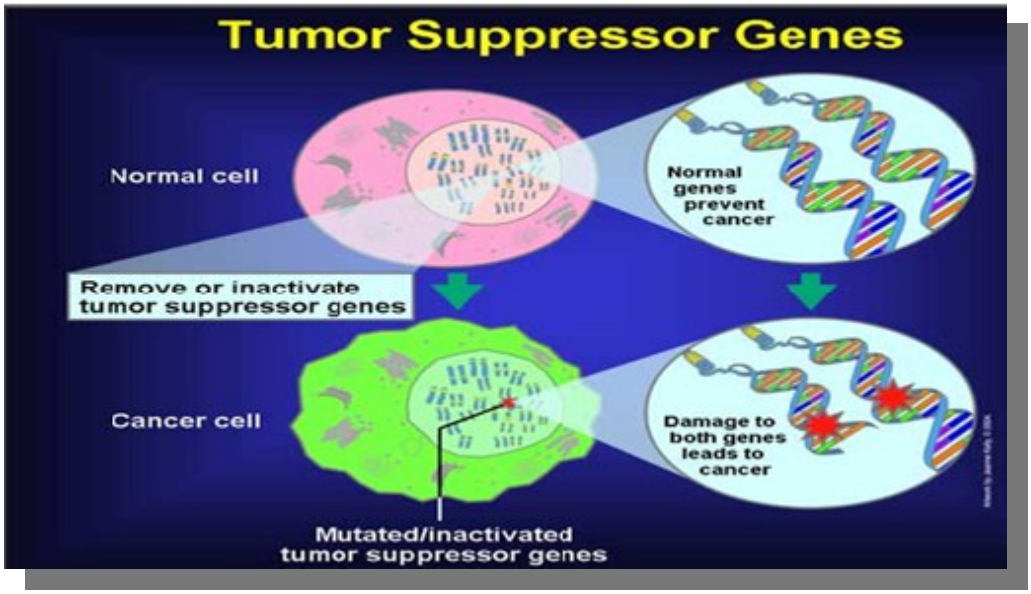


Figure 7.2 Tumor suppressor genes in cancer development

7.1.2 Apoptosis

Cell death, particularly apoptosis, is probably one of the most widely-studied subjects among cell biologists.Morphological hallmarks of apoptosis in the nucleus are chromatin condensation and nuclear fragmentation, which are accompanied by rounding up of the cell, reduction in cellular volume (pyknosis) and retraction of pseudopodes (Kroemer et al., 2005). Chromatin condensation starts at the periphery of the nuclear membrane, forming a crescent or ring-like structure. The chromatin further condenses until it breaks up inside a cell with an intact membrane, a feature described as karyorrhexis (Manjo and Joris, 1995). The plasma membrane is intact throughout the total process. At the later stage of apoptosis

some of the morphological features including membrane blebbing, ultrastructural modification of cytoplasmic organelles and a loss of membrane integrity are seen.

Mechanisms of apoptosis

Understanding apoptosis in disease conditions is very important as it not only gives insights into the pathogenesis of a disease but may also leave clues on how the disease can be treated. This in turn, may help in the development of drugs that targetcertain apoptotic genes or pathways. Caspases are central to the mechanism of apoptosis as they are boththe initiators and executioners. There are three pathways by which caspases can be activated. The twocommonly described initiation pathways are intrinsic (mitochondrial) and extrinsic (or death receptor) pathways of apoptosis. Both pathways eventually lead to a common pathway or the executionphase of apoptosis.

The extrinsic death receptor pathway

The extrinsic death receptor pathway, begins when death ligands bind to a death receptor. Although several death receptors have been described, the best known death receptors is the type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95) and their ligands are called TNF and Fas ligand (FasL) respectively (Hengartner, 2000). These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases like caspase 8. Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein and the whole ligand-receptor-adaptor protein complex is known as the death-inducing signalling complex (DISC). DISC then initiates the assembly and activation of pro-caspase 8. The activated form of the enzyme,

caspase 8 is an initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases(Karp,2008).

The intrinsic mitochondrial pathway

This pathway is closely regulated by a group of proteins belonging to the Bcl-2 family, named after the Bcl-2 gene originally observed at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma (Tsujimoto, 1984). There are two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1). While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release.

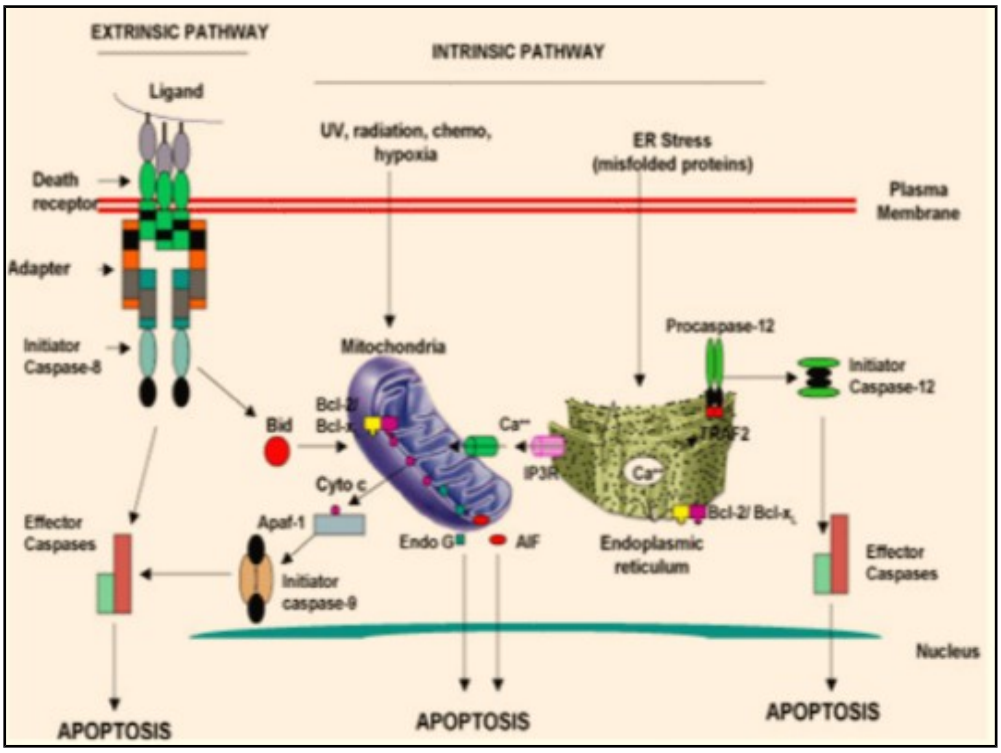


Figure 7.3Mechanism of apoptosis cascade

7.1.3 Ehrlich ascites carcinoma (EAC) cell

The intensive studies on the transplantable tumors were taken into consideration in the last two to three decades. Experimental tumors have great importance for the purposes of modeling, and Ehrlich ascites carcinoma (EAC) is one of the commonest. EAC is referred to as an undifferentiated carcinoma, and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also does not have tumorspecific transplantation antigen (TSTA) (Kaleoğlu and İşli, 1977). The reason for its load usage is that the suspension contained homogeneous free tumor cells of the Ehrlich ascites tumor, and in this way, it has a transplantable capacity for certain quantitative tumor cells to another mouse (Klein, 1951).

7.1.4 Alternative approaches in cancer therapy

The usage of plant products in medicine has agreat historical inheritance among people (Duke, 1985). Nature gives a great deal of effective anti-cancer agents such as dactinomycin and doxorubicin derived from microorganisms and vinblastine, irinotecan, topotecan, vincristine and taxanes from plants which are used frequently in recent years. Several plants were reportedto stimulate the immune system in different pathways. In addition, they increased specific cellular and humoral immune responses (Bhakuni et al., 1969). Moreover, there is a growing trend for herbal drugs because of low toxicity and high medical effectiveness of the extracts from these plants.

Securidaca longepedunculata caused a decrease in angiogenesis as observed in the reduction in weight of treated animals and a reduction in volume of ascitic fluid in treated mice. DNA fragmentation assay of Ehrlich ascites carcinoma cells from treated animals depicted a possible pro-apoptotic effect of the *S.longepedunculata* extract due to the ladder forming pattern which was comparable to that of the standard drug (fluorouracil). *S.longepedunculata* had a cytotoxic and pro-apoptotic effect on Ehrlich ascites carcinoma cells. The data revealed that EAC-aliquot volume, EAC-total and alive cell numbers were potentially decreased while dead cell number and percent were profoundly increased as a result of treatment with ulvan polysaccharide. The expression of proapoptotic and cell cycle arrest protein p53 in cytoplasm in the nuclei of EAC-cells in mice treated with ulvan polysaccharide were remarkably increased while the anti-apoptotic protein Bcl-2 expression was decreased (Lawal et al., 2012).

Morphological analyses revealed that *Synadeniumum bellatum treatment* induced EAC cell death by apoptotic pathway. The occurrence of reactive oxygen species (ROS) overgeneration, increased intracellular Ca^{2+} concentration, alteration in mitochondrial membrane potential, phosphatydylserine externalization, and activation of caspases 3, 8, and 9 had been showed.Methanol extract of *Eucalyptus camaldulensis* treated EAC cells showed membrane blebbing, chromatin condensation, nuclear fragmentation (apoptotic features) in Hoechst 33342 staining under fluorescence microscope. The DNA profile in agarose gel (1.5%) electrophoresis also confirmed that the extract caused EAC cell death by apoptosis (Islam et al., 2014).

The selective antineoplastic activity of *Calotropis species* is of great interest as an anticancer agent from plant origin because the species shows several pharmacological activities which are used in traditional treatment (Habib and Karim, 2013). Till now very little is known

regarding the anticancer activity of plant latex as well as its specific mechanism of action on different carcinoma. In the present chapter, attempts were undertaken to assess the antitumor and as well as cytotoxic, antioxidant and apoptotic potential of ethanol (EECGL) and water (WECGL) extract of *Calotropisgigantea* latex against Ehrlich ascites carcinoma (EAC) cells in *in vitro* and *in vivo* experimental conditions.

7.2 Materials and methods

7.2.1 Chemicals and reagents

RPMI 1640, penicillin and streptomycin were purchased from Sigma Aldrich Co, LLC, US. Fetal bovine serum (FBS) was purchased from GIBCO. 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Histopaque-1077, sodium dodecyl sulphate (SDS), sodium chloride (NaCl), potassium chloride (KCl), di-sodium hydrogen phosphate (Na_2HPO_4),2',7'-Dichlorodihydrofluorescein diacetate (H2DCFDA),propidium iodide (PI), 4',6'-diamidino-2-phenylindole (DAPI) , Rhodamine 123, 5-fluorouracil, 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB), Tris–HCl, Tris buffer, Titron X- 100, phenol, chloroform, iso-amyl alcohol, ethidium bromide (EtBr), Acridine orange (AO), 2-vinylpyridine, Ethylenediaminetetraacetic acid (EDTA), Tricarboxylic acid(TCA),thio- barbituric acid (TBA), 1-Chloro-2,4-Dinitrobenzene (CDNB),reduced glutathione (GSH), haematoxylin, eosin, xylene,bis-acylaramide, poly-acylaramide, antibodies (Cell signalling technology, Beverly,MA,USA), 4-Nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3'-indolyphosphate (BCIP) and other chemicals werepurchased from Sigma-Aldrich, St. Louis, MO, USA HimediaIndia, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai,India were purchased for the experimentation.

7.2.2. Animals maintenance

Adult male Swiss albino mice weighing 22-25 g were used for the maintenance of Ehrlich ascites carcinoma(EAC) and for the *in vivo* tumour regression study.The mice were housed in poly acrylic cage (38x 23x10 cm). The animals were kept on a 12 h light: 12 h dark regime at 25 °C for 7 days before commencement of the experiment. The animals had free access to standard diet and water. Mice were deprived of food but not water prior to administration of the test extracts. The study was approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests & Climate Change, and Govt. of India and performed in compliance with the relevant laws and guidelines of the CPCSEA.

7.2.3Cell lines

Ehrlich ascites carcinoma(EAC) cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. EAC cells were maintained by weekly intraperitoneal transplantation in the above said mice at the concentration of 1×10^6/cells per mouse.

7.2.4Mice lymphocyte cell (MLC) was isolated according to the method of Hudson and Hay (Hudson and Hay, 1989).

7.2.5Experimental design for *in vitro* study

Ehrlich ascites carcinoma(1×10^6 cells) cells were exposed to different concentrations (5, 10, 25 and 50 μg ml^{-1}) of EECGL and WECGL for 24 h where control cells did not receive any extract treatment.After the treatment schedule, the cells were collected separatelyand centrifuged at 1000–1200 rpm for 5min at 4^0C to separate cells and supernatants. The cells were washed with PBS (pH-7.4). A required amount ofcells was lysed and then thecells were processed for biochemical estimations.

7.2.6 *In-vitro* study

7.2.6.1 *In vitro* cell viability was measured by MTT assay (Mosmann T, 1983).

7.2.6.2 Oxidative stress parameters were assessed by estimating reduced glutathione (Griffith, 1981) and intracellular ROS level (Roy et al.,2008).

7.2.6.3 Nitric Oxide release and generation were estimated by the modified method of Chakroborty et al. (Chakroborty etal.,2011).

7.2.6.4EAC cell membrane integrity was measured by performing **Lactate dehydrogenase release assay** (Al-Qubaisi et al.,2011).

7.2.6.5Cellular morphology study was done by polarizing microscopy(Roy et al.,2008).

7.2.6.6Cellular morphology study by scanning electron microscopy (SEM)

Cells were treated with EECGL and WECGL for 24 h. After that cells were fixed with3.7% glutaraldehyde (Merck) for 1 h, followed by serial dehydration in alcohol (50%, 70%, 80%, 90% and 100% for 5 min at each step) and finally subjected in cover slip for critical point drying. Samples were then air dried in vacuumdecicator and mounted on stub. Next, they were placed in vacuum chamber of SEM gold coating apparatus and gold was coated at 2.5 kV, 20–25 mA for 120 s. The morphogram (surface features) of EAC cells were then observed using scanning electron microscope (Carl Zeiss, Germany,EVO18) using 20 kVacceleration voltage.

7.2.6.7 Study on apoptosis

Apoptotic morphology was assessed by performing **chromatin condensation study by PI and DAPI staining**(Prasad and Koch, 2014), **acridine orange–ethidium bromide staining**(Ho et al., 2009), **DNA fragmentation study by agarose gel electrophoresis**(Gong et al., 1994), **mitochondrial membrane potential by Rhodamine 123**(M'Bemba-Meka et al.,2006), **DNA fragmentation study by alkaline comet assay** (Alcantra et al., 2011) and **cell cycle study by flow cytometry** (Nunez, 2001).

7.2.6.8 Detection of apoptosis by Annexin V-FITC Apoptosis Detection Kit

Apoptosis detection was performed using Annexin V-FITC Apoptosis Detection Kit (Abgenex, Cat:1001K). 2×10^6 cells/ml EAC cells were plated and treated with EECGL and WECGL for 24 h. The cells were harvested, washed with PBS, resuspended in 1× Annexin V binding buffer, and stained with Annexin V-FITC and PI for 15 min at room temperature in the dark. Apoptosis was detected using flow cytometer (BD FACSVerse) and Cell Quest software.

7.2.6.9 Western blot analysis

Cells were washed with cold PBS and suspended in RIPA buffer (Sigma-Aldrich) supplemented with Cocktail Protease Inhibitor (SigmaAldrich Co, LLC, US.).Total protein were separated on sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and transferred to PVDF membranes. The membranes were stained with Coomassie blue to visualize the amount of total protein transferred in each lane.To reduce nonspecific binding, membranes were pre-incubated for 2 h on a rocker at room temperature in a blocking buffer containing 5% non-fat dried milk, 5 M NaCl, 20 mMTris-base, and 0.15% Tween-20, pH 8. Membranes were probed using a specific primary antibody against GAPDH (1: 1500

dilution),antiBcl2 (1:2000 dilution), anti-Bax (1:2000 dilution),anti-caspase-3 (1:2000 dilution) diluted in 5% BSA in Tris-buffered saline with Tween-20 (TBST) overnight at 4°C. After being washed three times (5 min each) in TBST, membranes were incubated at room temperature for 1 h with ALP-conjugated suitable secondary antibodies (1:10000) against the primary antibodies. Membranes were washed three times in TBST. Then proteins were visualized after staining with NBT-BCIP buffer. Then the picture were captured by Gel Doc (Bio-Rad). Densitometry of the appropriate sized bands was measured using molecular imaging software (Image J 148-jdk 6 software)(Mandal et al., 2006).

7.2.7*In-vivo* anticancer activities

7.2.7.1 Treatment schedule

Seventy two male mice were divided into sevengroups (n=11).

Group I : Saline Control

Group II : EAC Control

Group III : EAC + EECGL/WECGL (100 mg/kg body weight, i.p.)

Group IV : EAC + EECGL/ WECGL (200 mg/kg body weight, i.p.)

Group V : EAC + 5-FU (20 mg/kg body weight)

Except first group, all group animals were inoculated with 0.1 ml of 1×10^6 EAC cells/mouse intraperitoneally. The second group served as EAC control. After 24 h of tumour inoculation, III and IV groups received respective test extract once daily and the group V received reference drug 5-FU once daily for 14 consecutive days (Haldar et al., 2010). Twenty four

hours after last dose and 18 h of fasting, blood was collected from six mice of each group for the estimation of haematological parameters. Then the animals were sacrificed by cervical dislocation, ascites fluid wasdrawnfrom peritoneal cavity for the study of tumor regression parameters (Gupta et al., 2004) and liver and kidney tissue was collected to performand hepatic and renal oxidative stressparameters..

7.2.7.2 Body weight changes (Maiti Choudhury et al., 2010) and **host survival time** (Jacob and Latha, 2013) were performed.

7.2.7.3Tumor volume (Bala et al., 2010) and **tumor cell count** (Saha et al., 2011) were described previously.

7.2.7.4Red (RBC) and White (WBC) blood cell countwere performed by the method of Wintrobe (Wintrobe,1967).

7.2.7.5 Haemoglobin percentage was determined (Dacie and Lewis, 1958).

7.2.7.6 Determination of**malondialdehyde(MDA) content**, **reduced glutathione (GSH),and activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione s-transferase (GST)**in EAC bearing mouse**.**

MDA content was determined by the method of Ohkawa(Ohkawa et al.,1979). GSH content was measured by the method of Griffith. (Griffith, 1981).The specific activity of SOD was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Marklund and Marklund (Marklund and Marklund, 1974). CAT activity was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H_2O_2 according to the method of Aebi (Aebi, 1974). The activity of GPx was determined by the method of Rotruck et al. with slight modification (Rotruck et al.,1973).The absorbance of the reaction

product was assayed at 412nm.Glutathione-s-transferase (GST) activity was measured spectrophotometrically (Habig et al.,1974).

7.2.7.7Histological study

All the animals from each group were sacrificed for histopathological examinations. Organs such as liver, kidney were collected from all the animals. The collected organs were weighed and preserved in 10% neutral buffered formalin, then dehydrated in alcohols and embedded in paraffin.Five micron thickness of tissue sections were stained with haematoxylin and eosin (H and E) for histopathological study (Standish et al., 2006).

7.2.7.8 Study on peritoneal angiogenesis

After sacrificing the animals on the 15th day the peritoneum of the mice were removed and the inner lining of the peritoneum were observed for angiogenesis. Photographs of the peritoneal linings were captured (Augustine et al., 2014).

7.2.7.9 Immunohistochemistry (IHC) study

Immunohistochemical analysis of Ki-67, CD31 antigens of EAC–induced tumors in mice wasperformed using Ki-67 and CD31 antibodies (Sarkar et al., 2010). Tissue specimens were processed for immunohistochemical analyses. Neutral buffered formalin-fixed tissue was embedded in paraffin. Tissue sections were prepared with a microtome and mounted on glassslides, and immunohistochemical analysis was done within 24 hours. Sections were deparaffinized in xylene, rehydrated with graded alcohols (100%, 95% and 80% v/v) and washed in distilled water. Endogenous peroxidase activity was quenched with 0.01% H_2O_2. Sections for Ki-67 and CD31 analysis were treated further with 0.05% trypsin and 0.05% $CaCl_2$ in Tris-HCl (pH 7.6) for 5 minutes at 37ºC. Antigen retrieval was done by

microwaving the sections in 10 mM citric acid (pH 6.0) for 30 minutes. The slides were washed thrice in PBS and blocked with 2% BSA in TBST for 30 minutes. Tissue sections were then incubated with antiserum to Ki-67 and CD31 (1:50) for 3 hours at room temperature. After being washed thrice with PBS, the sections were incubated with appropriate secondary immunoglobulins (1:500) for 45 minutes at room temperature. The slides were then washed thrice in PBS, labelled with avidin-biotin peroxidase complexes (1:25) for 30 minutes at room temperature and then washed with PBS. Immunoreactivity was determined using diaminobenzidine as the final chromogen. Finally, sections were counterstained with haematoxylin, dehydrated through a sequence of increasing concentrations of alcohol,cleared in xylene and mounted with DPX (Merck).

7.2.8Statistical analysis

All the parameters were performed in triplicate manner. The data was expressed as Mean±SEM, Comparisons between control and treated groups were analysed by using the one-way ANOVA test, $p<0.05$ as a limit of significance.

7.3 Results

7.3.1 EECGL and WECGL induce the *in vitro* cytotoxicity in EAC cells

EAC cells were exposed to EECGL and WECGL at various concentrations for 24 h, and cytotoxicity was determined usingthe MTT assay (Fig. 7.4). The reduction in viability of EECGL and WECGL treated EAC cells occurs in a dose-dependentmanner.The IC_{50} values of EECGL and WECGL in EAC cells were 25±0.56, and 40.98±0.78 µg/ml respectively.EEECGL and WECGL significantly decreased EAC cell viability by 12%, 30%,

50%, 59% and 8%, 22%, 35%, 51% at 5, 10, 25 and 50 mg ml^{-1} doses respectively.On the other hand, no significant decline in lymphocyte viability was noted at the doses of EECGL and WECGL up to 50 µg ml^{-1}.

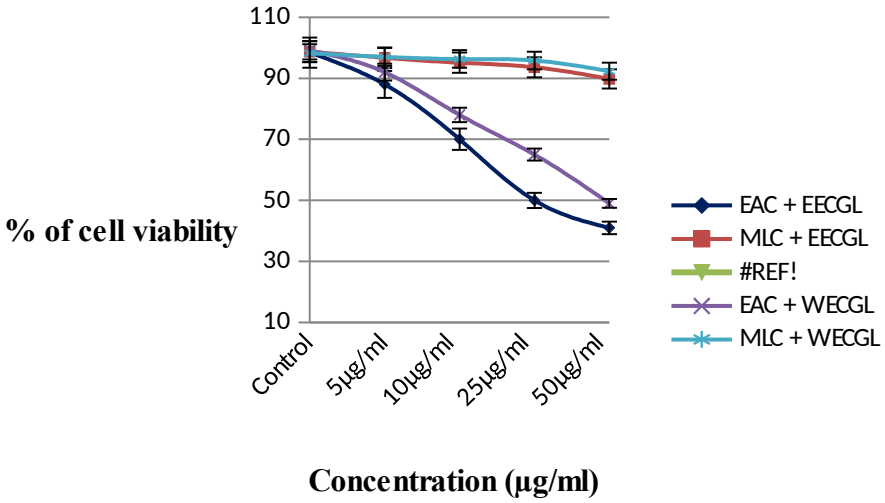


Figure 7.4*In vitro* cytotoxic effects of EECGL and WECGL on EAC cells and MLC. Values are expressed as the Mean± SEM of three experiments

7.3.2 Cellular redox status (GSH levels). The present study displayed decreased GSH content in EECGL and WECGL treated EAC cells at respective significance ($p<0.01$ and $p<0.05$) level (Fig. 7.5).

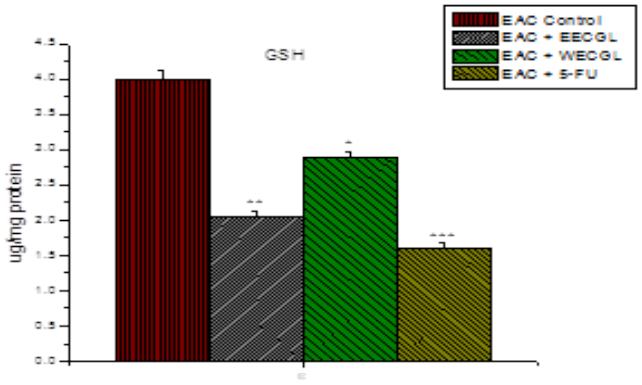


Figure 7.5 Intracellular reduced glutathione (GSH) levels of DLA Control and EECGL and WECGL treated EAC cell. Data are expressed as Mean ± SEM. Probability values are given in asterisks. * indicates p< 0.05, ** indicates p<0.01, *** indicates p< 0.001; values are taken in respect of control.

7.3.3 Intracellular ROS level

Fluorescent microscopic images ofmitochondrial membrane potential (MMP) in EAC control and treated EAC cell have been shown in Fig. 7.6 A. MMP was measured on the basis of rhodamine 123 fluorescence intensity. The fluorescence intensity (Fig. 7.7 A) was decreased significantly (p<0.01 and p<0.05) in EECGL and WECGL treated EAC cells respectively.

In EAC cell, DCF fluorescence intensity due to intracellular ROS generation was enhancedby EECGL and WECGL at their respective IC_{50} doses (Fig. 7.7 B). This result was interrelated by respective fluorescence microscopic images(Fig. 7.6 B).

A

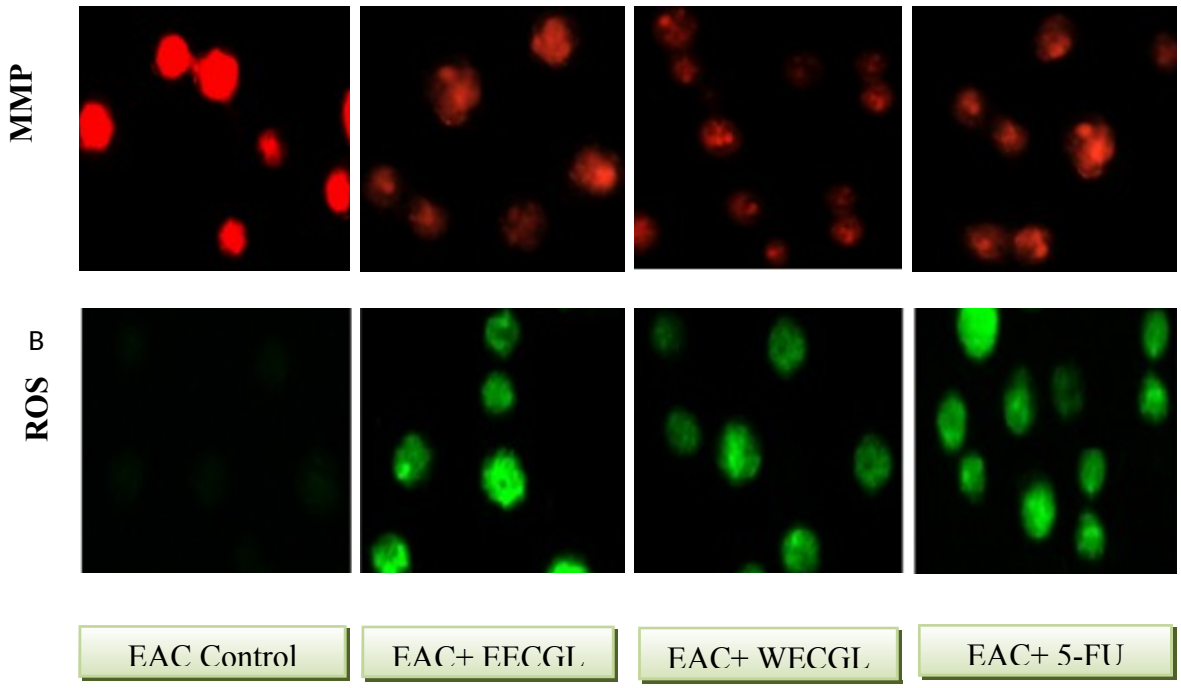


Figure 7.6 (A) Fluorescent microscopic image of EECGL and WECGL treated EAC cells after staining with Rhodamine 123.(B) Fluorescence microscopic image of Reactive oxygen species (ROS) formation by H_2DCFDA staining.

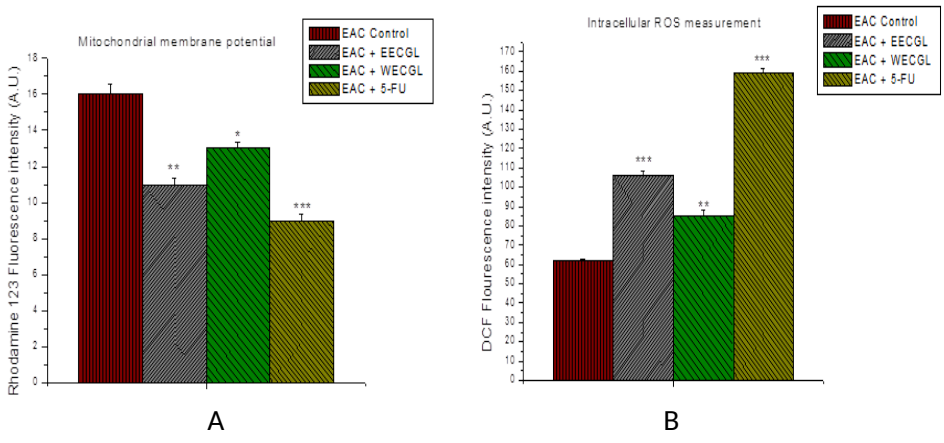
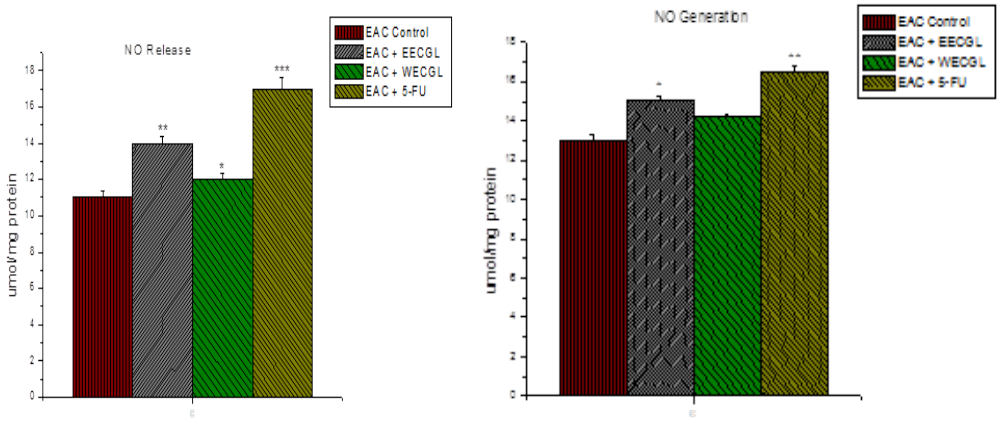


Figure 7.7(A) Effects of EECGL and WECGL on mitochondrial membrane potential in EAC cell. Data are expressed as Mean ± SEM. Probability values are given in asterisks. * indicates p< 0.05, ** indicates p<0.01; *** indicates p<0.001values are taken in respect of control.

(B) Effects of EECGL and WECGL on reactive oxygen species (ROS) induction in EAC cell. Data are expressed as Mean ± SEM. Probability values are given in asterisks. ** indicates p< 0.01, *** indicates p<0.001; values are taken in respect of control.

7.3.4 Nitric oxide release and generation level

Nitric oxide generation and releasewere elevated in EAC cellsafter the treatment of EECGL and WECGL. Increased NO release in EECGL and WECGL treated EAC cells at respective significant ($p<0.01$ and $p<0.05$) level (Fig. 7.8 A) was seen. The NO generationlevel in treated EAC cell was also increased significantly ($p<0.05$) at IC_{50} dose of EECGL (Fig. 7.8 B).



A B

Figure 7.8(A) Nitric oxide (NO) releaseand (B) nitric oxide generation levels of EECGL and WECGL treated EAC cell. Values are expressed as Mean ± SEM of three experiments; Data are expressed as Mean ± SEM. Probability values are given in asterisks. * indicates p< 0.05,** indicates p< 0.01, *** indicates p<0.001; values are taken in respect of control.

7.3.5 Lactate dehydrogenase (LDH) release level

In EECGL and WECGL treated EAC cellsLDH release level was elevated. The LDH level in treated EACcell was significantly (p<0.05) increased at IC_{50}dose of EECGL (Fig. 7.9).

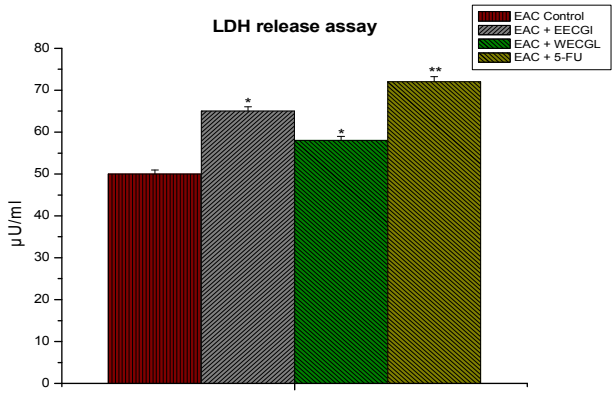


Figure7.9 Effects of EECGL and WECGL on LDH release assay in EAC cell. Values are expressed as the Mean ± SEM of three experiments; Results are expressed as Mean ± SEM. Probability values are given in asterisks. * indicates p< 0.05,** indicates p< 0.01; values are taken in respect of control.

7.3.6 EECGL and WECGL drive apoptosis in EAC cells

Polarizing microscopeand scanning electronmicroscopeeimages confirmed that the formationof typical pattern of apoptotic bodies wasinduced by EECGL and WECGL (Fig. 7.10).

Chromatin condensation was also detected under fluorescent microscopy after PI staining and DAPI staining (Fig. 7.11).Prominent chromatin condensation was noticed in EECGL treated EAC cells compared to WECGL.

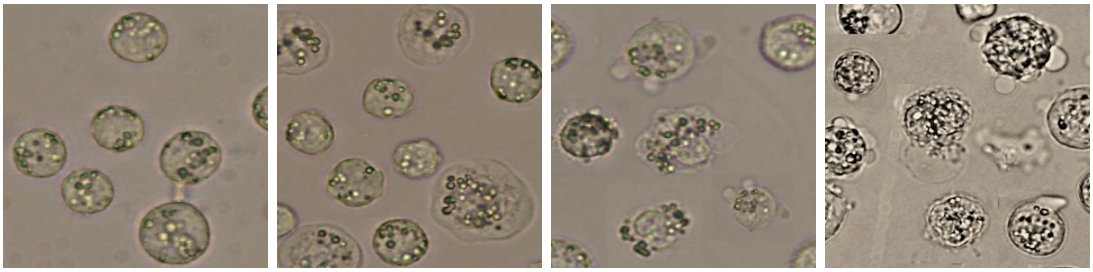
From AO-EtBr staining, the viable cells with intact DNA show a round and green nucleus. Early apoptotic cells with fragmented DNA appear as greenish white -colourednuclei. Late apoptotic and necrotic cell with fragmented DNA are displayed with orange and orange-red nucleus respectively (Fig. 7.12). Most of EECGL and WECGL treated EAC cells showed early and late apoptotic stages after AO-EtBr staining (Fig. 7.12).

Alkaline comet assay showed DNA fragmentation in EECGL and WECGL treated EAC cells (Fig. 7.13 A). The increased percentage of tail DNA intensity was observed in EECGL, WECGL and 5-FU treated EAC cells (Fig. 7.13 B).

The induction of programmed cell death was again confirmed by DNA ladder formation in agarose gel electrophoretic study (Fig. 7.13 C).DNA fragmentation was noticeable in EECGL, WECGL and 5-FU treated EAC cells.

EECGL and WECGL induced EAC cell cycle arrest at the G_2/M phase, as shown in Fig. 7.14.DNA content of treated EAC cells was analysed using PI staining, and cell distributions among sub-G_1, G_0/G_1, S and G_2/M phases were expressed. Results showed increasing accumulation of cells at the G_2/M phase. Accumulation of G_2/M phase cells was significantly greater in cells treated with extract than in control, supporting the results of the MTT cell proliferation assay.

microscopy



ectron microscopy

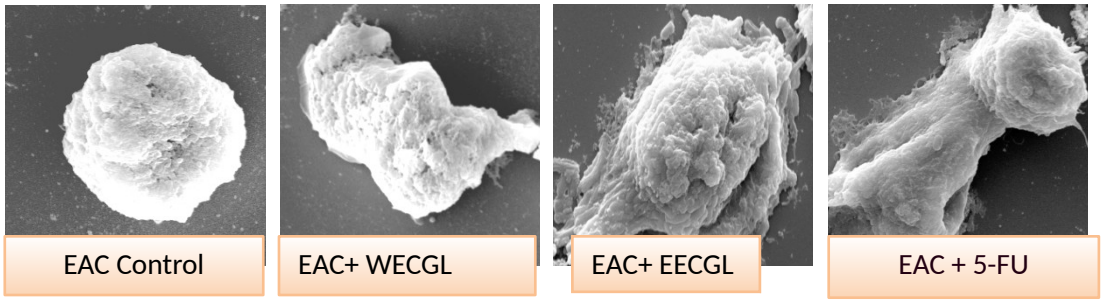


Figure 7.10.Photomicrographs of representative apoptotic EAC cells treated with EECGL and WECGL.

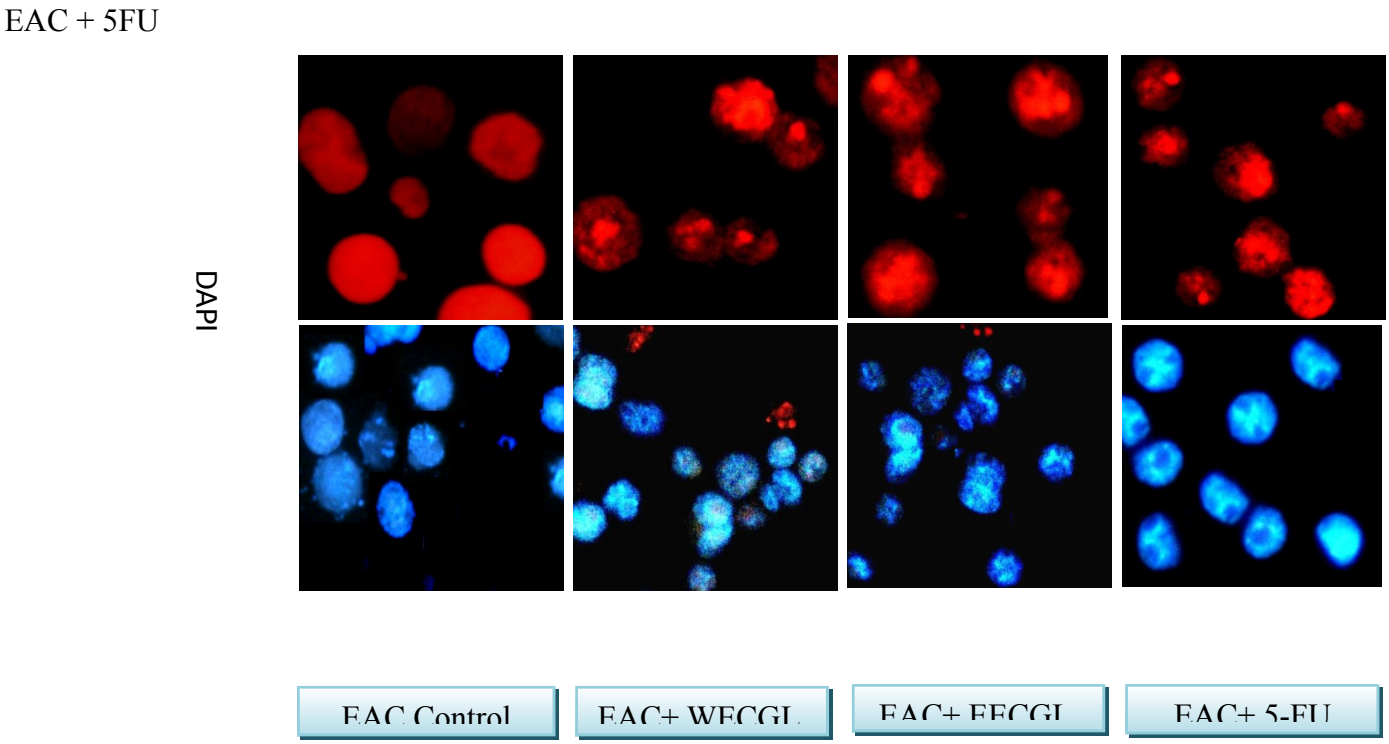


Figure 7.11.Fluorescent microscopic image of chromatin condensation in EAC cells treated with EECGL, WECGL and 5-FU along with EAC control. Cells were stained with PI and DAPI staining.

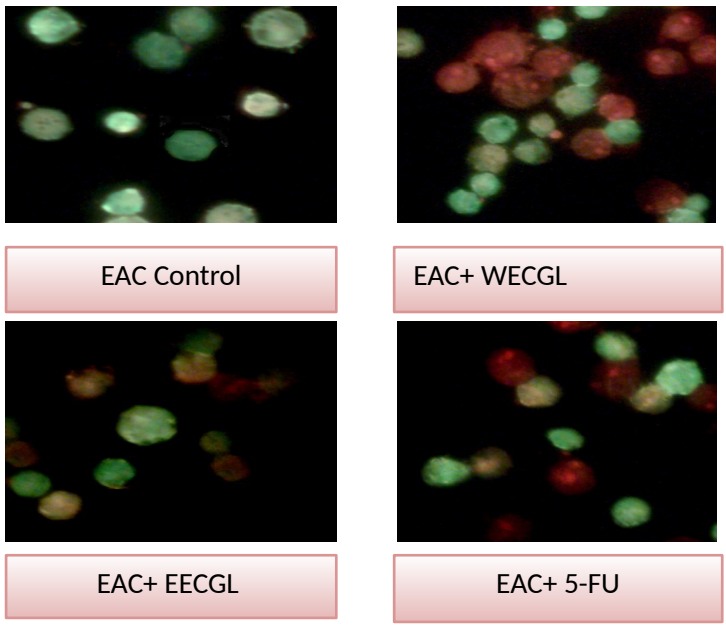
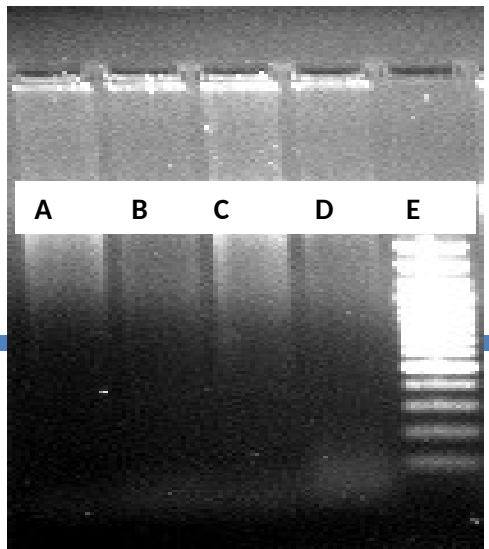
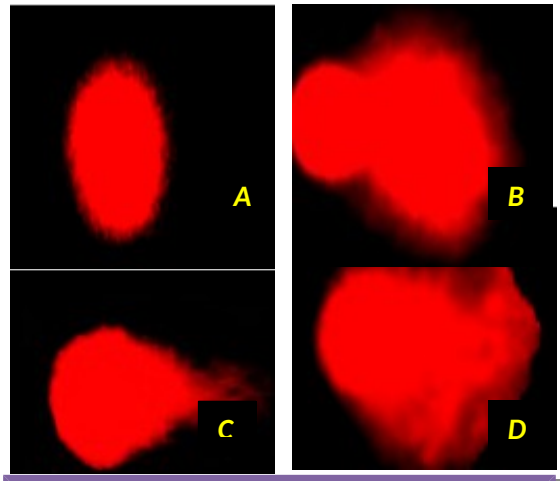


Figure 7.12.Fluorescent microscopic images of AO-EtBr stained EECGL and WECGL treated EAC cells. Staining shows a round and green nucleus with intact DNA in viable cells; early apoptotic cells show fragmented DNA with greenish white-colour nuclei; late apoptotic and necrotic cell represent fragmented DNA with orange and orange-red nucleus respectively.



A



C

A-EAC Control, B- EAC + EECGL, C- EAC + WECGL, D- EAC + 5-FU

A-DNA Ladder, B-EAC Control, C- EAC + EECGL, D- EAC + WECGL, E- EAC + 5-

B

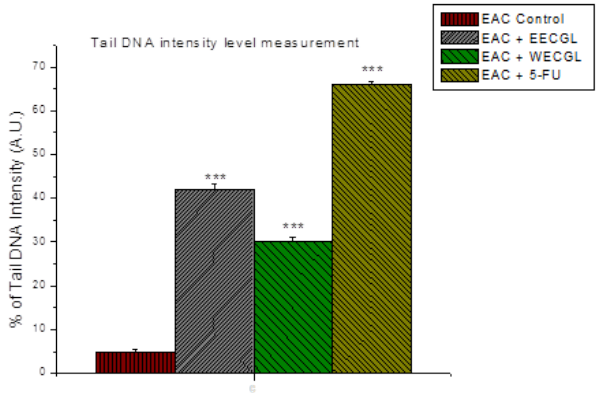
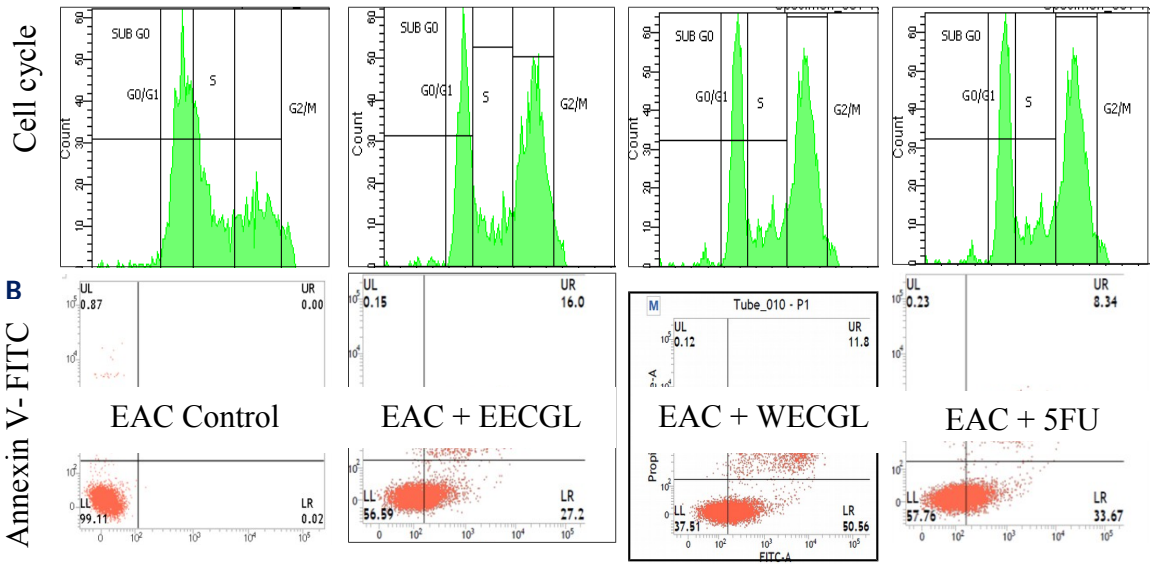


Figure 7.13.(A) Fluorescent microscopic image of DNA damage by alkaline comet assayin EECGL and WECGL treated EAC cells.

(B)The percentage of tail DNA intensity in alkaline comet assay as measured in treated EAC cells. Results are expressed as Mean ± SEM. Probability values are given inasterisks. *** indicates p< 0.001; values are taken in respect of control.

(C) DNA laddering inEECGL and WECGL treated EAC cells.

A



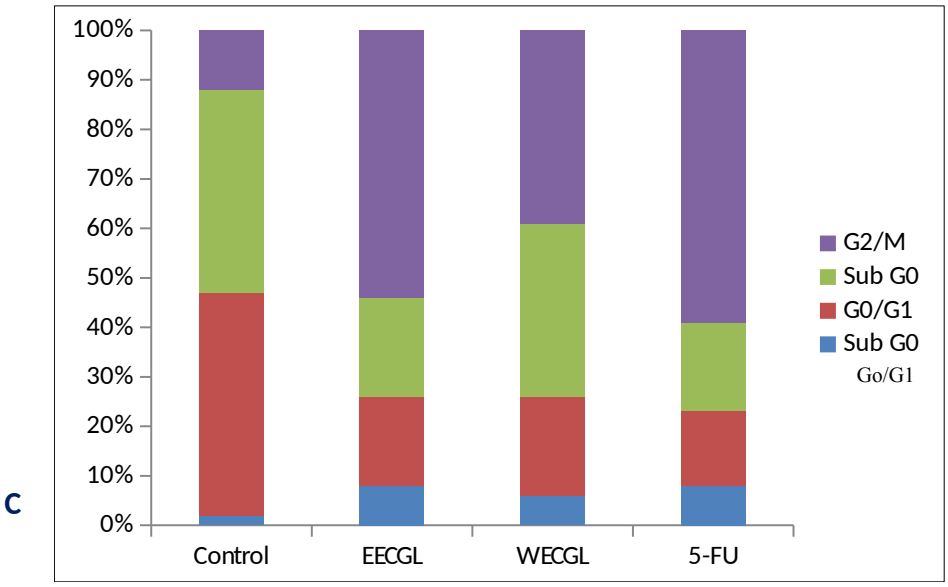
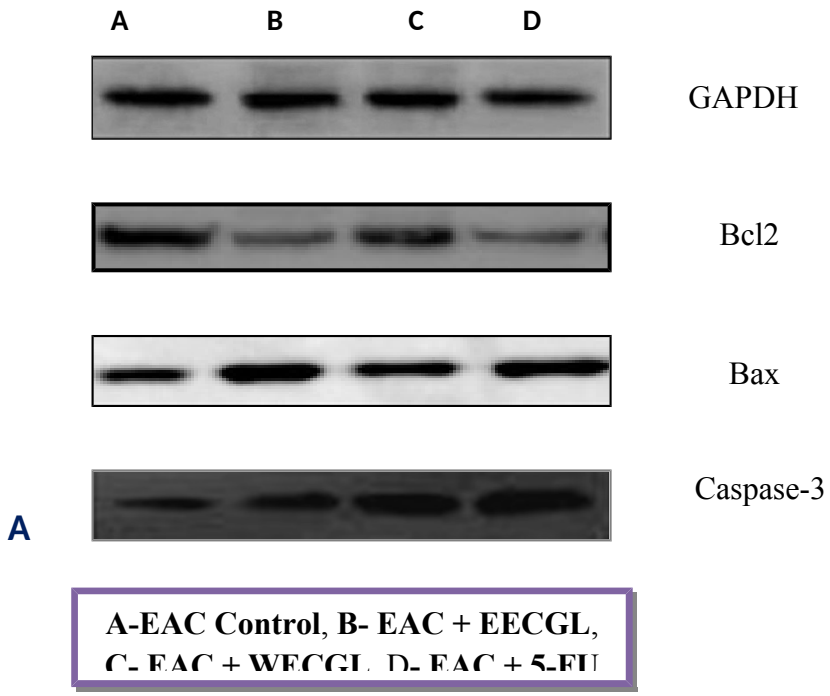


Figure 7.14.Flow cytometer-based apoptosis assay. (A) EAC cells were treated with respective IC_{50} of EECGL and WECGL for 24 h, and stained with PI and measured by flow cytometry.(B) Flow cytometric detection of apoptosis by Annexin V-FITC and PI staining of treated EAC cells. (C) Accumulationof G_2/M cells plotted from the cell cycle-based apoptosis assay. Data are presented as Mean ± SEM from three independent experiments.

7.3.7 EECGL and WECGL treatment increased Bax and caspase-3 expression but decreased Bcl-2 expression

There was an upregulation of pro-apoptotic bax protein, and downregulationofantiapoptotic Bcl-2 protein in EAC cells (Fig. 7.16). An increase in caspase 3expression was also observed, indicating the role of caspase 3 in DNA fragmentation and apoptosis induction in EAC cells.



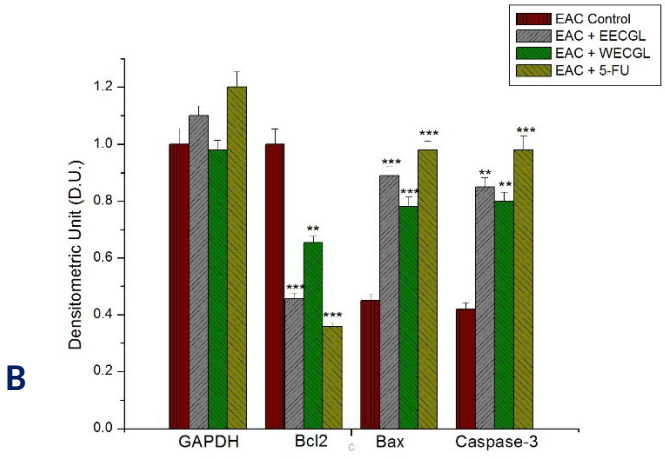


Figure 7.15. Expression of BCl-2, Bax, Caspase-3 in EECGL and WECGL treated EAC cells. Cell lysates were prepared and subjected to Western blot analysis. These proteins were detected by anti-Bcl2, anti-Bax and anti-Caspase 3 antibodies. The results were represented by using Gel-Doc apparatus (Bio Rad) and GAPDH was the internal control. The results were obtained from at least three independent measurements.

7.3.7 Effect of EECGL and WECGL on tumor regression of EAC bearing mice

The result in Fig. 7.17 indicates that control EAC-bearing mice had a gradual increase in body weight from the day zero. When compared to the body weight of control EAC-bearing mice on day 15, the body weight of the treated mice decreased significantly by about 50%, indicating the effect of EECGL and WECGL in preventing the growth of EAC cells.

Inhibition of tumor growth in vivo expressed by Increase life span (ILS) and mean survival time (MST) have been shown in Fig 7.18(A,B) and 7.19 (A,B). In case of EAC control, mean survival time is 15±0.57 whereas with high dose of EECGL (200 mg/kg of body weight), mean survival time is 25 ± 0.62 days indicating 66.66% increase in longevity of the treated group with respect to EAC control. High dose of WECGL (200 mg/kg of body weight), mean survival time is 16.66 ± 0.88 days indicating 11.06 % increase in longevity of the treated group with respect to EAC control EECGL and WECGL have demonstrated enhanced effect on the mean life span by 72.33% and 33.33%.

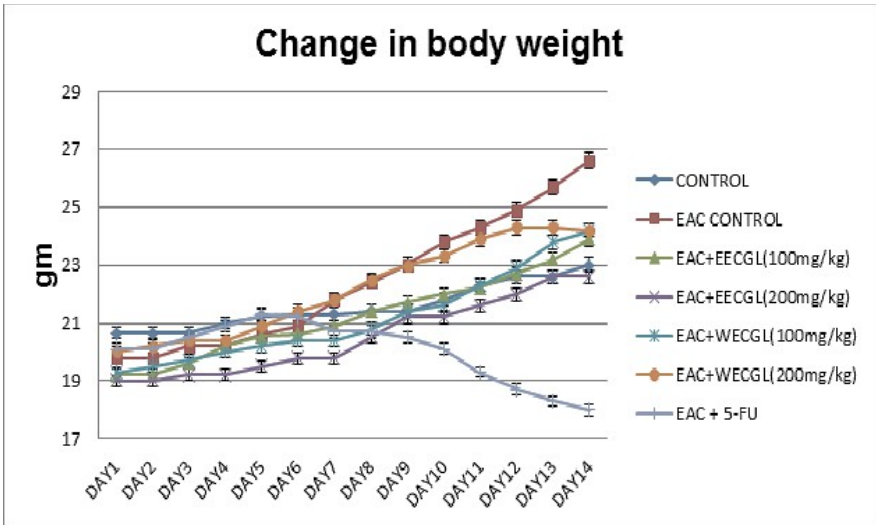


Figure 7.16. The effect of EECGL and WECGL on change of body weight in EAC bearing mice. Data are expressed as Mean± SEM.

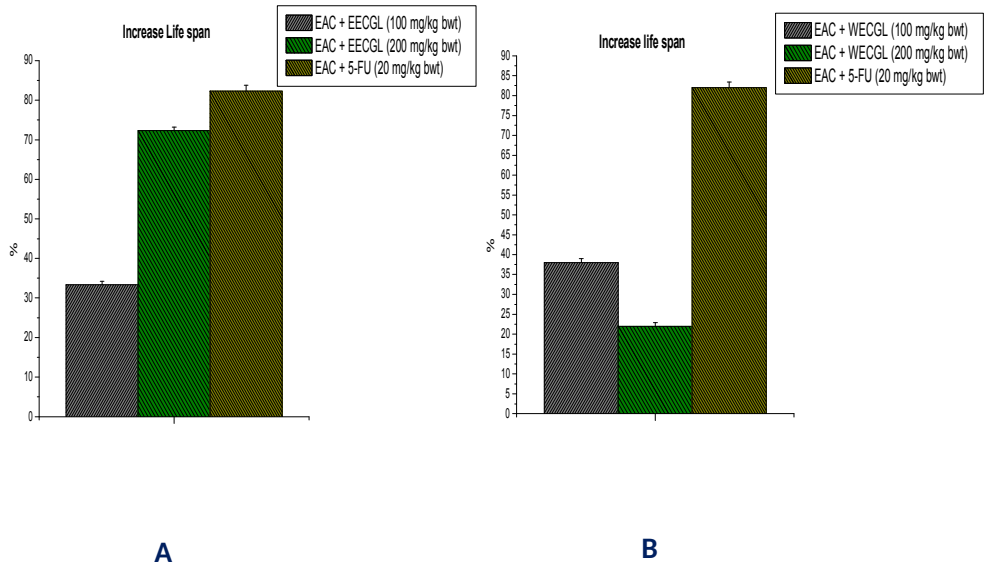


Figure 7.17. The effect of EECGL and WECGL on Increase life span (ILS) in EAC bearing mice. Data are expressed as Mean± SEM.

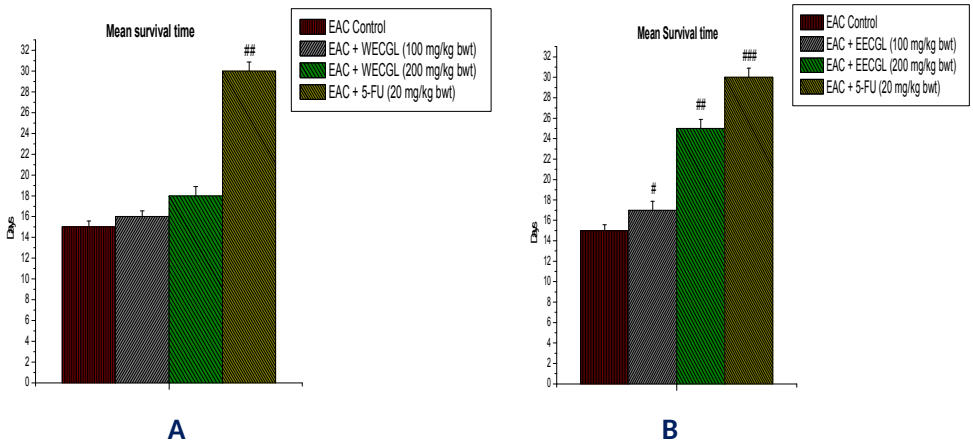


Figure 7.18.The effect of EECGL and WECGL onMean survival time (MST) in EAC bearing mice. Data are expressed as Mean ± SEM. Probability values are given in asterisks. ## indicates p< 0.01, ### indicates p<0.001; values are taken in respect of EAC control.

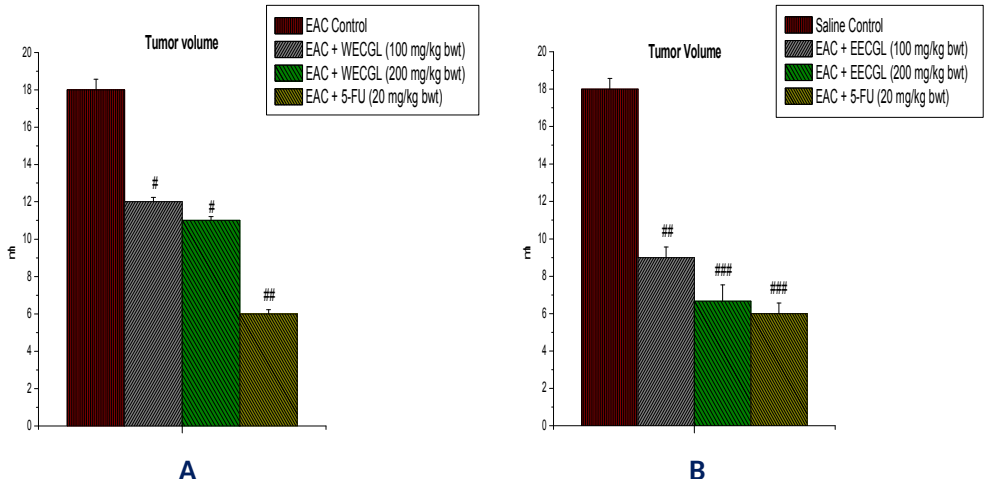


Figure 7.19.The effect of EECGL and WECGL onTumor volume in EAC bearing mice. Data are expressed as Mean ± SEM. Probability values are given in asterisks. # indicates p< 0.05,## indicates p< 0.01, ### indicates p<0.001; values are taken in respect of EAC control.

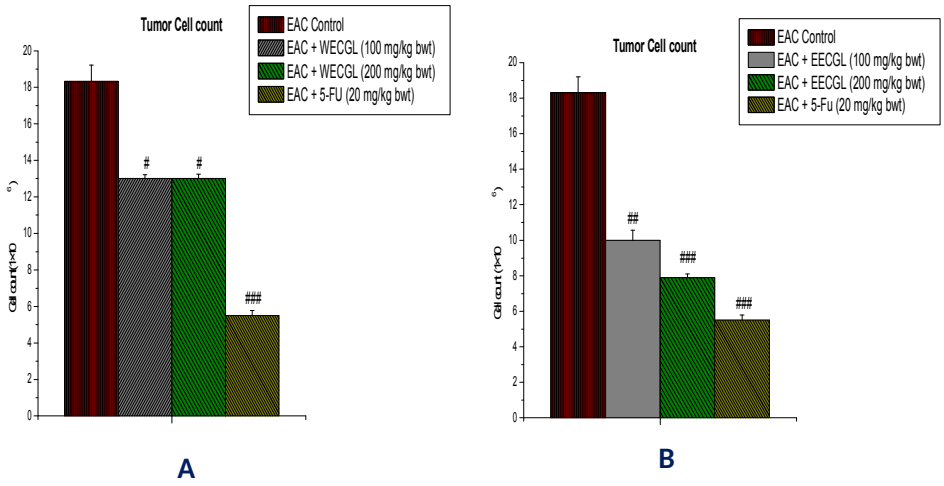


Figure 7.20. The effect of EECGL and WECGL on Tumor cell count in EAC bearing mice. Data are expressed as Mean ± SEM. Probability values are given in asterisks. # indicates p< 0.05, ## indicates p< 0.01; values are taken in respect of EAC control.

7.3.8 Effect on haematological parameters

Haematological parameters of EAC-bearing mice on day 15 were found to be significantly changed from normal (saline control) group (Table 7.1 and 7.2). Haemoglobin content and RBC count in the EAC control group was significantly ($p<0.01$)and($p<0.001$) decreased in comparison to the normal group. EECGL and WECGL at100 and 200 mg/kg body wt. dose levels increasedthe haemoglobin content and RBC counts significantly. The total WBC counts were found to be increased significantly in the EACcontrol group ($p<0.001$). Administration of EECGL and WECGL at the above said doses reduces theWBC counts.

Table 7.1 Haematological parameters after EECGL administration on EAC bearing mice

Haematological Parameters	Saline control	EAC Control	EAC + EECGL (100mg/Kg bwt.)	EAC + EECGL(200mg/Kg bwt.)	EAC + 5-FU (20 mg/Kg bwt.)
Hb Percentage	**15±0.577**	**9.66±0.33 ****	**11.87±0.233##**	**12.43±0.145##**	**13.3±0.29##**
Total RBC Count (cu.mm)	**6.23±0.145**	**1.83±0.066*****	**4.8±0.115#**	**5.2±0.057##**	**5.6±0.057###**
Total WBC Count(cu.mm)	**5100±58**	**9600±153*****	**8667±115 ##**	**8200±88 ##**	**5767±88 ###**

Table 7.2Haematological parameters after WECGL administration on EAC bearing mice

Haematological Parameters	Saline control	EAC Control	EAC + WECGL (100mg/Kg bwt)	EAC + WECGL(200mg/Kg bwt)	EAC + 5-FU (20 mg/Kg bwt)
Hb Percentage	**15±0.577**	**9.66±0.33 ****	**10.87±0.154##**	**11.53±0.21##**	**13.3±0.29##**
Total RBC Count (cu.mm)	**6.23±0.145**	**1.83±0.066*****	**4.1±0.034###**	**4.9±0.057##**	**5.6±0.057###**
Total WBC Count(cu.mm)	**5100±58**	**9600±153*****	**8845±120 ##**	**8340±88 ##**	**5767±88 ###**

Data are expressed as Mean ± SEM. Probability values are given in asterisks and #, ** indicates p< 0.01, *** indicates p< 0.001; values are taken in respect of saline control.# indicates p< 0.05, ## indicates p< 0.01, ### indicates p< 0.001; values are taken in respect of EACcontrol

7.3.9 Effect on antioxidant parameters

MDA contents were significantly ($p<0.001$) increased in EAC control animals compared to that of normal saline control animals. After treatment with EECGL at the dose levels of 100, 200 mg/kg body wt MDA level was decreased significantly ($p<0.05$) in kidney and liver it was decreased significantly ($p<0.01$) at the dose of 200 mg/kg (Fig 7.22). In case of WECGL, MDA level was reduced significantly ($p<0.05$) in kidney and liver compared to EAC control group.

Prominent ($p<0.001$) decrease in liver and kidney GSH level was observed in EAC control animals. The treatment with EECGL at the dose levels of 100and 200 mg/kg body wt reversed GSH level to near normal values in the liver and kidney samples (Fig 7.23).

WECGL also restored these values towards the normal status; same as at the dose level of 200mg/kg body wt.($p<0.05$) decrease in the activity of liver and kidney superoxide dismutase (SOD) was observed of EAC control animals.WECGL at both dose levels (Fig 7.24) normalized the SOD activity to a good extent ($p<0.05$) compared to EAC control.

The treatment with EECGL at 100 and 200 mg/kg body wt. doses reversed these changes to near normal values in the liver and kidney of treated mice (Fig 7.24). EECGL at 200 mg/kg body wt. dose level was found to be more potent.

Catalase (CAT)activity was significantly ($p<0.001$) decreased in EAC control animals compared to normal control animals. After treatment with EECGL at the dose levels of 100 and 200 mg/kg body wt., CAT activity was increased significantly ($p<0.01$) and($p<0.001$) in the kidney and liver respectively (Fig 7.25). After treatment with the dose levels of 100, 200 mg/kg of WECGL CAT activity was increased significantly ($p<0.05$) in the kidney and liver compared to EAC control.

The activity of glutathione peroxidase (GPx) was significantly ($p<0.001$) decreased in EAC control animals when compared with normal control animals. After treatment with EECGL, GPx activity increased significantly ($p<0.05$) in the kidney but more pronouncedly ($p<0.01$)in the liver at 200 mg/kg dose level (Fig 7.26). In case of WECGL, GPx activity was increased significantly ($p<0.01$) and ($p<0.05$) in the kidney and liver compared to EAC control at 200 mg/kg body wt dose.

Prominent ($p<0.001$) decrease in liver and kidney GST activity was also seen in EAC control animals. The treatment with EECGL at the above mentioned dose levels showed significant changes ((Fig 7.27). After treatment with WECGL, GST activity was increased significantly ($p<0.01$) in the kidney and livercompared to those of in EAC control group.

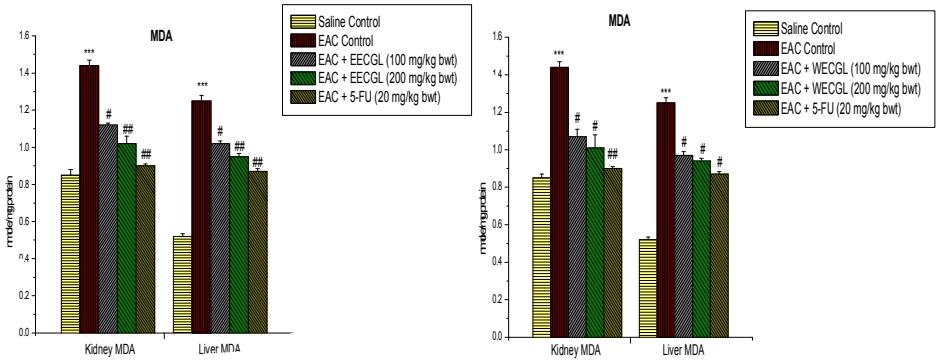


Figure 7.21. The effect of EECGL and WECGL on liver and kidney malondialdehyde in EAC bearing mice. Data are expressed as Mean± SEM; Probability values are given in asterisks and #.'***'represents significant difference at ($p<0.001$) compared to saline control; # indicates $p< 0.05$, ## indicates $p<0.01$; ### indicates $p< 0.001$; values are taken in respect of control.

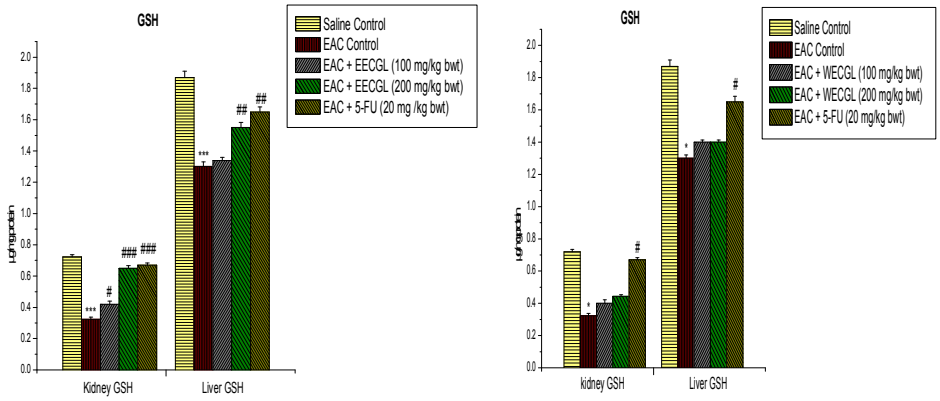


Figure 7.22Liver and kidney reduced glutathioneshows the effect of EECGL and WECGL in EAC bearing mice. Data are expressed as Mean± SEM; Probability values are given in asterisks and #.'*' 'represents significant difference at ($p<0.05$) compared to saline control '***'represents significant difference at ($p<0.001$) compared to saline control; #

indicates p< 0.05, ## indicates p<0.01; ### indicates p< 0.001; values are taken in respect of control.

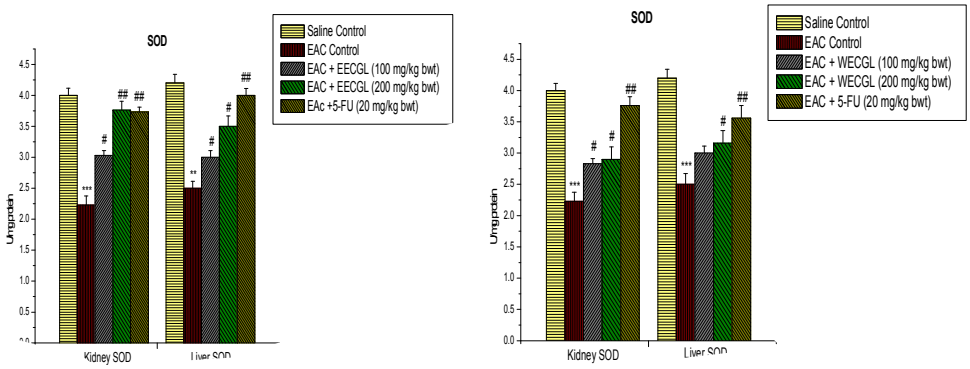


Figure 7.23. The effect of EECGL and WECGL on liver and kidney superoxide dismutase in EAC bearing mice. Data are expressed as Mean± SEM; Probability values are given in asterisks and #. '***'represents significant difference at (p<0.001) compared to saline control; # indicates p< 0.05, ## indicates p<0.01; values are taken in respect of control.

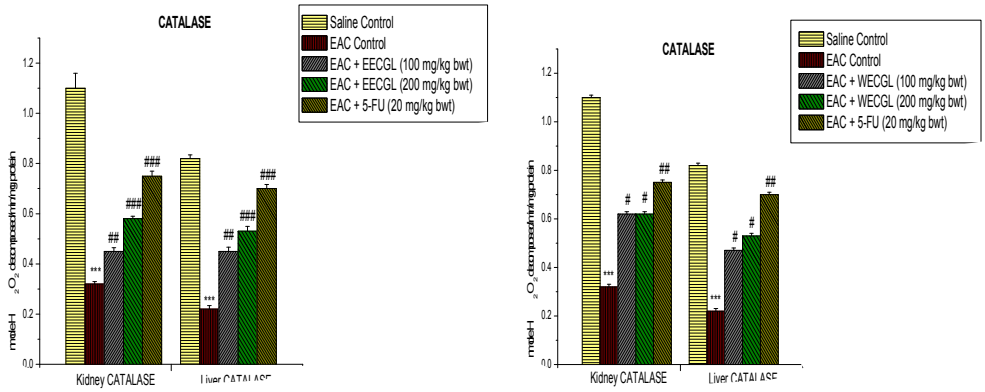


Figure 7.24. The effect of EECGL and WECGL on liver and kidney Catalase in EAC bearing mice. Data are expressed as Mean± SEM; Probability values are given in asterisks and #. '***'represents significant difference at (p<0.001) compared to saline control; #

indicates p< 0.05, ## indicates p<0.01; ### indicates p< 0.001; values are taken in respect of control.

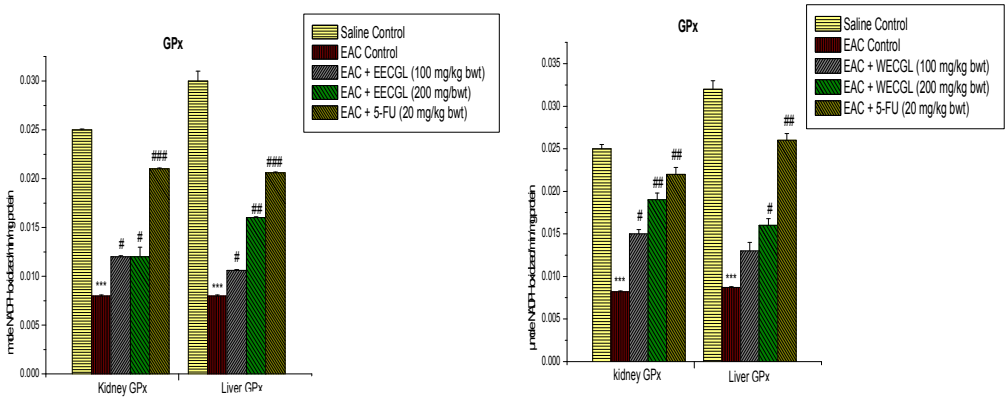


Figure 7.25.The effect of EECGL and WECGL on liver and kidney glutathione peroxidase in EAC bearing mice. Data are expressed as Mean± SEM; Probability values are given in asterisks and #. '***'represents significant difference at (p<0.001) compared to saline control; # indicates p< 0.05, ## indicates p<0.01; ### indicates p< 0.001; values are taken in respect of control.

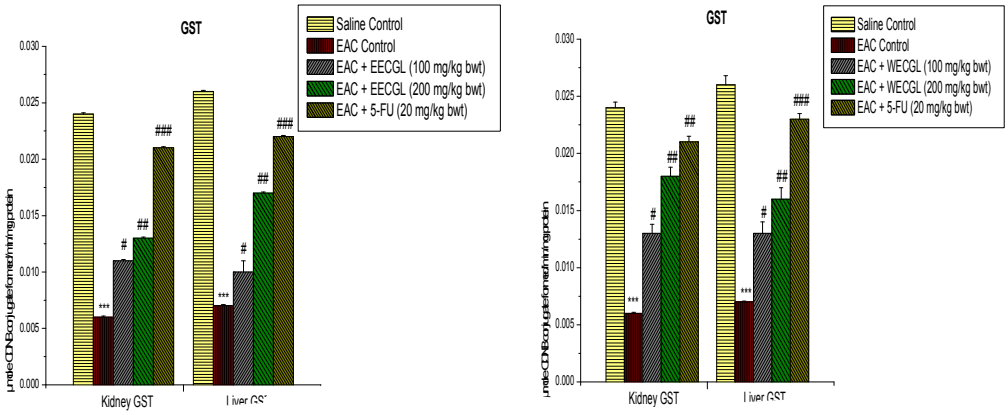


Figure 7.26. The effect of EECGL and WECGL on liver and kidney glutathione peroxidase in EAC bearing mice. Data are expressed as Mean± SEM; Probability values are given in asterisks and #. '***'represents significant difference at ($p<0.001$) compared to saline control; # indicates $p< 0.05$, ## indicates $p<0.01$; ### indicates $p< 0.001$; values are taken in respect of control.

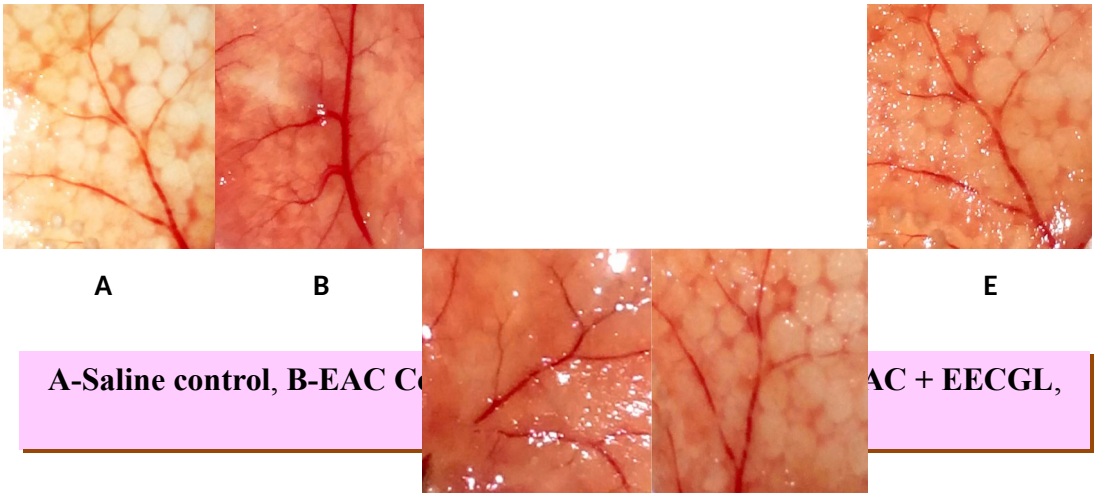
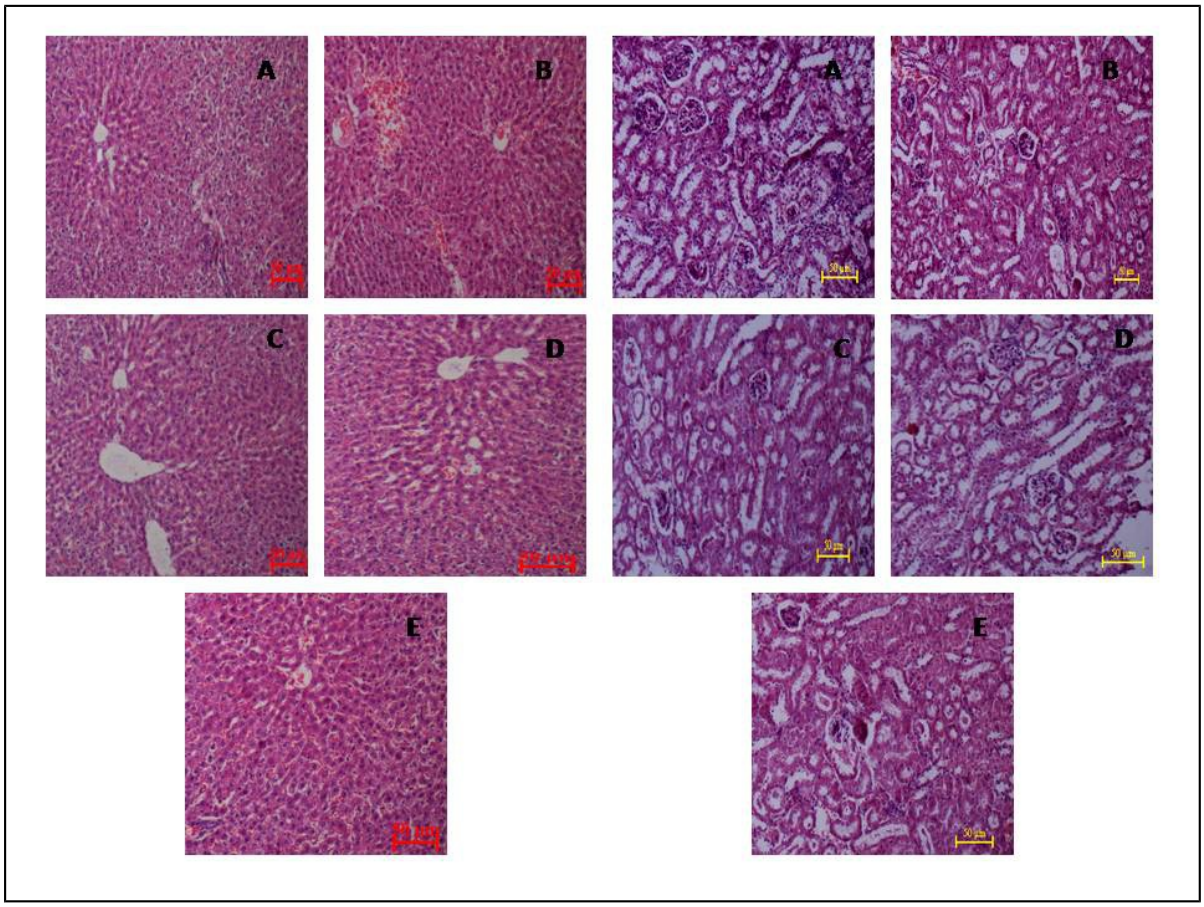


Figure 7.27. Effect of EECGL and WECGL on peritoneal angiogenesis. The figures (A–E) represent the inner peritoneum lining ofuntreated, treated and standard drug treated EAC induced animals, respectively.



A-Saline control, **B-EAC Control**, **C- EAC + WECGL**, **D- EAC + EECGL**, E- **EAC + 5-FU**

Figure-7.28.Hematoxylin and eosin staining (H & E) in mice liver and kidney of control and experimental groups of mice for histopathological analysis.

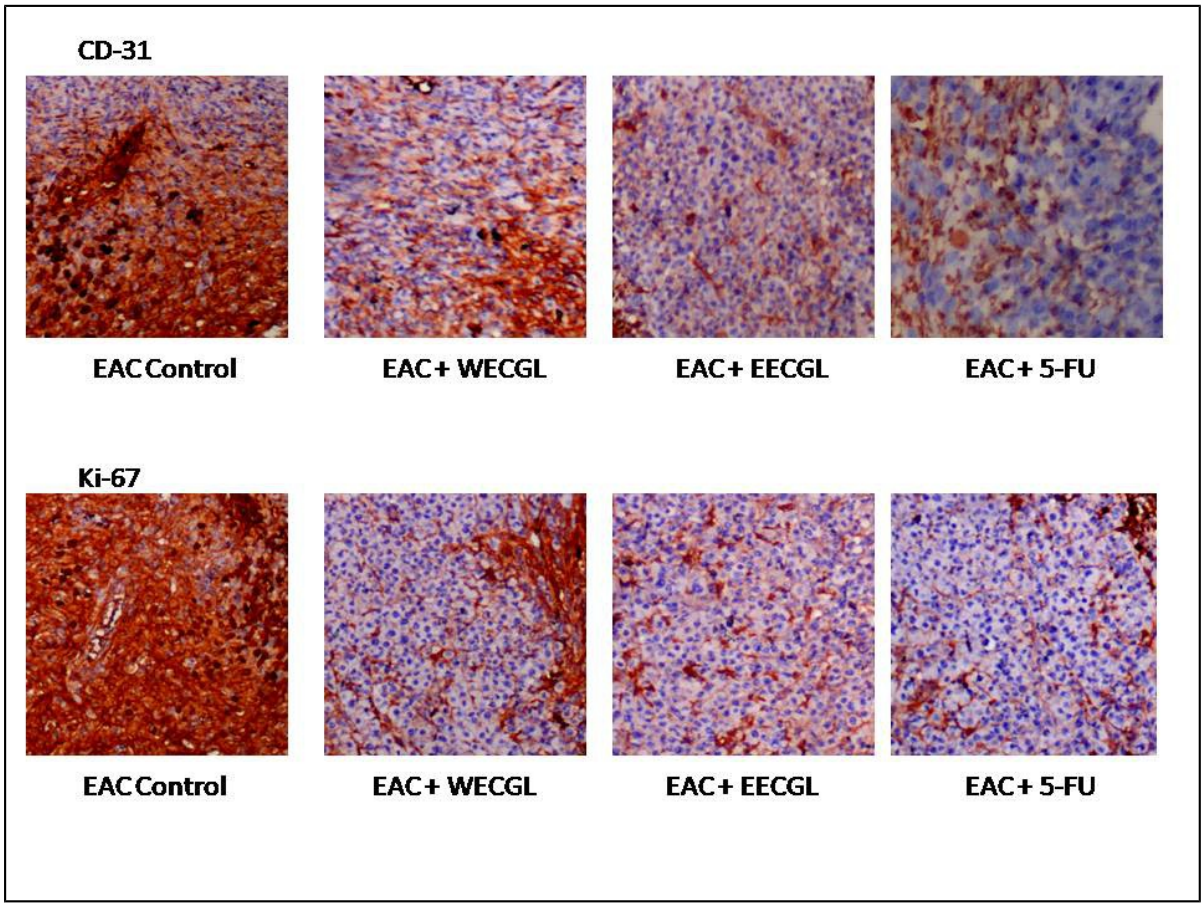


Figure 7.29.Antineoplastic activities of EECGL and WECGL in EAC-induced solid tumors in a mouse model. Immunohistochemical analysis of EAC-induced solid tumors from mice treated with EECGL and WECGL. Paraffin-embedded sections of tumors are processed and immunohistochemical assays of Ki-67 and CD-31 are performed. Bar 20 µm.

7.4 Discussion

In the present chapter,in vitro cytotoxic, antioxidant, apoptotic, antiangiogenic and antineoplastic potential of ethanol (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex were investigated against Ehrlich ascites carcinoma (EAC)cells in *in vitro* and *in vivo* experimental conditions. The above said effects of *Calotropis gigantea* latex extracts onEAC cellshave been demonstratedhere.

EECGL and WECGL exert there in vitro cytotoxic effects against EAC cells showing IC_{50}values of 25 and 40.98 µg/ml respectively.

Glutathione is an important intracellular reductant and dealswith the protection against free radicals, peroxide and toxic compounds. Quite a lot ofpathological conditions markedly reduce cellular GSH concentrations by non-enzymatically oxidization of GSH to glutathione disulfide (GSSG) caused by oxidative stress (Grăvilă et al., 2010). Decreased levels of GSH might be asa result of the effective conversion of GSH to GSSG by increasedfree radicals induced by ECGL and WECGL in EAC cells.

Diminution of mitochondrial membrane potential is another marker of apoptosis-induced cell death (Adrie et al.,2001). The present study showed that EECGL and WECGL decreased mitochondrial membrane potential as compared to EAC control. Alteration of mitochondrial membrane potential in extracts treated lymphoma cells may result in malfunction in ATP synthesis and maintenance of the ATP level that leads to either apoptosis or necrosis. Likewise, the results indicate that a significant ($p<0.01$) increase of ROS level is found due to EECGL exposure (Fig. 7.6). The fluorescence images are highly correlated with the fluorescence intensity level. As there is increasing evidence that ROS at elevated levels act as critical signaling molecules in the induction of apoptosis induced by many different

stimuli (Carmody and Cotter,2001; Yang et al.,2006), studies were performed to determine if EECGL and WECGL-induced cytotoxicity occurs via an apoptotic pathway. The results presented in Fig. 7.6 B provide strong evidence that induce apoptosis in cancer cells with the production of ROS. Collectively, these studies indicate that the primary mechanism of cytotoxicity may go on by EECGL and WECGL inducing the generation of ROS, which then underlies the induction of apoptosis.Therefore, it is possible that EECGL and WECGL-induced changes in the electron transport chain result in increased ROS formation.

The free radical, NO is an intra- and inter-cellular messenger (Jaffrey and Snyder, 1995). NO reacts with superoxide to form more toxic peroxynitrite (ONOO), which has important tumoricidal functions. The excess production of NO shows massive oxidative injury . In the study, a higher level of nitrite was released and generated in EECGL-treated carcinoma cells owing to the high production of free radicals, mainly NO.LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged (Burd et al.,1973). To investigate the anticancer mechanism of EECGL and WECGL, the cytotoxicity was assessed using the LDH release assay. EECGL and WECGL were found to disrupt the membrane integrity of the lymphoma cells and thereby increase LDH release.

Formation of apoptotic bodies and bluish/orange condensed chromatin in DLA cells after treatment with EECGL and WECGL at IC_{50} doses clearly indicated the apoptotic properties of EECGL and WECGL in EAC cells.During apoptotic condition, chromatin undergoes a phase change from a heterogeneous, genetically active network to an inert highly condensed form that is fragmented and packaged into apoptotic bodies.Fluorescence image of EAC

cells stained by PI and DAPI confirmed that EECGL and WECGL have the ability to inhibit the cell proliferation via apoptosis. Treatment of EECGL and WECGL exhibited typical apoptotic morphology (Fig. 7.12) with condensed nuclei in treated EAC cells compared to EAC control. As membrane integrity became compromised and PI and DAPI stain leaked into intact membrane, even in shrunken cell, the apoptotic nuclei appeared bright pink chromatin and bright blue chromatinthat are highly condensed and fragmented (Bortner and Cidlowski,1998).

Staining cells with fluorescent dyes, including acridine orange and ethidium bromide, is used in evaluating the nuclear morphology of apoptotic cells. To corroborate that apoptosis has been induced by EECGL and WECGL, EAC cells were analysed in the presence of acridine orange and ethidium bromide staining (AO/EB staining). Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity (Jayadev et al.,2004). Cells stained green represent viable cells, whereas yellow staining represented early apoptotic cells, and orange staining represents late apoptotic cells, reddish staining represents necrosis cells.

The increase in the tail DNA (%) can be as a result of direct induction of DNA strand breaks or disruption of DNA backbones by compounds or its byproducts, such as free radicals (Yang and Schaich, 1996).

Formation of DNA fragmentation is one of the characteristic features observed in apoptotic cells and it is generally considered as hallmark of apoptosis (Fink and Cookson, 2005). The formation of a DNA ladder correlates with the early morphological signs of apoptosis (Ariffin et al.,2009). The ladder-like appearance of DNA observed in EECGL and WECGL treated EAC cells, was also another indicator of the apoptosis inducing capability of the latex.

Several phytochemicals have been reported to inhibit the cancer cellgrowth through cell cycle arrest In the present study it was found that EECGL and WECGL could induce G_2/M phase arrest in EAC cells. The G_2/M phase cell cycle arrest serves to prevent the cell from entering mitosis. Mitosis is regulated by Cdc2 and binding to cyclin B. This result suggests that DNA damage may extrude cyclin B1 from the nucleus, which promotes G_2/M phase arrest.(Aggarwal et al., 2004).

Annexin V-FITC binding is a major marker of apoptosis since phosphatidyl serine translocation to the cell surface precedes nuclear breakdown, DNA fragmentation (Radhika et al.,2010). The intensive staining on the cell membrane surface resulted after the treatment with EECGL and WECGL prove that EECGL possess highest apoptotic potential (59.27% early apoptosis) than WECGL.

Changes in Bax/Bcl-2 ratio and activation of caspase cascade have been reported to be caused by downregulation of Bcl-2 with upregulation of Bax. In this investigation, it is found that Bax expression was significantly elevated in EECGL and WECGL treated EAC cells while Bcl-2 expression depleted.

We further confirmed the anticancer activity of *C. gigantea* by treatment of EAC bearing mice with EECGL and WECGL extracts. The body weight of the EAC induced mice gradually increases with passage of time due to accumulation of ascitic fluid in the belly. Hence change in weight is considered as a parameter to determine the intensity of the disease. EECGL and WECGL at 200 mg/kg caused significant decrease in body weight of the animal, respectively, while in the untreated group of animals the increase in weight was about 32g.Prolongation of mean life span of the animals was seenin EAC bearing mice after administration ofEECGL and WECGL at 100 and 200 mg/kg b wt. In EAC bearing mice peritoneal fluid is the direct nutritional source for tumor cells (Prasad and Giri, 1994).

Treatment with EECGL significantly reduced the tumour volume probably by lowering the ascitic fluid volume. Decrease in EAC cell count in EECGL and WECGL treated group of animals indicates that the extracts are having *in vivo* anti proliferative activity.

The tumor control group was marked by increase inWBC count and decrease in RBC count. It is found from our results that treatment with EECGL and WECGL extract could restore the haematological parameters in the treated groups. In this case it can be postulated that due to progression of cancer, WBC levels increased in the EAC induced animals. On treatment with the EECGL and WECGL, the WBC and RBC levels were significantly restored. It was observed that the level of WBC severely raised up for the EAC induced untreated group of animals whereas the RBC count and hemoglobin count of these group drastically dropped down as compared to normal animals. Interestingly, EECGL and WECGL could restore the haematological parameters of the EAC induced mice.Treatment with EECGL significantly reduced the tumour volume and body weight increase probably by lowering the ascitic fluid volume.

It is reported that implication of free radicals occurred in carcinogenesis (Player, 1982).The increased level of lipid peroxidation may be due to the inactivation of antioxidant enzymes in cancerous tissues. A significant decrease in MDA levels by EECGL and WECGL treatment indicated their role in reducing oxidative stress, thus indicating its protective potential against EAC cell.

Reduced glutathione (GSH) shows a key role in cell defense mechanisms by acting as an antioxidant or by reacting with many toxic agents to form conjugates that are eliminated from the cell (Deleve and Kaplowitz,1991). The observed depletion of GSH level in the EAC bearing mice cells may suggest its involvement in the scavenging of excess free radicles in tumor cells.

Body's antioxidant defense systems operate for scavenging ROS to prevent the oxidative stress and antioxidant enzymes, namely, SOD and CAT acts as the first line of cellular defense against oxidative damage (Ferreccio et al.,1998). EECGL and WECGL administration decreased the activities of these antioxidant enzymes in the liver and kidney tissue, which may be related to saturation of SOD with superoxide radicals in tumor cells or a decrease or loss of expression of SOD. A decrease in SOD activity will result in decreased production of H_2O_2 which in turn affects CAT activity.

The present data showed significant decrease in the activities of GPx and GST in mice liver and kidney after EECGL and WECGL exposure, resulting in considerable decline in the activities of these enzymes. Thus, decrease in GPx activity may be implicated in both free radical dependant inactivation of enzyme and depletion of its co-substrates, i.e., GSH and NADPH. The observed decrease in GPx activity may also be due to reduced availability of GSH. A significant restoration in the activities of these enzymes after EECGL and WECGL administration indicated that they act as an effective antioxidant.

The histopathological results that revealed the EAC hosts had loss of increased lymphoid aggregation and architecture in the liver, glomerulous alteration in the kidney. But in the case of EECGL and WECGL treated mice liver and kidney showed a reduction in hepatocyte necrosis and glomerulous alteration respectively. Liver and kidney section of EAC cell induced mice showed structural alteration in nucleus. Histopathological observations in EAC bearing mice showed severe damage in hepatocytes.The kidney histopathology micrograph of EAC induced mice showed marked tubular and glomerular damage. This damage is partially reversed by EECGL and WECGL extract treatment.

The present study evaluated the effect of EECGL and WECGL on cell proliferation and apoptosis by Ki-67 (proliferation) andCD-31(angiogenesis), respectively. The results reveal significant reductions in of Ki-67 and CD-31compared to the untreated controls (Fig. 7.30). Control tumors contain hypercellular areas with a high expression of Ki-67 (Das et al.,2012).CD31 is highly expressed on tumor tissues in a general state of angiogenesis (Yang et al.,2008). This suggests that the extracts have antiangiogenic properties.

7.5 Conclusion

In conclusion, the present study demonstrated that EECGL and WECGL could inhibit the *in-vitro* proliferation of EAC cell and suppressEAC tumor growth *in-vivo*.*Invitro*ROS generation, LDH release assay, flow cytometric analysis indicated that EECGL and WECGL primarily induced apoptosis in the cancer cells.Moreover, the extract, through morphological analysis using AO/EB staining procedures, showed that the extract was able to trigger EAC cell death through apoptosis. EECGL and WECGL induced caspase-dependent apoptosis via up-regulation of Bax, Caspase-3 of the intrinsic apoptotic pathwayand down-regulation of Bcl-2. The present study demonstrated that the EECGL and WECGL increased the life span of EAC tumor bearing mice and it enhanced the antioxidant status and reduced the lipid peroxidation. Therefore, the results from this study provided important experimental facts to suggest that *C. gigantea* latex extracts may be therapeutic agents for treating cancer.

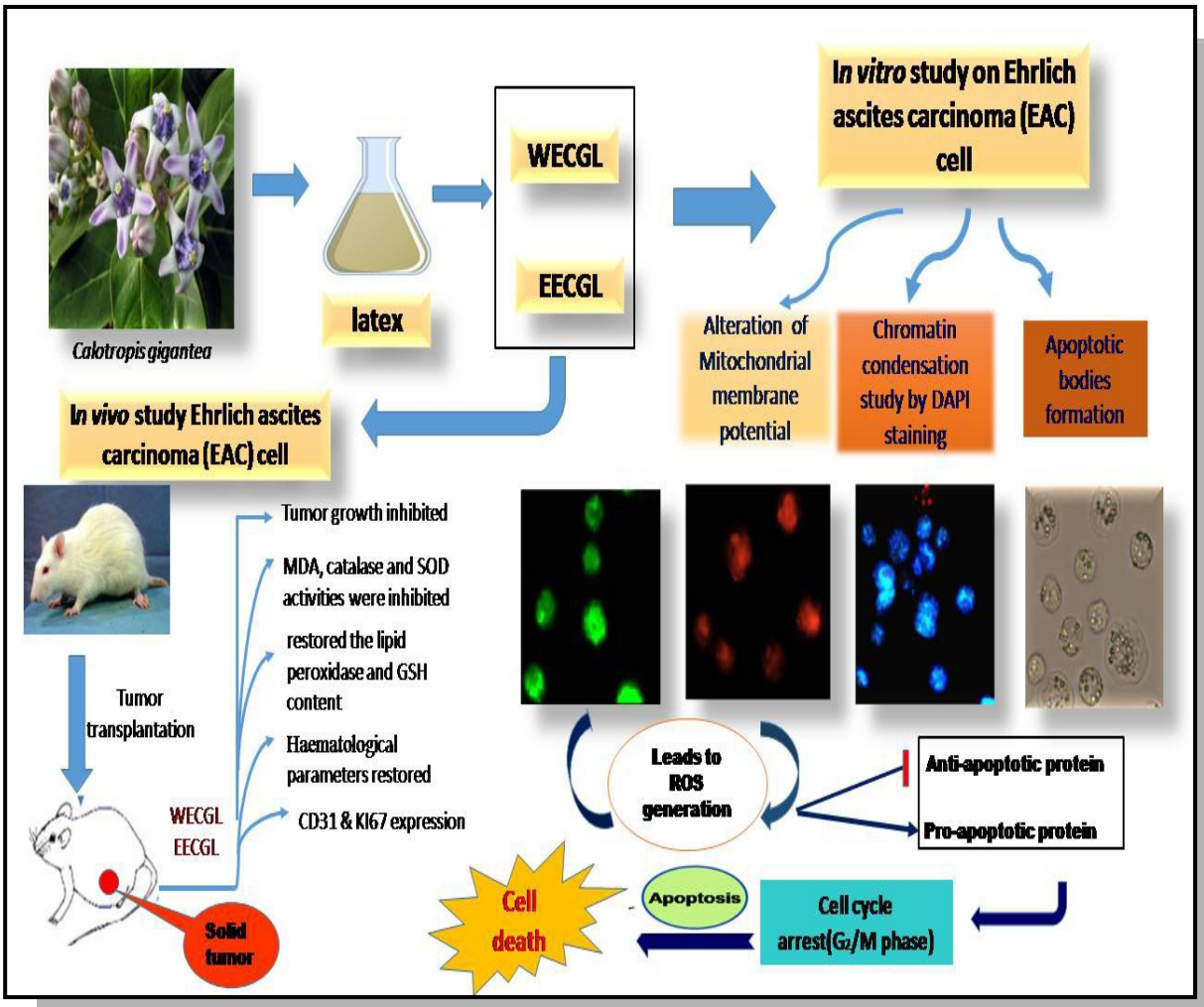


Figure 7.30. Mechanistic view of antineoplastic activity of *Calotropis gigantea* latex

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