Chapter 5 Evaluation of *in-vitro* cytotoxic and apoptosis-inducing properties of *Calotropis gigantea* Linn. latex in Jurkat cells and antimitotic potential in *Allium cepa* root tip cells.

Abstract

Since antiquity plants have been used as a source of therapeutic agents and are playing an important role in the indigenous system of medicine to fight against diseases. The present study carried out to evaluate the *in-vitro*cytotoxic activity of ethanol (EECGL) and water (WECGL) extracts *Calotropis gigantea* Linn. latexon human acute T cell leukemia producing Jurkat cells and also to investigate the antimitotic potential of the extracts in *Allium cepa* root tip cells.

EECGL and WECGL produced cytotoxic effects on Jurkat cells at 25 and 50µg/ml (Fig. 5.1). The IC_{50} values of EECGL and WECGL for Jurkat cells were 416±0.57, and 50±0.98 µg/ml respectively producing no significant reduction in cell viabilityin human lymphocytes up to the dose level of 50µgml^{-1}. In Jurkat cell, DCF fluorescence intensity due to intracellular ROS generation was elevated by EECGL and WECGL at their respective IC_{50} doses.The extract caused pronounced chromatin condensation after PI and DAPI staining and DNA fragmentation. *C.gigantea* latex extracts were found to be antimitotic in *Allium cepa* root tip cells at 100mg/ml. EECGL treated group showed significantly less number of dividing cells compared to the control group. The mitotic index of EECGL, WECGL treated group and control group were 27%, 47% and 57% respectively. These findings also reveal that *Calotropis gigantea* latex extracts exhibit *in-vitro*cytotoxic and apoptotic potential against human acute T cell leukemia producing Jurkat cells and pronounced antimitotic potential in *Allium cepa* root tip cells.

Saswata Sanyal, Pralay Maity, Ananya Pradhan, Madhubanti Bepari and Sujata Maiti Choudhury*.*Calotropis gigantea linn.* latex:phytochemical screening

,antimitotic potential in *Allium cepa* root tip cells and in-vitro cytotoxicity sarcoma-180 cells. World Journal of Pharmacy and Pharmaceutical Sciences. 2016; 5:1180-1193.

5.1 Introduction

Cancer is a multi-mechanistic second leading disease in theworld and needs a multidimensional approach for itstreatment, control and prevention. Plant based drugs, forms animportant constituent of total medicines available for handlingvarious human diseases. The practice of phytochemicals in cancer prevention has received considerable interest in the past few decadesdue to certain discoveries with specific properties including anti-oxidant, anti-inflammation and apoptotic ability.

5.1.1 Leukaemia

Leukaemia states to a group of acuteor chronic malignant diseases that comprise the bloodformingorgans, characterized by an anomalous proliferation, differentiation, and disproportionate production of whiteblood cells and their precursors in the bone marrow, withor without a corresponding enhancement of those in thecirculating blood. Though theswift growth of advanced technologies and new discoveries in the diagnosis andtreatment of leukemia have increased the survivingrateofleukemiapatientsmore likely than in the past, an estimated 67,870 deaths will result from blood cancer in this year 2016(Leukemia Research Foundation,2016). Due to this new approaches have been made to discover novel anti-leukemic compounds from phytomedicine.

Jurkat cells

Jurkat cells are an immortalized line of human T lymphocyte cells. The cells are used to study acute T cell leukemia, T cell signaling, and the expression of various chemokine receptors. The primary useofJurkat cells, however, is to determine the mechanism of differential susceptibility of cancers to drugs and radiation.

The Jurkat cell line (originally called JM) was established in the late 1970s from the peripheral blood of a 14-year-old boy with T cell leukemia.

Antileukaemic phytochemical compounds

Many novel components are reported, among them few are documented here;curcuminhave the ability to induce cell death in two leukemic celllines: K562 and Jurkat cells (Duvoix et al., 2003). Crude plant extract prepared from*T. welwitschii*exhibitsanticancer activity against Jurkat T cells(Moyo B, Mukanganyama S, 2015). Ethanol extract of aerial parts of *Convolvulus arvensis*was found to have cytotoxic effect against lymphoblastic leukemia, Jurkat cells(Saleem et al., 2014). The drugs vinblastine and vincristine, isolated from *Catharanthusroseus,*have significant antiproliferative and cytotoxic role against blood cancer (Glass et al. 2003, Gaines, 2004).

5.1.2 Mitosis

Mitosis is a vital process in actively multiplying cells, resulting in the division of replicated sets of chromosomes and two genetically identical daughter cells. Failure in cellcycle checkpoint regulation frequently leads to aneuploidy and genetic instability, resulting in either cell death or cancer.In the same manner, cancer defines abnormal, deregulated cellsthat undergo unrestricted divisions (Kastan and Bartek, 2004). In spite of being the shortest phase of the cell-cycle, mitosis organizes major changes in multiple cellular components.Signalling

pathways are activated in a complex way and hushed onsignals, and timely prompting of protein degradation processesleads to gross and activerearrangement of the cell construction.Hence, it is considered to be the most delicatephase of the cellcycle,during which it is highly subjected to cell death whenexposed to various attacks (Rieder and Maiato, 2004). Damages sustained by any kind of cellular stress activate the spindle assembly checkpoint (SAC), which breaks progression and assures a prolongedmitotic arrest. Such breaks are prospective to signal induction of adeath program, known as mitotic cell death (MCD), and now it isusually expected as an antiproliferative strategy for the development of chemotherapeutic agents against different types of cancer.

Microtubules are produced during interphase and are important for correct chromosome segregation and cell division undergoing mitosis. Microtubule dynamics is more rapid during mitosis in comparison to interphase, and thus microtubules are anperfect drug target since cancer cells havehyperproliferative activity (Dumontet and Jordan, 2010).On the basis of their mechanism of actions, antimitotic drugs are mainly assorted into microtubule-stabilizing drugs such aseribulin, spongistatin, rhizoxin and Microtubule-destabilizing drug such as vinflunine, vincristine, vinorelbine, vindesine etc (Liu et al., 2014). Antimitotic drugs inhibit polymerization dynamics ofmicrotubules by stimulatingthe spindle assembly checkpoint (SAC) blocking transitionfrom metaphase to anaphase (Masawangetal., 2014).Afterward, cells undergo mitotic arrest and since the compound interrupts in spindle formation and chromosome alignment, cells remain either in a prolonged arrest state with subsequentapoptosis induction or in a senescence-like G_1 state (Mitchison,2012).

5.1.2 Antimitotic drugs from phytochemicals and their antimitotic potential on *Allium cepa*

Several new highly active natural products have been introduced and their therapeutical potential for anti-cancer treatments is testedover the last decade.Different types of anticancer drugs are available, among them antimitotic drugs are used frequently (Edelman, 2006). Among the antimitotic agents plant-derived compounds constitute a major class of cytotoxic drugs such as paclitaxel, vincristine, and combretastatinetc (Iwasaki,1993). A compound isolated from flower of *P. juliflora* exerts clastogenic, antimitotic and cytotoxic effect, tested on*Alliumcepa* root tip cells (Singh,2012). Ethanol extract of the stem bark of *Streblusasper* possess antimitotic, cytotoxic and antitumor activities (Alamgiret al.,2013). *In vitro* antimitotic and genotoxiceffect of cucurbitacin enriched extract of *T. dioica*root was also assessed using *Allium cepa*root meristems (Bhattacharya andHaldar, 2012).Several extracts of *R.hypocrateriformis*showed antimitotic activities on *Allium cepa*and cytotoxic potential on MCF-7, HCT-15, MOLT-4 cell lines (Saboo et al., 2013). Hence *Allium* has been proven as a rapid, reliable, and inexpensive toolto evaluate antimitotic effects of various chemical compounds (Leme and Marin-Morales, 2008). Because of its homogenous meristematic cells, very large chromosomes and only sixteenchromosome numbers, the *Allium cepa*sspecies is ideal for use in bioassays(Havey et al., 2002). It is widely used fordetection of cell cycle block, cytotoxic and mutagenic properties ofdifferent compounds, including anticancer drugs of plantorigin (Kura's, 2006). The results obtained by *Allium* test could be useful incorrelating the antimitotic effect with mammalian test systems (Fiskesjo, 1997).

Recently, a number of anti-cancer agents have become considerable therapeutices in the clinical settings which include: vinca alkaloids, taxols, camptothecin,podophyllotoxin, and its derivatives. The plant *Calotropisgigantea* is a well-known a common wasteland weed, grows widely throughout the Indian subcontinent, which has been proved for many pharmacological properties and its roots and leaves are used traditionally for the treatment of abdominal tumours. Therefore, this plant latex was selected for screening of its anticancer activity in *in*

*vitro*human cancer cell line as well as in plant cells. Hence, the present chapter was designed to evaluate the *in vitro* cytotoxic and apoptotic potential of ethanolic (EECGL) and water (WECGL) extract of *Calotropisgigantea* latex in Jurkat cells and also to explore the antimitotic activity of the latex extracts in *Allium cepa* root tip cells.

5.2 Materials and methods

5.2.1 Chemicals

RPMI 1640, Penicillin, Streptomycin (Sigma Aldrich Co, LLC, US), Fetal bovine serum (GIBCO), Dimethyl sulfoxide (DMSO), Sodium dodecyl sulphate (SDS), Propidium iodide, 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-tetrazolium bromide (MTT) reagents, 5-fluorouracil,2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA),propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), agarose, Tris buffer, ethidium bromide (EtBr), Titron X-100, sodiumdodecyl sulphate (SDS), phenol, chloroform, iso-amyl alcohol, ammonium acetate, RNAse, Acetorcine, Hydrochloric acid, acetic acid wereand other chemicals were purchased from Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

5.2.2 Collection of plant material and preparation of extracts (Maity et al., 2015)

The method has been discussed in chapter-2.

5.2.3. Cell lines

Jurkat cells were obtained from National Center for Cell Science (NCCS), Pune. Jurkatcells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), and antibiotic, antimytotic solution (100 U ml^{-1} penicillin, 10 mg ml^{-1} streptomycin and 4 mM L-glutamine) under 5% CO_2 and 95% humidified atmosphere at 37˚C in an incubator.1×10^6 ml^{-1} viable cells were used for different experiments in the present study.

The red variety of *Allium cepa* was obtained from local market. The bulbs of *Allium cepa* were sprouted for 3 to 4 day in water saturated sand tub at room temperature and the developed root tip cells were used for the study of antimitotic activity.

5.2.4 Isolation of human lymphocyte cell (HLC)

Blood samples were collected from healthy individual for the separation of lymphocytes. The lymphocytes were isolated from heparinized blood samples according to the method of Hudson and Hay. About 3 ml of blood were layered onto same amount of Histopaque 1077 (Sigma-Aldrich Co. LLC, US) and centrifuged at 2000 rpm for 30 min at room temperature. The upper buffy coat layer containing lymphocytes was transferred to a clean centrifuge tube and washed three times in phosphate buffer (PBS). The mice lymphocytes (MLCs) were resuspended in RPMI complete media supplementedwith 10% FBS and incubated for 24 h at 37 ºC in a 95% humidified and 5% CO_2 atmosphere in a CO_2 incubator.

5.2.5 Experimental design of *in vitro*anticancer study on Jurkat cells

Jurkat (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. Control cells did not receive anyabove said extract exposure.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 werewashed with phosphate buffer saline (PBS, pH 7.4). A required amount ofcells was lysed and then processed for biochemical estimations.

5.2.5.1 *In-vitro* cytotoxic study

Jurkat, human lymphocyte cells (1×10^6 per well) were plated in 96 well plates and then the cells were incubated after the treatment of EECGL and WECGL for 24 h at 37˚C. Then

washing with PBS (pH 7.4), 5 mg ml^{-1} of MTT in phosphate-buffered saline solution were added in each well. After 3 h of incubation, DMSO was added to each well. Viable cells were measured at the absorbance of 540 nmon ELISA Analyser (Bio-Rad, Model 680)(Mosmann, 1983).

5.2.5.2 Measurement of intracellularreactive oxygen species (ROS) (Roy et al., 2008)
The method has been discussed in chapter-3.

5.2.5.3 Chromatin condensation by propidium iodide and DAPI staining

Jurkat cells (1×10^6cells ml^{-1}) were seeded inpetriplateand incubated with EECGL and WECGL for 24h at 37°C and 5% CO_2. The cells were then fixed by 70% ethanol incubation at -20°C for 2 hours. After fixation, cells were washed and stained with DAPI at 37°C for 5 minutes and RNAse-propidium iodide (PI) mixture (1mg ml^{-1}) at 37°C for 15 minutes. The cells were washed with phosphate buffer and observed under fluorescence microscope (LEICA, Germany) (Prasad and koch,2014).

5.2.5.4DNA fragmentation study by agarosegel electrophoresis

At first, 1×10^6 ml^{-1}Jurkatcells were subcultured in the culture medium and incubated for 24 h at 37˚C in a humidified 5% CO_2 atmosphere and EECGL, WECGL and standard drug 5-FU were then added to culture medium for 24 h. After 24 h cells from treated groups were centrifuged at 1200 rpm to obtain the pellet containing both intact and apoptotic cells. Before overnight lysis, lysis buffer was mixed to the cell pellet. After 16 h, RNAse solution was added to it and incubated at 37 °C for 2 h. DNA was then isolated by phenol–chloroform-isoamyl alcohol extraction method. DNA in the aqueous phase was collected and precipitated by 7.5 M ammonium acetate and ice cold dehydrated ethanol, then incubated overnight at -20^0C and DNA was dissolved in TE buffer (pH 7.4). The total DNA solution on DNA electrophoresis was performed in 1.5% agarose gel containing 1 μg ml^{-1}ethidium bromide at

75 volt, and DNA fragments were visualized by exposing the gel to UV light and image was captured by Gel-doc apparatus (Biorad) (Gong et al., 1994).

5.2.6 Antimitotic activity

5.2.6.1 Study of mitotic index

The bulbs of *Allium cepa*were sprouted for 3 to 4 day in water saturated sand tub at room temperature. The developed roots were treated by dipping these in the ethanolic (EECGL) and water extract (WECGL) of *C.gigantea*latex at the concentration 100mg/ml for 24 hours. The treated dipped root tips were separated by cutting and were transferred at room temperature to aceto-alcohol fixing solution (1:3v/v) for 1 h. Then the root tips were preserved in 70% alcohol in refrigerator. At the time of staining, the preserved root tips were immerged in 2% acetorcine and 1N HCl and boiled at the smearing point. The root tips were placed on grease-free clean slide and 2% acetic acid was added. Then covered with cover slip, the root tips were squashed to prepare smear and observed under microscope. The cell-numbers in each stage of cell division were counted in different microscopic fields of for each group(Pardesi et al.,2008).

5.2 Results:

5.3.1. EECGL and WECGL showed *in vitro*cytotoxicityonJurkat cells

EECGL and WECGL exhibited ***in vitro*** **cytotoxic effects** onJurkat cells at the concentrations of 25 and 50µg/ml (Fig. 5.1). The IC_{50} values of EECGL and WECGL for Jurkat cells were 41.6 ± 0.57, and 50±0.92µg/ml respectively. On the other hand, no significant reduction of cell viability in human lymphocytes was noted by EECGL and WECGL up to the dose level of 50 µg ml^{-1}.

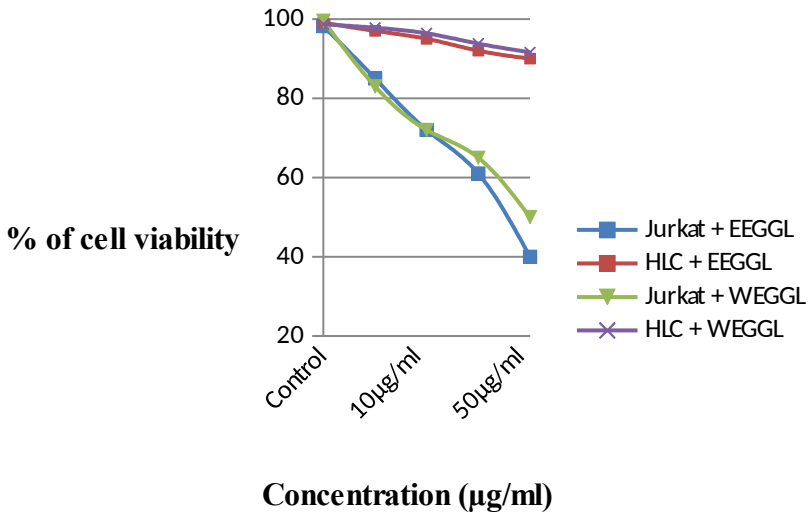


Figure 5.1*In vitro* cytotoxic effects effect of EECGL and WECGL on Jurkat cells and HLC. Values are expressed as Mean ± SEM of three experiments.

5.3.2 Intracellular ROS level

In Jurkatcell, DCF fluorescence intensity due tointracellular ROS generation was elevated by EECGL and WECGL at their respectiveIC_{50}doses(Fig. 5.2). These wereprominently seen in respectivefluorescence microscopic images.

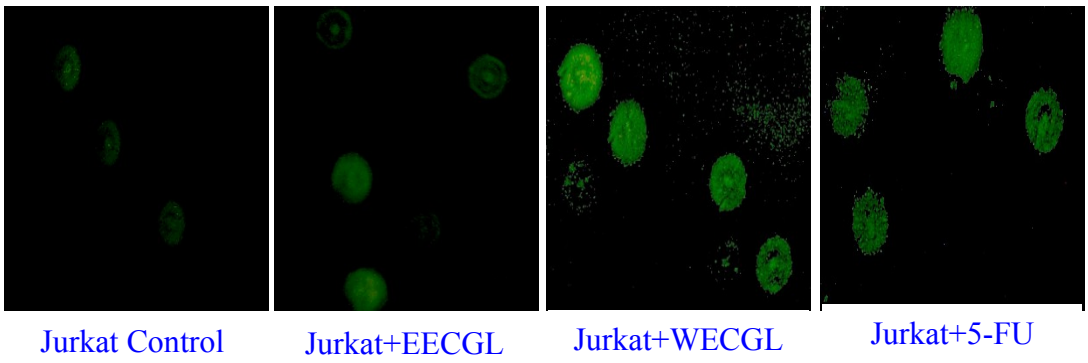


Figure 5.2 Fluorescent microscopic image of intracellular ROSgeneration by H_2DCFDA staining.DCF fluorescence intensity was expressed in term of ROS production.

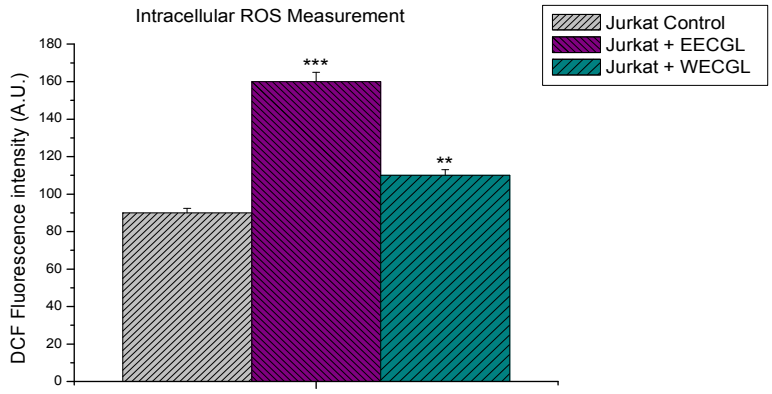
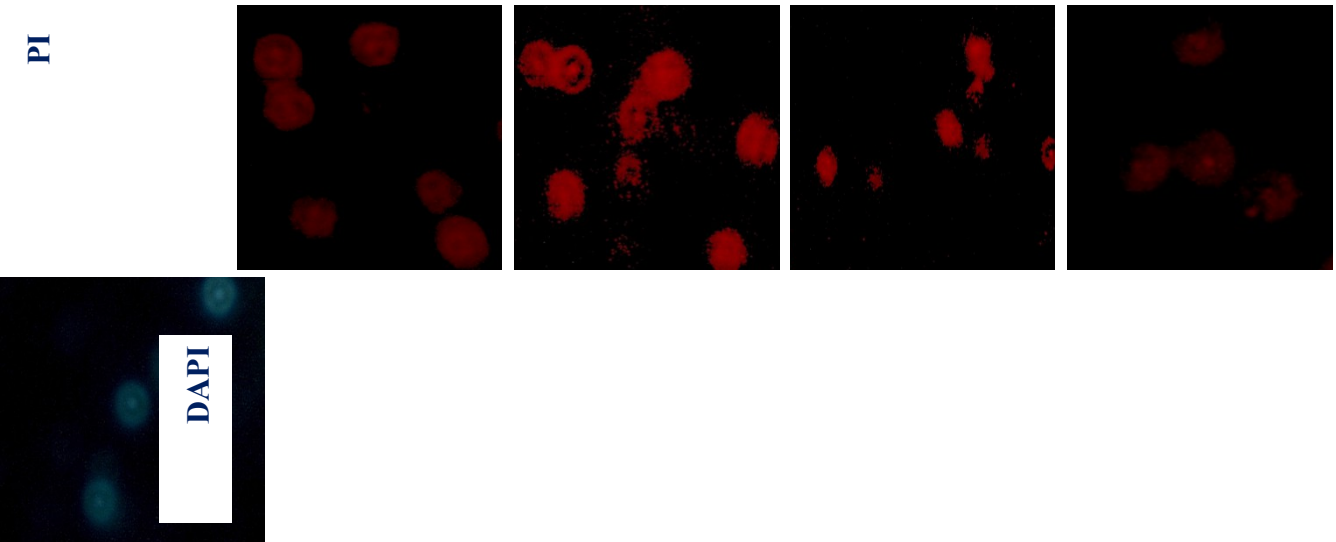


Figure 5.3 Effects of EECGL and WECGL on DCF fluorescence intensity in Jurkat cells. Data are expressed as Mean ± SEM. Probability values are given in asterisks. ** indicates p< 0.01, *** indicates p<0.001; values are taken in respect of control.

5.3.3 EECGL and WECGLproduced chromatincondensationinJurkat cells

Chromatin condensation was observed under fluorescent microscope after PI and DAPI staining (Fig. 5.4).EECGL produced pronounced chromatin condensationafterPI staining compared toWECGL and standard drug 5-FU.



Jurkat Control Jurkat + EECGL Jurkat + WECGL Jurkat+5 FU

Figure 5.4Fluorescentmicroscopic image of chromatin condensation in Jurkat cells treated with EECGL, WECGL and 5-FU. Cells were stained with PI andDAPI staining.

5.3.4EECGL and WECGL- induced apoptosis as measured by DNA laddering

In the present study, the induction of apoptosis was revealed by DNA ladder formation by agarose gel electrophoretic study. DNA fragmentation was prominent in EECGL, WECGL and 5-FU treated Jurkat cells (Fig. 5.5)

A – Ladder; B - JurkatControl;C –Jurkat + WECGL; D - Jurkat+ EECGL;E - Jurkat+ 5-FU

Fig. 5.5 DNA laddering of EECGL ECGL and WECGL for 24 hours

5.3.5 Antimitotic effect of EECGL and WECGL

The effects of EECGL and WECGL on percentage of cells in different stages of mitosis and mitotic index in *Allium cepa*root tips are shown in Fig. 5.5, 5.6, 5.7 and 5.8. *C.gigantea* latex extracts were found to be antimitotic in *Allium cepa* root tip cells at the concentration of 100mg/ml.EECGL treated group shows significantly less number of dividing cells compared

to the control and WECGL group. The mitotic index of EECGL, WECGL treated group and control group were 27%, 47% and 57% respectively.

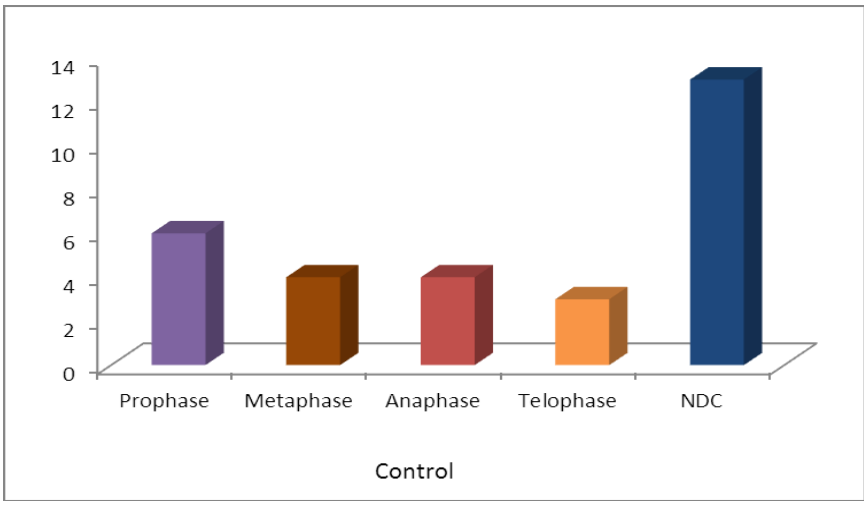


Figure 5.6 shows the percentage of cells in different stages of mitosis in control (water treatment) group.

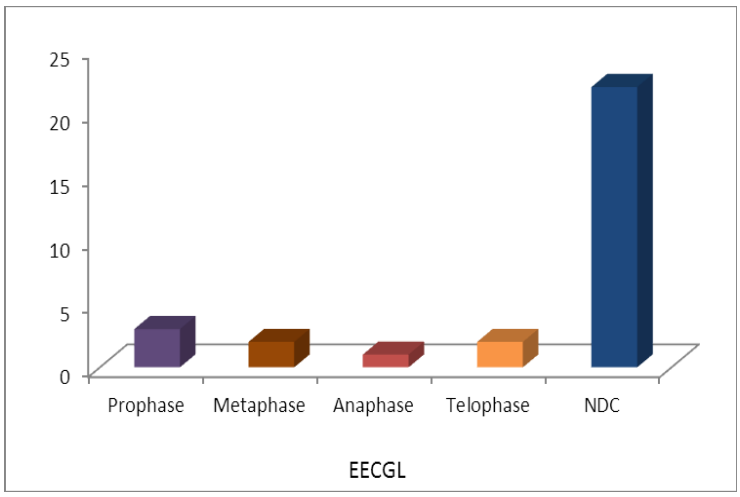


Figure 5.7The effect of EECGL on percentage of cells in different stages of mitosis.

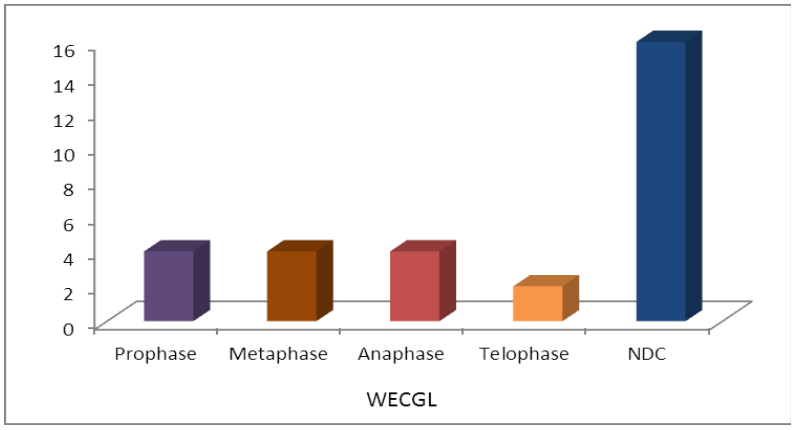


Figure 5.8The effect of WECGL on percentage of cells in different stages of mitosis.

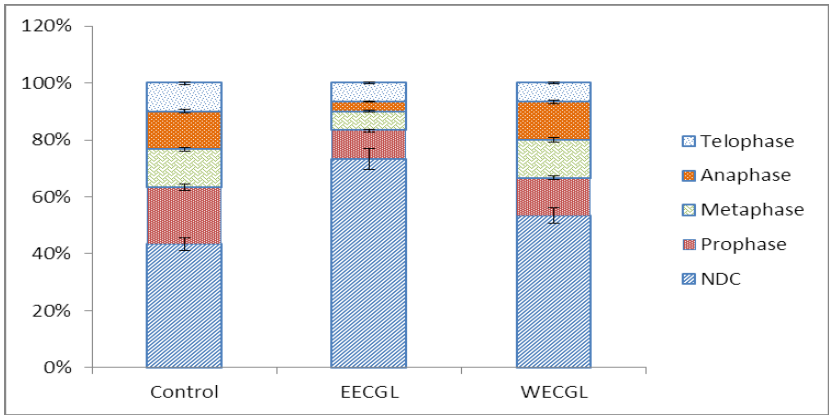


Figure 5.9. The comparative percentage of cells in different stages of mitosis

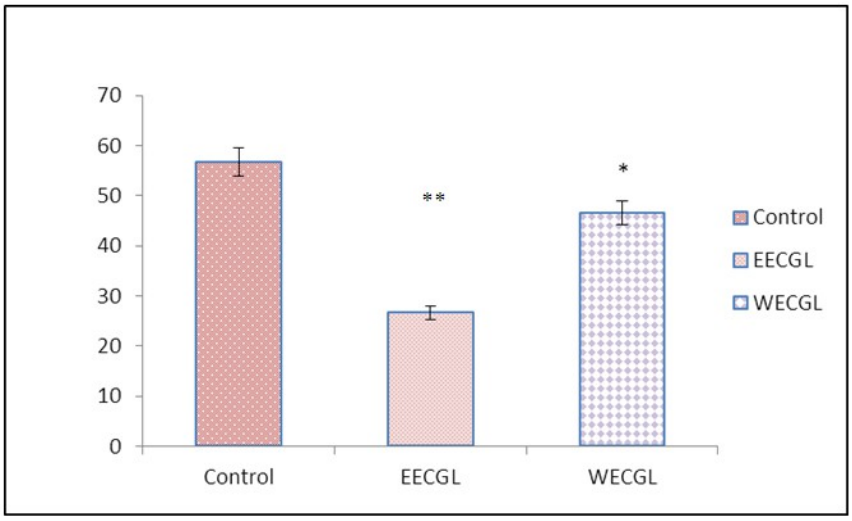


Figure 5.10shows the effect of EECGL and WECGL on mitotic index in *Allium cepa*root tips. Data are expressed as Mean ± SEM. Probability values are given in asterisks. * indicates $p< 0.05$, ** indicates $p<0.01$; values are taken in respect of control.

5.4Discussion

The present chapter was designed to assessthe in-*vitro*cytotoxic,antiproliferative and apoptotic potential of ethanolic (EECGL) and water (WECGL) extract of latex of *Calotropisgigantea*on human acute T cell leukemia producing Jurkat cells and antimitotic potential in *Allium cepa* root tip cells.

Both of EECGL and WECGL exert their in-*vitro*cytotoxicactivity showingrespectiveIC_{50} at 41.6 and 50µg/mlin case of EECGL and WECGL.The impairment of mitochondrial electron transport chains may elevate ROS generation (Jeyaraj et al.,2013). The results indicate that a significant ($p<0.01$) increase of ROS level is found due to EECGLand WECGL exposure (Fig. 6.4). The fluorescent microscopic images (Fig. 6.3) are highly correlated with the fluorescence intensity level. Increase in intracellular ROS in cancer cell line can induce their cell cycle arrest, senescence and apoptosis. This can be achieved in cancer chemotherapy or by depletion of cells from antioxidant proteins or generation of ROS by immune cells. Apoptosis is linked to an increase in mitochondrial oxidative stress that causes cytochrome C release, an irrevocable event that leads to the activation of caspases and cell death. From our result it was obvious that EECGL and WECGL are able to damage the cancer cell via ROS generation.

Incidence of membrane blebbing and bluish/orange condensed chromatin in Jurkat cells after treatment with EECGL and WECGL at IC_{50} doses noticeablypointed out the apoptotic properties of EECGL and WECGL in Jurkat cells. Fluorescence microscopic image of Jurkat cells using PI and DAPI staining established that EECGL and WECGL have the ability to

preventJurkatcell proliferation via apoptosis. Application of EECGL and WECGL exhibited distinctive apoptotic morphology (Fig. 6.8) with condensed nuclei in treated Jurkat cell. The membrane integrity became lost and PI and DAPI stain leaked into intact membrane. The apoptotic nuclei appeared as bright pink or bright blue chromatin that are highly condensed and fragmented (Bortner and Cidlowski, 1998).

Apoptosis induction by EECGL and WECGL is verified by DNA ladder assays. EECGL and WECGL-treated Jurkat cells show characteristic ladder-like DNA patterns, unlike untreated control cells. The study revealed that the extracts induce apoptosis in Jurkat cells by stimulation of DNA fragmentation.

The present study revealed that EECGL and WECGL had prominent antimitotic activity. The maximum percentage of root tips cells in EECGL and WECGL treated groups were seen to be in prophase of mitosis. It indicates the transition of inhibition from prophase to metaphase and subsequent phases. Maximum numbers of non-dividing cells were found in the extracts treated groups compared to the control group. Mitotic index of *Allium cepa*root tips treated with EECGL showed significantly lower mitotic index. Among the two *C. gigantea*latexextracts, EECGL showed the highest degree of suppression in cell division. A group of cytotoxic anticancer drugs suppress the cell cycle by inhibiting the enzymes that participates in DNA and RNA synthesis (Ravi et al.,2002).*Calotropis*is a medicinal plant which contains various phytochemicals like flavonoids, alkaloids, phenols; those may exhibit antimitotic property (Kubo et al.,1993) by inhibiting the cell division of another species like *Allium cepa*.

5.5 Conclusion

In conclusion, our findings demonstrate that EECGL and WECGL display*in-vitro*cytotoxic, and antileukemic potential againstJurkat cells. *Invitro* analysis of ROS generation, DAPI and

PI staining indicated that EECGL and WECGL primarily induced apoptosis in the cancer cells. The present investigation also confirms the antimitotic activity of ethanol and water extracts of *C.giganteal*atex in *Allium cepa*root tip cells. So, these findings establish the antiproliferative, apoptosis-inducing abilities of EECGL and WECGL in human acute T cell leukemia producing Jurkat cells and antimitotic potential in *Allium cepa* root tip cells.

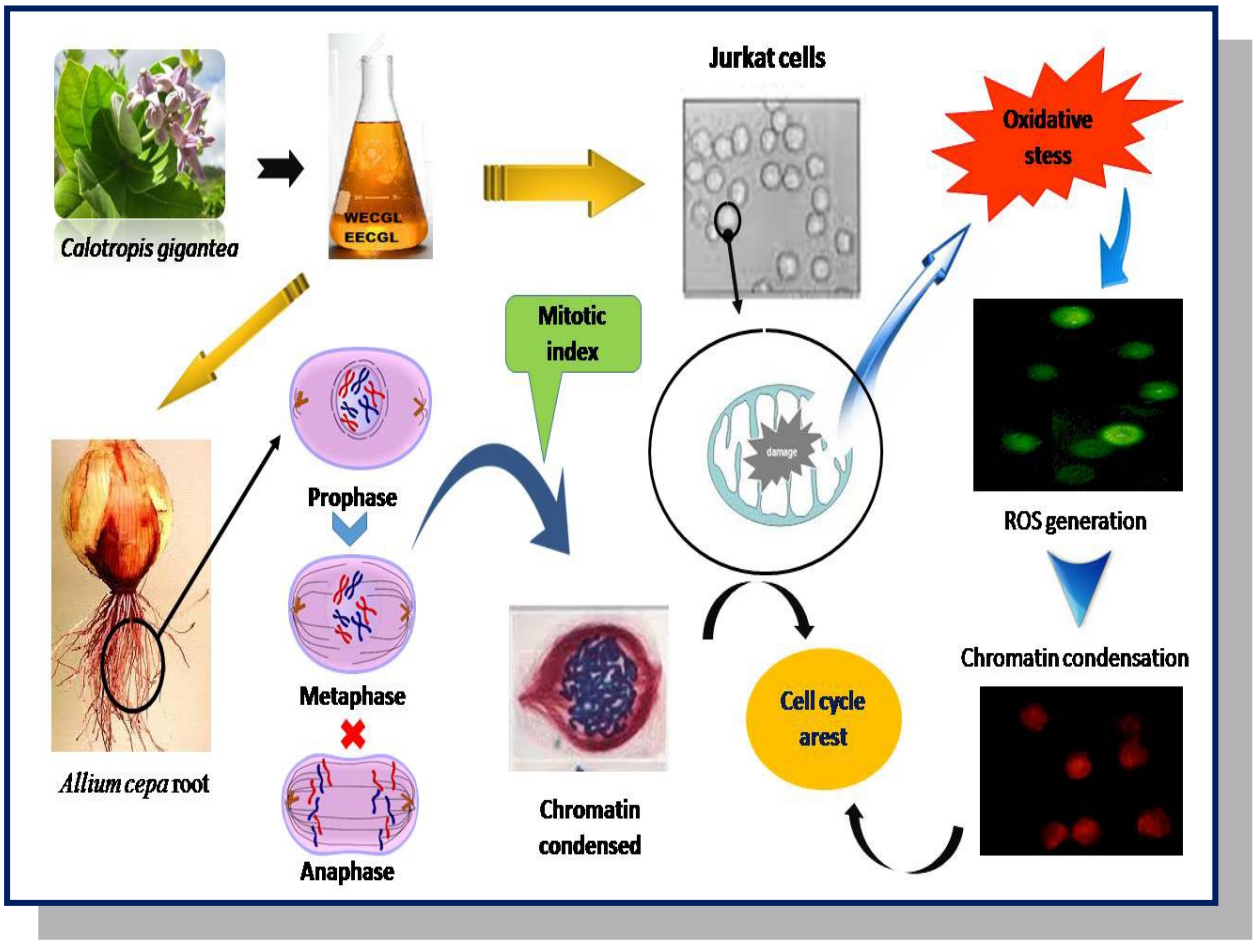


Figure 5.11 Schemeticrepresntation of antimitotic and antiproliferative activity of *Calotropisgigantea* latex

Moyo B, Mukanganyama S. Antibacterial Effects of *Cissuswelwitschii* and *Triumfettawelwitschii* Extracts against Escherichia coli and Bacillus cereus2015; 2015:10