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# Russell's Viper Venom Purified Toxin Drct-II Inhibits the Cell Proliferation and Induces G1 Cell Cycle Arrest in Human Leukemic Cancer Cells

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## Abstract

The present study was an effort to establish the anticancer activity of the purified protein toxin (drCT-II) from Indian Russell's viper *(Daboia russelli russelli)* venom in leukemic cell line and animal model. Isolation and purification of drCT-II was done through CM-cellulose ion exchange chromatography and RP- HPLC. SDS- PAGE molecular weight and first 20 amino acid sequence of drCT-II was done. The anti-leukemic activity using U937 and K562 cell line was established through cytotoxicity, apoptosis, cell cycle study, morphology, cancer marker proteins. The mean survival time of EAC induced male albino mice was established. Human lymphocyte cytotoxicity was done. drCT-II was eluted with 0.1 M NaCl on CM-cellulose ion exchange chromatography. On RP-HPLC, drCT-II produced single peak with retention time of 14.6 min. SDS-PAGE molecular weight was found to be 6.6 KDa and the first 20 amino acid sequence was found to be LQXNKLVPIASKTXPPGKNL. drCT-II produced time and dose dependent cell (U937 and K562) growth inhibition. The IC50 was found to be 35.5 µg/ml for U937 cell and 48.2 µg/ml for K562 cells. drCT-II produced membrane disruption, blebbing and nuclear disintegration in U937 and K562 cells observed through confocal and scanning electron microscopy. It exhibited DNA fragmentation and comet formation in leukemic cells. drCT-II produced apoptosis, cell cycle arrest at $G_1$ phase and increased the expression of $P_{21}$, $P_{27}$ and $P_{53}$. drCT-II induced apoptosis in leukemic cells was followed through caspase 3 and 9 pathway activation. EAC cell growth in male albino mice was significantly inhibited by drCT-II, thus increased the mean survival time. drCT-II significantly reduced the human lymphocyte count (in culture). It may be concluded that drCT-II, a 6.6 KDa protein purified from *Daboia russelli russelli* venom would be a novel pro-apoptotic agent that induced cancer cell killing through p53 and caspase pathway.

**Keywords:** Snake venom; Russell viper; Cytotoxicity; Apoptosis; Cell cycle; Caspase

## Introduction

Cancer, one of the largest killer diseases contributed a projected death toll of more than eight million lives per year, across the globe [1]. It remained as the second most deadly disease over the last millennium but expected to surpass heart disease early in the present century [2]. In India, improved nutrition, mass awareness, modernization of sanitation and availability of advanced diagnostic and therapeutic option enabled an overall decline in mortality and morbidity from other class of diseases. However in case of cancer, wider availability of diagnostic steps facilitating an early diagnosis has been reflected in proportionate rise in the relative and absolute cancer incidence [3]. Cancer remains the most severe problem of the biomedical world. The biological hallmark of cancer is the uncontrolled aberrant growth which invades the surrounding tissue and beyond (metastasis) [4]. The therapeutic and epidemiological perspectives have spurred the quest for newer diagnostic and treatment options towards cancer treatment and prevention. The application of current treatment technique, which includes surgical, radiation therapy, chemotherapy, biological therapy, results in the cure / remission in only half of these patients [5].

The search for biological anti-cancer agents has been pursued over a century. Various plant and animal products, including snake venom toxins have been thoroughly investigated for anticancer efficacy. Venoms and toxins have found a niche in the pharmaceutical market. Several isolated toxins with a known mode of action have practical applications as pharmaceutical agents, diagnostic reagents or tools. Captopril, the inhibitor of the angiotensin I converting enzyme isolated from Brazilian snake (*Bothrops jararaca*) has been used in the treatment of lung cancer [6]. From the past few decades, research on isolation and characterization of anticancer agents from the snake venom has been ventured. The anti-metastatic activities of the venom of Indian monocellate cobra (*Naja kaouthia*), Indian cobra (*Naja naja*) Russell's viper (*Daboia russelli russelli*) and Banded krait (*Bungarus fasciatus*) were studied on carcinoma, sarcoma and leukemia models [7-9].

The present study was an effort to establish the anticancer activity of purified fraction of Indian Russell's viper *(Daboia russelli russelli)* venom in cell line and animal model. It is expected that this study may add new information on anticancer effects of *Daboia russelli russelli* snake venom, which may be utilized for future drug development clue against cancer.

## Methods and Materials

### Collection of snake venom

Live captive adult (either sex) *Daboia russelli russelli venom* was purchased commercially from Calcutta Snake Park, Kolkata, India. The venom was lyophilized, stored in amber coloured bottle at 8 ± 1°C in vacuum desiccators and expressed in terms of dry weight/protein concentration.

### Chemicals

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Solvents and chemicals used were of analytical grade unless otherwise mentioned. Primary antibody (Caspase 3 kit, Caspase 9 kit, p53, p21, p27 and β-actin) and HRP-conjugated goat anti-rabbit secondary antibody were procured from BD Biosciences, CA, USA.

## Animals

Adult male Swiss albino mice (20 ± 2 g) were purchased from authorized animal supplier of Calcutta University. Experiments using animals conforms to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No.85-23 revised 1996) and had the approval of the animal ethics committee of the Institute (accreditation No. of the institute 147/1999/CPCSEand A, Government of India).

## Collection / Culture of cancer cell lines

**Maintenance of Ehrlich ascites carcinoma cell line:** Ehrlich ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. Cells were maintained in male albino Swiss mice by intraperitoneal inoculation of $10^5$ cells/ mouse bi-weekly.

**Maintenance of cell lines:** Human leukemic cells (U937, K562) were purchased from National Facility for Animal Tissue and Cell Culture, Pune, India. The leukemic cells were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (10 µg/ml) and gentamycin (40 µg/ml). The cells were routinely cultured in $CO_2$ incubator at 37°C in 5% $CO_2$ atmosphere and 95% humidity.

**Purification of drCT-II by ion exchange chromatography:** *Daboia russelli russelli* venom (100 mg) after heat treatment (70°C for 30 minutes) was centrifuged (900 g×20 minutes) and the supernatant was adsorbed on a CM-cellulose column (80×15 mm) equilibrated with 0.02M phosphate buffer (pH 7.2). Elution was accomplished at room temperature (22 ± 2°C). Initially, the column was washed with 0.02M phosphate buffer (pH 7.2), then stepwise elution was carried out with 0.02, 0.05, 0.1, 0.2 and 0.5M NaCl in 0.02M phosphate buffer (pH 7.2). 5 ml fractions were collected at room temperature (22 ± 2°C) and expressed in terms of protein measured after Lowry et al. [10]. The active fraction (tube no. 26= drCT-II) obtained from ion exchange chromatography was further purified by RP-HPLC on a Nova Pak $C_{18}$ column (Shimadzu, Japan) equilibrated with 100 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. Methanol and water solvent system (60:40 v/v) was used isocratically. Elution of the protein (drCT-II) was monitored at 280 nm. The molecular weight of drCT-II was determined through SDS-PAGE [11]. N terminal amino acid sequence of drCT-II was determined by a gas phase amino acid sequencer coupled with phenylthiohydan to in amino acid analyser after hydrolysing the protein with 6N HCL at 110°C for 24 hour.

## Anticancer studies

**Cytotoxicity studies on cell lines (*in vitro*):** MTT assay was performed according to Chen et al., with slight modifications [12]. $10^6$/ml of cancer cells (U937, K562) were treated with different concentration of drCT-II for 48 hrs. 40 µl of MTT (5mg/ml of PBS, pH 7.2) was added to each well and kept in the $CO_2$ incubator for 4hr. The culture medium with MTT was carefully removed and 100 µl of dimethyl sulfoxide were added to each well to dissolve the purple colored formazan crystals. Absorbance was recorded at 570 nm in an ELISA reader (Bio Rad. Model no: 680XR).

**Determination of IC50 dose of drCT-II on U937 and K562 cell lines:** The 50% of inhibition concentration ($IC_{50}$ dose) of drCT-II in U937 and K562 cells were determined using different concentrations of drCT-II (10-60 µg/ml) at 24 h, 48 h and 72 h. The respective viable cells were counted in the phase contrast microscope using Trypan blue [13]. The respective concentration of drCT-II and the percentage of cell death were plotted and $IC_{50}$ was determined.

**Morphological studies:** Analysis of apoptotic features of drCT-II treated cells was done by confocal and scanning electron microscopy. Cells were treated with IC50 dose of drCT-II for 48 hrs [8]. Control and treated cells (U937 and K562) were collected after washing twice with cold PBS (pH 7.2). Then cells were treated with 10 µl of Hoechst (100 µg/ml of PBS) and were placed on grease free glass slides and covered with cover slip. Cells were then observed under a confocal microscope (Leica TCS-SP2 system).

For scanning electron microscopy, U937 and K562 cells after treatment with drCT-II were washed twice with cold PBS (pH 7.2) and fixed with 2.5% glutaraldehyde in PBS at 4°C for 3 h in the dark. The cells were again washed with cold PBS and fixed in 1% osmium tetroxide in cacodylate buffer for 1 h. Dehydration was done with ascending concentrations of ethanol in deionized water. After drying, the cells were embedded in polylysine coated thick cover glass. The prepared cells on cover glass were gold coated (S150-Sputter coater, Edward, UK) and observed under a scanning electron microscope (Leica, Model S440) with 15 kV accelerating voltage. Photographs were taken with a digital camera [14].

## DNA fragmentation study

DNA was isolated from control cells and drCT-II treated cells using instructions of Apoptotic DNA Ladder kit. DNA ladder pattern was observed using horizontal agarose gel electrophoresis with ethidium bromide [15].

**Single-cell gel electrophoresis (alkaline comet assay):** Comet assay of U937 and K562 cells was performed under alkaline condition following method of Singh et al., with minor modifications [16]. U937 and K562 cells ($1\times10^5$) were cultured in presence and absence of drCT-II ($IC_{50}$ doses) for 48 h. The cells were then washed twice with cold PBS by centrifuging at 1500 rpm for 5 min in cold centrifuge. Concentration ($1–2\times10^4$/10 ml) of different cell groups was standardized by changing the dilution of cell suspension. Microscope slides were covered with 400 µl of 0.75% normal melting point agarose in PBS pre-warmed to 50°C. A cover glass was placed over the agarose solution and the agarose was allowed to solidify. The cover glass was then removed and 85 µl of cell–agarose suspension (10 µl of cell suspension containing about $10^4$ cells was mixed with 75 µl of 0.5% low melting point agarose, in PBS) was placed over the first agarose layer and allowed to solidify under a clean cover glass. After removing the cover glass 100 µl of 0.5% low melting point agarose was added and allowed to solidify in a chilled condition. After the cover glass was removed, the slides were gently immersed in a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100, pH adjusted to 10 with NaOH) and kept at 4°C in the dark for 1 h. The slides were placed on the horizontal gel electrophoresis unit filled with fresh, cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.5) for 20 min. Electrophoresis was conducted for the next 20 min at 18 V (1.0 V/cm, 250 mA). The slides were then drained, placed on a tray and flooded slowly with three changes of neutralization buffer (0.4 M Tris–HCl, pH 7.5), each for 5 min. The slides were stained with ethidium bromide (10 mg/ml), covered with a cover glass, and analyzed within 1 h at 100X magnification using a fluorescent microscope (Motic BA400, Germany) with green filter [17].

**Apoptosis study by flow cytometry:** Annexin-V FITC and propidium iodide (PI) was used for distinguishing live/apoptotic/necrotic cells. After treatment with drCT-II ($IC_{50}$ dose, 48 hrs), cells were washed in ice cold PBS (pH 7.2). Apoptosis assay was performed using Annexin V- FITC kit and BD FACS double laser flow cytometer [18].

**Cell cycle study by flow cytometry:** The phase of cell cycle arrest due to drCT-II treatment was determined using flow cytometer. Cells (U937 and K562) were treated with drCt-II ($IC_{50}$ dose) for 48 hrs. Detection of cell cycle arrest was done by using the instruction obtained from The Cycle Test™ Plus DNA Reagent Kit. Finally intracellular DNA content of the cells was analyzed in a BD FACS calibre single laser flow cytometer [7].

**Caspase 3 and caspase 9 assay:** Production of caspase 3 and caspase 9 of cells were estimated after treatment of drCT-II ($IC_{50}$ dose, 48 hrs) on U937 and k562 cells according to manufacturer's instruction of caspase 3 and caspase 9 assay kit [19].

**Western immunoblot analysis:** Expression of different proteins (p21, p27, p53 and β-actin) of U937 and K562 cells were measured by western blot analysis after treatment with drCT-II ($IC_{50}$ dose, 48 hrs) [20].

**In vivo anticancer activity of drCT-II on Ehrlich ascites carcinoma (EAC)–bearing mice:** EAC cells were inoculated on day 0 ($10^5$cells/mouse intraperitoneal i.p.) and animals were divided into three groups (n=12). Group-I was control and received intraperitoneal injection of same amount of vehicle (0.9% saline); Group-II (0.75 mg/kg/day, i.p of drCT-II) and Group-III was treated with standard drug, 5-FU (5 mg/kg/day i.p.) for 15 days. 6 mice of each group were sacrificed by cervical dislocation on the 16th day. Intraperitoneal tumor cells were collected and counted by Trypan blue exclusion method [21]. For determination of mean survival time, other 6 mice of each group were allowed to natural death [22,23].

**Effect of drCT-II on healthy human lymphocytes**

Human ethical clearance was availed from Department of Physiology, University of Calcutta, Kolkata (Ref. No. IHEC/AG/HUM/P17/12). After taking informed consent, whole blood was collected in heparinised vial aseptically by venipuncture of healthy adult volunteers. Using Ficoll-histopaque, lymphocytes were collected from whole blood. It was cultured in sterile complete RPMI 1640 media. $1\times10^6$ cells were treated with sterile PBS, drCT-II (twice $IC_{50}$ dose of U937 cell) and Imatinib mesylate (standard drug – 100 µg/ml). Cells were grown in a $CO_2$ incubator at 37°C with 5% $CO_2$ for 72 h in humidified condition. Effect of drCT-II on blood lymphocyte was assessed through MTT assay [24].

## Statistical analysis

For statistical analysis, GraphPad InStat software (La Jolla, CA, USA) was used. Oneway ANOVA was done for determination of difference between control and treated group. Dunnett's multiple comparison tests was done for post test analysis (significant level). Here, data were shown as mean ± SEM (n=6), P≤ 0.05 were considered to be significant.

# Results

## Purification of drCT-II by Ion exchange chromatography

*Daboia russelli russelli* venom was resolved into four major adsorbed protein peaks on CM cellulose column. Peak III eluted with 0.1 M NaCl in phosphate buffer possessed lethality and cytotoxic activities (Figure 1). The purified fraction (thereafter called as *dr*CT-II, *dr* stands for *Daboia russelli russelli*, CT for cytotoxin, II for the cytotoxic fraction) constitutes about 2.75 ± 0.05% protein of the venom applied onto the column. The fraction was desalted by dialysis against distilled water and it was subsequently lyophilized. The purity of *dr*CT-II was reconfirmed by RP-HPLC on $C_{18}$ Nova Pak column, where it showed a single symmetric peak with a retention time of 14.6 min (Figure 1 and inset). The calculated molecular weight of *dr*CT-II was found to be 6.6 kDa on SDS-PAGE (Figure 2). The first 20 amino acid sequence in the amino terminal of drCT-II was found to be LQXNKLVPIASKTXPPGKNL.

## Cytotoxicity studies

drCT-II at concentration of 10-60 µg/ml (U937 cell and K562 cell line) significantly inhibited the growth of the respective leukemic cells compared with the control cell in time and dose dependent manner. MTT assay revealed that drCT-II showed 18.2%-69.5% U937 cell and 12.3%-58.9% K562 cell proliferation arrest (Figure 3A and B).

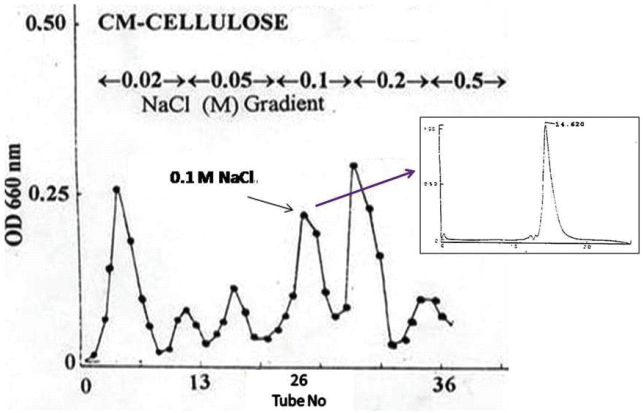


**Figure 1:** Purification of drCT-II through CM-cellulose ion-exchange chromatography. Heat treated venom (50 mg) was loaded on CM-cellulose equilibrated with 0.02M phosphate buffer, pH 7.2. The proteins were eluted with stepwise gradient of NaCl (0.02M-1.0M). Among the six peaks shown in the chromatogram, peak 4 was eluted with 0.1M NaCl, showed anticancer activity. [In Inset] RP-HPLC chromatogram of drCT-II on C18 column. drCT-II was eluted in main peak having retention time 14.620 min.

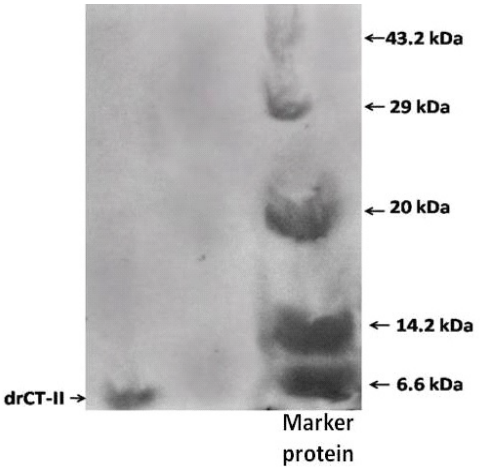


**Figure 2:** Determination of molecular weight of drCT-II by SDS-PAGE.

## Determination of IC50 value of drCT-II

After 72 h, IC50 value of drCT-II in U937 cells was 35.5 µg/ml and in K562 cells was 48.2µg/ml (Figure 4A and B).

## Morphometry study

Confocal and SEM image of drCT-II (IC50 dose) treated K562 and U937 cell showed membrane disruption, blebbing, nuclear disintegration in both the leukemic cell line as compared with the untreated control cells (Figure 5 A-H).

**DNA fragmentation and comet assay:** The gel pattern of DNA samples isolated from untreated control cell lines showed intact banding pattern. Whereas, the gel pattern of DNA samples isolated from drCT-II($IC_{50}$ dose) treated U937 and K562 cells showed smeary banding pattern in individual experiments (n=4) (Figure 6A).

drCT-II ($IC_{50}$ dose) treated U937 and K562 cells showed typical comet formation in individual experiments (n=4), which might conclude that drCT-II worked through the nuclear localization (Figure 6B). The intensity of the comet tail relative to the head reflects the number of DNA breaks.

**Apoptosis study by flow cytometric analysis:** After 24 h treatment with drCT-II ($IC_{50}$ dose) in U937 cell, it was observed in flow cytometry that 45.14% cells were in LL quadrant, 44.52% in LR quadrant, 10.06 % in UR quadrant and 1.06% in UL quadrant, whereas control U937 cells showed 94.37% in LL quadrant, 1.72% in LR quadrant, and 2.35% in UL quadrant. Flow cytometric data revealed that drCT-II ($IC_{50}$ dose) treated K562 cell showed 11.63% live, 0.63% early apoptotic, 63.96% late apoptotic and 23.78% necrotic cell. The control untreated (K562)

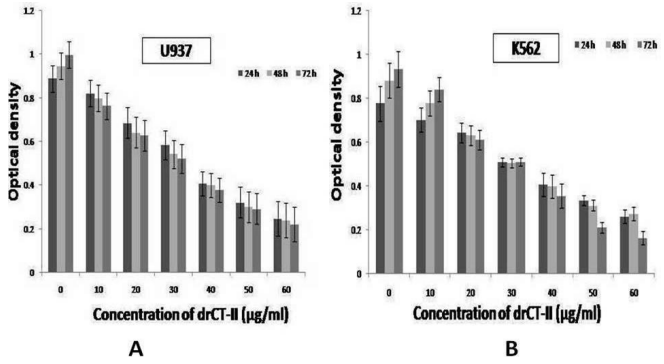


**Figure 3:** Dose and time dependent cytotoxicity of drCT-II on (A) U937 cell and (B) K562 cell.

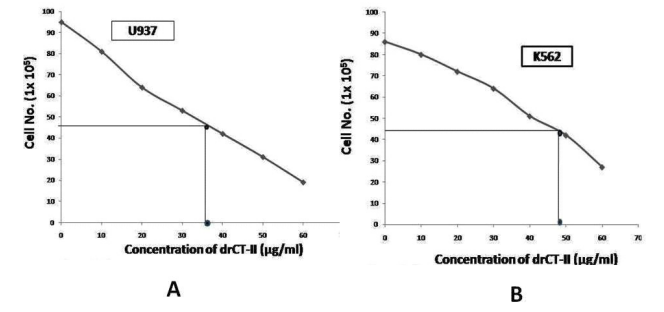


**Figure 4:** $IC_{50}$ dose determination of drCT-II on (A) U937 cell and (B) K562 cell.

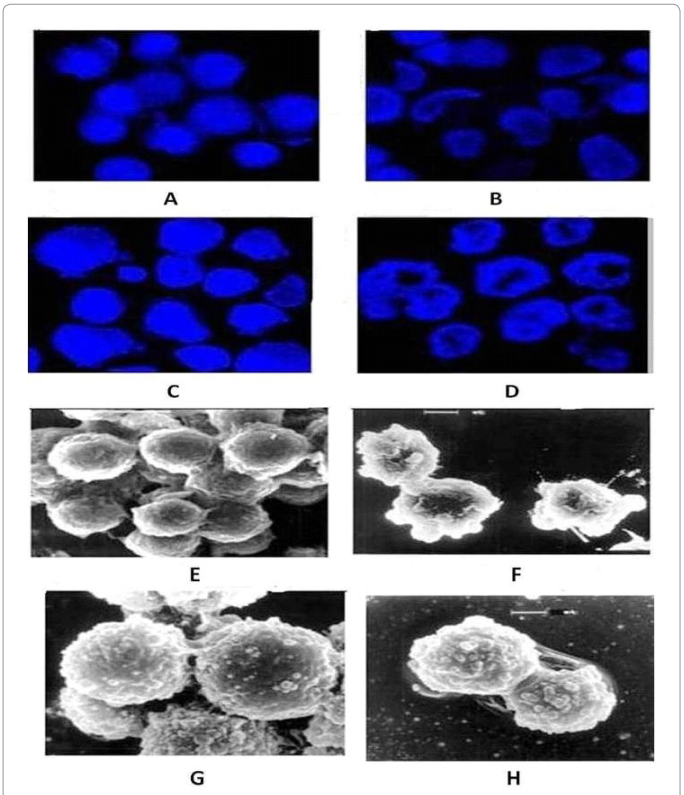


**Figure 5:** Morphometry study of leukemic cell line by confocal microscopy and SEM.
Confocal studies: A- Control U937 cell, B- drCT-II treated U937 cell, C- Control K562 cell and D- drCT-II treated K562 cell.
SEM studies: E- Control U937 cell, F- drCT-II treated U937 cell, G- Control K562 cell and H- drCT-II treated K562 cell).

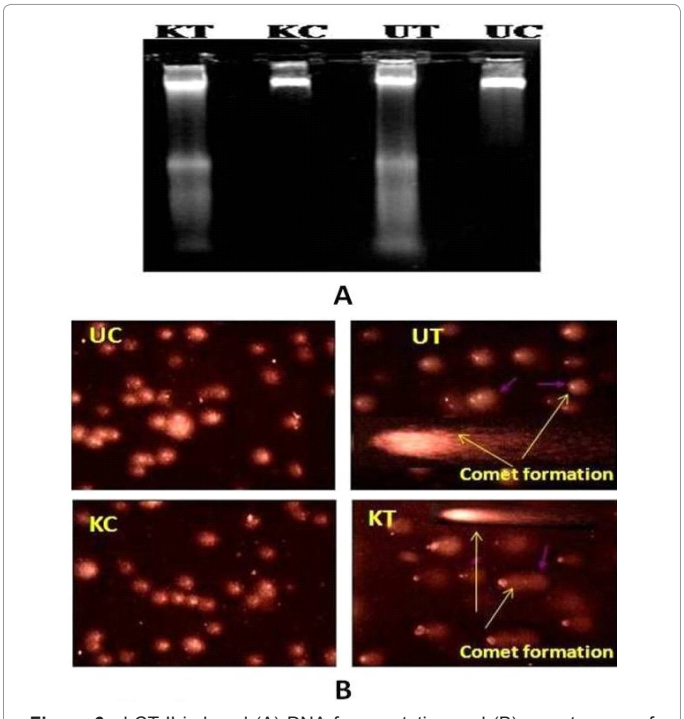


**Figure 6:** drCT-II induced (A) DNA fragmentation and (B) comet assay of U937 and K562 cell. UC- control U937 cell, UT- drCT-II treated U937 cell, KC- Control K562 cell and KT- drCT-II treated K562 cell.

plate showed 95.18% live, 0.03% early apoptotic, 1.28% late apoptotic and 3.51% necrotic cell (Figure 7). The data exhibited that drCT-II induced leukemic cell were mostly in early apoptotic condition.

**Cell cycle study by Flow cytometric analysis:** The flow cytometric analysis of cell cycle showed that drCT-II ($IC_{50}$ dose) treated U937 cells showed 59.72% G1+G0, 34.72% S and 3.24% G2+M cell population, whereas the control cell showed 25.68% G1+G0, 61.49% S and 13.07% G2+M cell population. In K562 cell, there was 21.57% G1+G0, 75.53% S and 1.65% G2+M cell population, whereas the control cell showed 9.99% G1+G0, 85.62% S and 6.16% G2+M cell population (Table 1). From the result it could be concluded that U937 and K562 cells were arrested at G1+Go phase after treatment with drCT-II.

**Caspase 3 and caspase 9 assay:** drCT-II ($IC_{50}$ dose) treatment caused 2 fold and 1.3 fold increase in caspase 3 activity in U937 and K562 cell line, respectively as compared with the untreated control cells. Caspase 9 expression was increased 2.15 fold after treatment with drCT-II ($IC_{50}$ dose) in U937 cell. No change in caspase 9 expression was observed in K562 cell line when compared to control. Both of the leukemic cells followed caspase 3 mediated apoptosis pathway and U937 followed caspase 9 regulation when treated with drCT-II ($IC_{50}$ dose).

**Analysis of apoptotic protein by western immunoblotting analysis:** Treatment with drCT-II ($IC_{50}$ dose) increased the expression

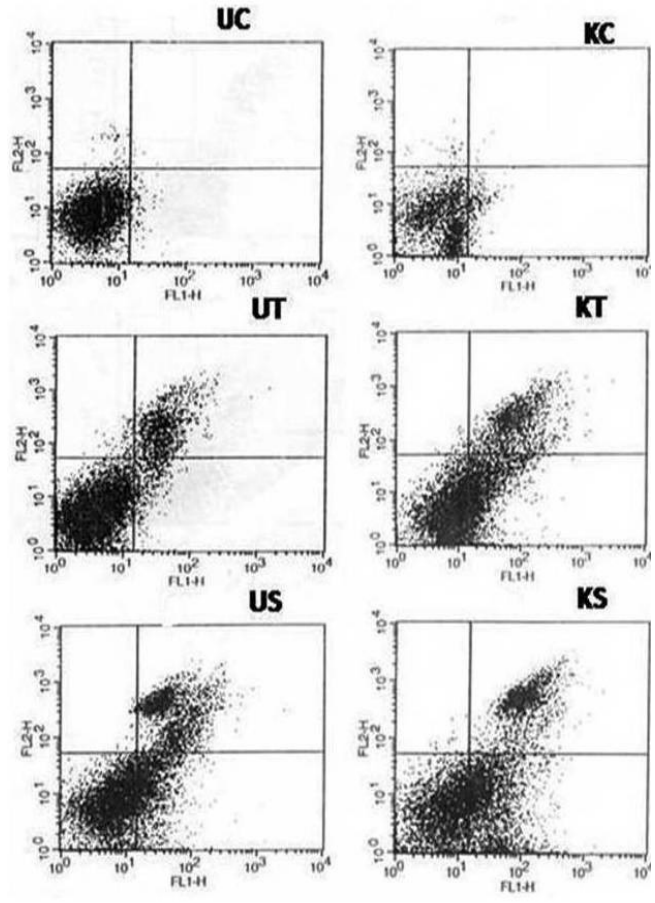


**Figure 7:** drCT-II induced Apoptosis on leukemic cell line showed by flow cytometry using AnnexinV-FITC. UC- control U937 cell, UT- drCT-II treated U937 cell, US- standard treated U937 cell, KC- Control K562 cell and KT- drCT-II treated K562 cell, KS- standard treated K562 cell.

| Leukemic cell type | G0 Phase | G1 Phase | S Phase | G2 Phase |
|---|---|---|---|---|
| Control U937 | 4.81 0.39 | 36.4 0.72 | 11.64 0.53 | 47.161.55 |
| drCT-II treated U937 | 6.99 0.13 | 50.02 1.0* | 10 0.7 | 35.4 1.38* |
| Control K562 | 6.72 0.33 | 32.22 0.99 | 19.34 0.55 | 41.73 1.35 |
| drCT-II treated K562 | 8.3 0.4 | 45.3 0.8 * | 17.8 0.6 | 32.4 0.88* |

Values are % inhibition of cell count (mean ± SEM, n=4), = increased value and = decreased value (* indicates p<0.01 significant)

**Table 1:** drCT-II induced Cell cycle arrest on leukemic cells determination by flow cytometry.

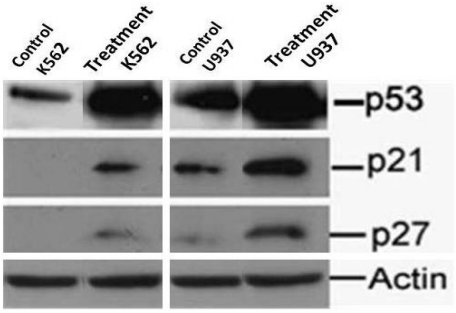


**Figure 8:** Western blot analysis of cell cycle regulator proteins induced by drCT-II on U937 and K562 cell.

of p53, p21 and p27, which indicated that drCT-II arrested the cell at G1 phase (Figure 8).

***In vivo* anticancer activity of drCT-II on EAC mice:** In EAC model, drCT-II ($IC_{50}$ dose) significantly reduced the EAC cell count by 30.4 ± 0.32% when compared to control group. 5-FU treated group showed reduced EAC cell count by 53 ± 0.44% when compared to control group. Mean survival time was increased by 83.3% and 105.5% in drCT-II treated and 5-FU treated group, respectively (Figure 9).

**Effect on human lymphocytes:** MTT assay showed that treatment with drCT-II (2 $IC_{50}$ dose) inhibited human lymphocytes growth by 35 ± 6.2% as compared with untreated control. Standard drug (imatinib mesylate) produced 49.0 ± 3.3% lymphocyte growth inhibitions as compared with control (Figure 10).

## Discussion

In the present study, drCT-II a protein (6.6 KDa) toxin was isolated from Indian *Daboia russelli russelli* venom which showed significant cytotoxic activity on leukemic cell lines and EAC bearing mice. Earlier, Gomes et al., isolated protein toxin drCT-I from the same snake venom which had molecular weight of 7.2 KDa and showed anticancer activity on leukemic cell having $IC_{50}$ value of 8.9 µg/ml and 6.7 µg/ml in U937 and K562 cell, respectively [7]. Both drCT-I and drCT-II were cataionic protein, yet there was difference in their first 20 N-terminal amino acid sequence (drCT-I: LKCNKLVPLFKTCPAGKNL; drCT-II: LQXNKLVPIASKTXPPGKNL). drCT-II showed dose dependent reduction of viable cell count and increased cytotoxicity on U937 and K562 cells *in vitro*. The $IC_{50}$ dose of drCT-II on U937 and K562 cell line were 35.5 µg/ml and 48.2 µg/ml respectively. The cytotoxicity of drCT-II was less than the cytotoxicity of drCT-I on U937 and K562 cell lines.

Yang et al., identified CTX-III from *Naja naja atra* which showed 1.7 µg/ml $IC_{50}$ value on K562 cells and Lee et al. showed cytotoxicity of OH-LAAO (isolated from King cobra) against MCF7 cell [25,26]. drCT-II induced apoptosis in both leukemic cell lines were measured by fluorescence activated cell cytometry. Externalization

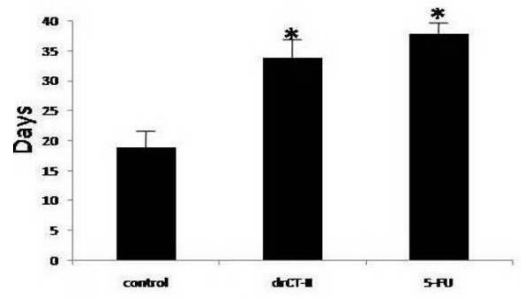


**Figure 9:** Effect of drCT-II on Ehrlich ascites carcinoma (EAC)–bearing mice.

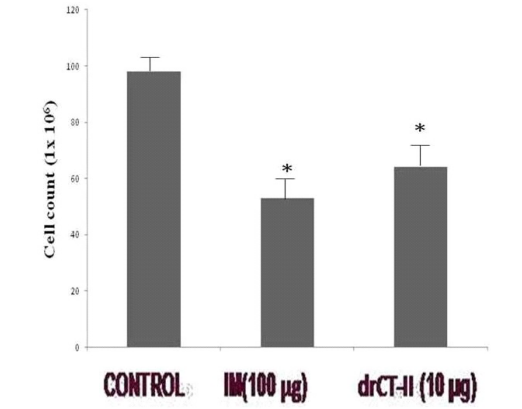


**Figure 10:** Effect of drCT-II on human lymphocyte cell count.

of phosphatidylserine on cell membrane during apoptosis is the main principle for this Annexin-V/PI binding assay. Dual staining with annexin-V-FITC and propidium iodide made it possible to identify live cells, early apoptotic cells and late apoptotic cells [27]. The increased number of early apoptic cells and late apoptotic cells (both U937 and K562 cell lines) after drCT-II treatment confirmed that it inhibited cancer cell growth by inducing apoptosis. Confocal microscopy and scanning electron microscopy showed membrane disruption, blebbing and nuclear disintegration in drCT-II treated U937 and K562 cells. Comet formation and fragmentation of DNA due to apoptogenic trigger requires activation of nucleases, e.g. caspases. Caspase 3 shares both caspase 9 and caspase 8 mediated pathway of apoptogenic signalling. Caspase activation presents a late and common stage to all cells undergoing apoptosis. In the present study, drCT-II was able to upregulate caspase 3 and caspase 9 in leukemic cells. Extrinsic and intrinsic apoptotic pathways can be activated separately by the activation of caspases [28]. Caspase 9 is the initiator caspase for apoptosis in the intrinsic pathway, which then activates caspase 3. Caspase 3 subsequently cleaves and activates several caspases resulting in apoptosis [29]. Thus, the antiproliferative effects observed in drCT-II treated leukemic cells were due to intrinsic apoptotic pathway. The same mechanism was observed in case of drCT-I [7]. Corroborating this finding, CTX-III (*Naja naja atra*) also induced apoptosis in human leukemic cells by caspase 3 activation as observed by Yang et al. [25].

Cell cycle progression is regulated by the sequential events that include activation and subsequent inactivation of cyclin dependent kinases (Cdk) and cyclins. Cdks are a group of serine/threonine kinases that form active heterodimeric complexes following binding to their regulatory subunits, cyclins. Several Cdks, mainly Cdk2, Cdk4, and Cdk6 work cooperatively to drive cells from G1 into S phase. Cdk4 and Cdk6 are involved in early G1 phase and initiate the S phase. The members of the WAF/Kip family, i.e., WAF1 (p21), Kip1 (p27), and Kip2 (p57) from heterotrimeric complexes with G1/S Cdks. Their major action has been reported to inhibit kinase activity of Cdk/cyclin-E complex. p21 proteins bind to cyclins and prevent Cdks from phosphorylating retinoblastoma (Rb) proteins. p21 can also bind and inhibit PCNA, a subunit of DNA polymerase delta synthesized during S phase. In the present study, drCT-II increased the expression of p21 which inhibited the cell cycle to enter in S phase. When normal cells are out of control, apoptosis occurs and may cause diseases such as autoimmunity, immunodeficiency and cancer etc. Apoptosis plays an important role under normal physiological conditions [30]. Impaired apoptosis is a crucial phenomenon in the process of cancer development [31]. The majority of anticancer drugs in clinical trials exerted their effects on cancer cells via arrest in cell cycle and induction of apoptosis. The present study confirmed that the protein toxin drCT-II from Indian Russell's viper (*Daboia russelli russelli*) venom possessed anti cancer activity in leukemic cancer cell line through apoptosis and cell cycle arrest. drCT-II also killed normal lymphocytes (35%) as compared with imatinib mesylate (49%). drCT-II showed higher $IC_{50}$ value towards cancer cell which is 6-7 fold more than drCT-I, 10-15 fold higher than NN32 (purified cytotoxic protein from *Naja naja*) and 15-30 fold increase than NKCT1 (purified cytotoxic protein from *Naja kaouthia*). One of the major limitations of drCT-II is that it shows lethality, cardio and neurotoxicity (data not shown) that should not be overlooked before further trials.

To conclude, a protein toxin drCT-II (6.6 kDa) was purified from Indian viper (*Daboia russelli russelli*) venom by ion exchange chromatography and reverse phase HPLC. drCT-II exerted cytotoxicity against human leukemic cancer cell line (U937, K562). drCT-II induced apoptosis through intrinsic pathway and arrested cell cycle at sub G1 phase on U937 and K562 cells. Further detailed studies at molecular level are required on drCT-II, which may lead to development of a new therapeutic tool for the management of cancer in the near future.

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