International Journal for Pharmaceutical Research Scholars (IJPRS)

V-2, I-4, 2013 ISSN No: 2277 - 7873

RESEARCH ARTICLE

Apoptosis Influencing Activity of Latex of *Euphorbia antiquoram* Linn in Mice

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Manuscript No: IJPRS/V2/I4/00222, Received On: 03/12/2013, Accepted On: 06/12/2013

ABSTRACT

Programmed cell death (PCD) describes a physiological and pathological process of cell deletion that plays an important role in maintaining tissue homeostasis. It is a highly regulated cellular suicide process essential for growth and survival in all eukaryotes. Mouse spleen cells have been successfully used to analyze mechanisms of cytotoxicity for a variety of anticancer drugs. In the present study we analyzed the apoptosis influencing effect of latex of *Euphorbia antiquoram* using mice spleenocytes revealed by staining techniques such as AO and EtBr, PI, EtBr and DAPI. The extent of DNA damage was assessed on exposure to etoposide with or without latex milk of *Euphorbia antiquoram* by agarose gel electrophoresis. The present study revealed that the latex was able to induce apoptosis as evidenced by staining techniques Etoposide caused a maximal DNA damage in spleen cells, which are non cancerous and untransformed cells. This effect was effectively counteracted by the latex milk of *Euphorbia antiquorum.* These findings potentiate the use of latex milk of *Euphorbia antiquorum* in combination therapy when this extract can overcome the toxic side effect of chemotherapeutic drug, because it is safe to normal cells.

KEYWORDS

Apoptosis, Cytotoxicity, DNA Damage, *Euphorbia Antiqoram*

INTRODUCTION

Apoptosis becomes a major tool in cancer research in the view of the fact that some of gene mutations responsible for the induction of cancer pertain to loss of function mutation of tumor suppressor genes which are otherwise pro-survival genes. The other mutations relate to pro-apoptotic genes whose mutations prevent death of cells through apoptosis. Both kinds of mutation are essential in the onset, establishment and metastasis of cancer. Therefore, one of the most advocated modalities for the therapy of cancer is induction of apoptosis through therapeutic interventions.[1] Characterization of apoptosis mainly derives from morphological and ultra structural observations. Intracellular and plasma membrane structural modifications have been widely recognized as crucial factors involved in cell injury and death. Changes in nuclear morphology and in organelle structure as well as specific phenomena at the cell surface level, namely surface smoothing and surface blebbing, are often considered as markers associated with cell pathology. In addition, it must be recalled that these structural findings are intimately related to the cascade of biochemical and physiological events leading to changes in cellular homeostasis, to the loss of cell volume

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regulation to some modifications of macromolecule synthesis and, finally, to the loss of cell viability. Various staining methods are employed to visualize cells undergoing morphological changes.[2]

MATERIALS AND METHOD

Plant Material

Euphorbia antiquorum plant specimen was identified and authenticated (Specimen No 365) by Dr. G.V.S Murthy, Scientist E, Director, Botanical survey of India, Tamilnadu. The latex milk was collected from plant by breaking up the stem of *Euphorbia antiquorum*. The latex milk was collected in the morning hours between 7 to 8 a.m in a glass container and maintained in an ice - cold condition till the use of latex for extraction. The collected latex milk was extracted with methanol. The methanolic extract was prepared by dissolving 1.0ml of latex in 5.0 ml of methanol and allowed to evaporate at 60ºC in a in a water bath. The residue was dissolved in minimal volume of DMSO, stored and used for the assays. Spleenocytes were used as a source of primary cells. Spleenocytes were isolated from three to four weeks old mice and cultured in DMEM with 10% fetal calf serum. Inorder to assess whether the latex milk induces apoptosis various staining techniques like AO/EtBr, EtBr, PI, DAPI, were done. DNA fragmentation was also assessed.

Acridine Orange/ Ethidium Bromide Double Staning

AO/EtBr staining technique was used in order to differentiate between quiescent and actively proliferating cells. It is also used to measure apoptosis.[3]

The nuclear changes such as chromatin condensation and fragmentation in the spleenocytes in the presence and absence of latex milk and with or without etoposide was observed by Ethidium bromide staining as proposed by Mercille and Massie[4] with some minor modifications, propidium iodide staining as described by Sarker[5] DAPI staining as described by Rashmi.[6] DNA fragmentation, which generally occurs at the last phase of apoptosis, was analyzed by using agarose gel electrophoresis as proposed by Yin.[7]

RESULTS AND DISCUSSION

Morphological Study of Apoptotic Cells

Using acridine orange/ethidium bromide (AO/EtBr) dye mixture staining for apoptotic cells, apoptotic nuclei were identified by their distinctively marginated and fragmented appearance under the fluorescence microscope. The normal and apoptotic cells can be assessed by acridine orange and ethidium bromide staining.

Staining of apoptotic cells with fluorescent dyes such as AO/EtBr is considered as the correct method for evaluating the changed nuclear morphology as demonstrated in plate I. The results showed control cells fluoresce green and etoposide treated cells fluoresce red color bound to single stranded DNA in dead cells. Cells treated with latex milk alone showed few apoptotic cells and when co-treated with etoposide showed decreased dead cell number. The results are shown in (Fig.1)

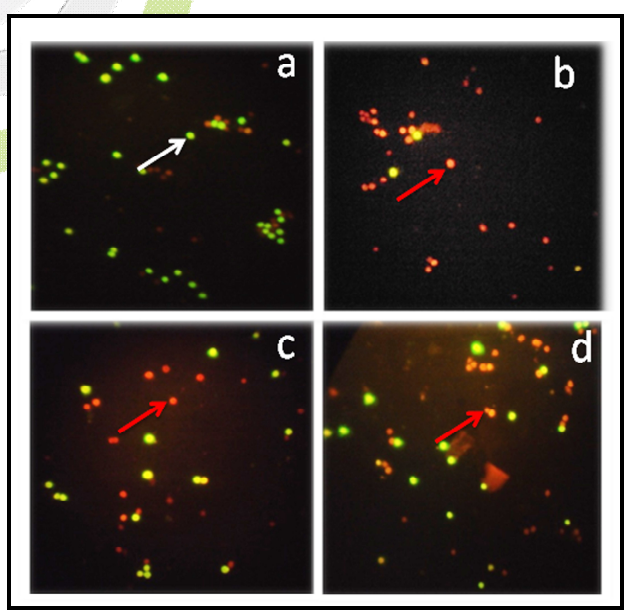

Figure1: Acridine orange, Ethidium bromide double staining of spleenocytes; a) Control; b) Cells and latex milk; c) cells and Etoposide; d) Cells, latex milk and Etoposide. White arrow indicates normal cells and red arrow indicates apoptotic cells

Similar results were seen when Gnidilatimonoein induced apoptosis among K562 cells based on AO/EtBr and Annexin – V/PI double staining observations showed which were abrogated by the addition of guanosine, became evident when the intracellular GTP level decreased to approximately 20-30% of the untreated control level.[8]

Apoptotic activity of petroleum ether and ethanolic extracts of *Rhinacanthus nastus* roots from *in vivo*, *in vitro* and *Agrobacterium rhizogenes* transformed plants in Hep2 cancer cell line showed that treatment with transformed root extracts caused typical apoptotic morphological changes in Hep2 cells by acridine orange and ethidium bromide staining.[9] Morphological changes with acridine orange and ethidium bromide staining proved that ethanolic extract of the flesh of *C. tuberosus*, ethanolic extract of the peel of *C. tuberosus*, ursolic acid and oleanolic acid have shown antiproliferative activity through induced apoptosis in MCF-7 cells.[10] Nouri and Yazdanparast \[11] confirmed apoptotic cell death by Gnidilatimonoein (Gn), a new diterpene ester from *Daphne mucronata* - treated HL-60 cells. From the present observations we were able to differentiate normal and apoptotic cells which were in different colour.

Ethidium Bromide Staining

Ethidium bromide (EtBr) is a molecule that intercalates into nucleic acids and can be used to visualize the nuclear changes in apoptotic cells. The cells were subjected to etoposide treatment in the presence and the absence of the latex milk extract and were stained with Ethidium bromide and the nuclear changes were observed. (Fig.2.) represents the morphological changes during apoptosis.

Several studies have used EtBr, a staining agent to quantify the number of cells showing nuclear changes. The extract of the Chinese herb *Sargentgloryvine* stem has *in vitro* anticancer effects including inhibition of proliferation and induction of apoptosis in the hepatoma cell line HepG-2 by mechanisms involving expression of Bcl-2 family proteins activating the intrinsic mitochondria apoptosis pathway.[12] Isocurcumenol was characterized as the active compound of *Curcuma zedoaria* by spectroscopy and was found to inhibit the proliferation of cancer cells without inducing significant toxicity to the normal cells. EtBr staining exhibited the morphological features of apoptosis in the compound-treated cancer cells.[13] The insulin secreting β-cells were incubated with increasing concentrations of olive leaf polyphenols or oleuropein for 24 h followed by exposure to H_2O_2 (0.035 mM) for 45 min. H_2O_2 alone resulted increased apoptotic and necrotic cell death as evidenced by EtBr staining [14] which was similar to the results of our present study which showed etoposide treatment showed increased apoptosis which was reversed by latex milk extract to some extent.

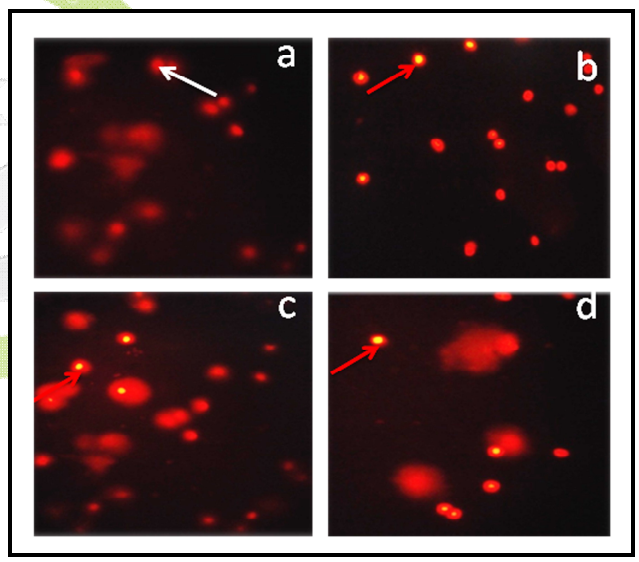

Figure 2: Nuclear changes observed in spleenocytes treated with etoposide and latex milk of *Euphorbia antiquorum* by Ethidium bromide staining; a) Control; b) Cells and latex milk; c) cells and Etoposide; d) Cells, latex milk and Etoposide. White arrow indicates normal cells and red arrow indicates apoptotic cells.

Propidium Iodide and DAPI Staining

Propidium iodide (PI) was used to corroborate viability studies with the most potent extracts. PI is a nucleic acid stain The spleen cells treated with or without etoposide and in presence or

absence of latex milk extracts were stained with propidium iodide and DAPI and the morphology of the normal and apoptotic cells were observed under a fluorescent microscope.

Etoposide induction in spleen cells brought about a steep increase in the number of apoptotic cells. Latex milk with etoposide treatment showed slightly decreased apoptotic cells compared to control cells. The results obtained are shown in (Fig. 3 and 4).

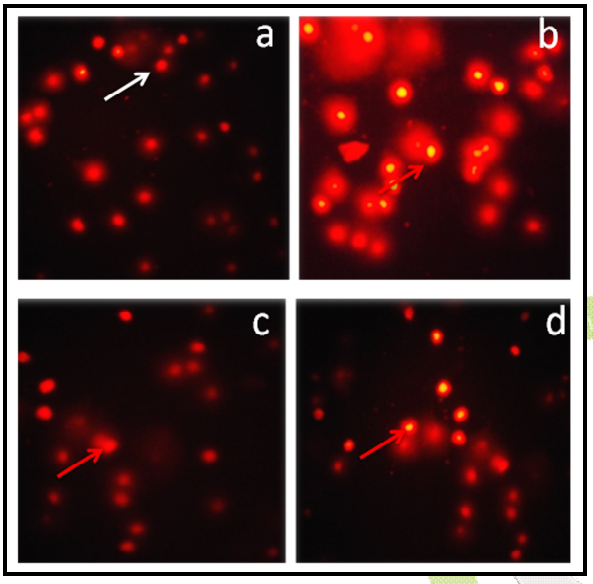

Figure 3: Effect of latex milk of *Euphorbia antiquorum* on spleen cells subjected to apoptosis by etoposide by propidium iodide staining; a) Control; b) Cells and latex milk; c) cells and Etoposide; d) Cells, latex milk and Etoposide. White arrow indicates normal cells and red arrow indicates apoptotic cells

Panaxynol and Panaxydol markedly inhibited proliferation of Human Promyelocytic Leukemia HL60 cells in a time and dose-dependent manner via an apoptotic pathway was shown by annexin V-FITC/ PI staining using flow cytometry.[15] *Scutellaria litwinowii* inhibited the growth of malignant cells in a dose-dependent manner. Among solvent fractions of *S. litwinowii*, the methylene chloride fraction was found to be more toxic compared to other fractions.[16] Osthole inhibited the growth of human lung cancer A549 cells by inducing G2/M arrest and apoptosis. The results suggest that Osthole may have a therapeutic application in the treatment of human lung cancer.[17] In the present study, PI and DAPI staining was effectively used to support the data that apoptosis is induced by etoposide but normal cells were protected by latex milk of *Euphorbia antiquorum*. The results were similar to those revealed by propidium iodide and ethidium bromide staining. The morphological changes of the spleen cells subjected to latex milk and etoposide as visualized by DAPI staining.

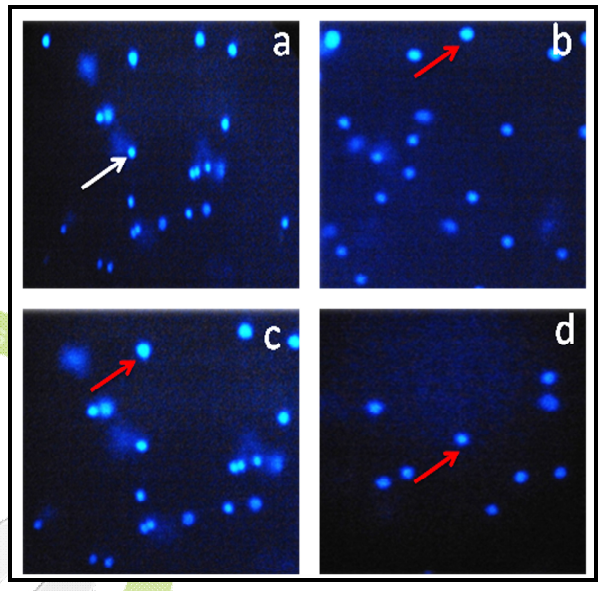

Figure 4: Effect of latex milk of *Euphorbia antiquorum* on spleenocytes subjected to apoptosis by etoposide by DAPI staining; a) Control; b) Cells and latex milk; c) cells and Etoposide; d) Cells, latex milk and Etoposide. White arrow indicates normal cells and red arrow indicates apoptotic cells

Apoptosis is the predominant form of programmed cell death and occurs under a variety of physiological and pathological conditions. One of the most prominent biological features of apoptosis is nucleosomal DNA fragmentation. DNA fragments are considered to be the hallmark of apoptosis. In the present examination, the extent of DNA damage by fragmentation was analyzed in the spleen cells exposed to etoposide with or without latex milk of *Euphorbia antiquorum*. The extent of DNA damage was documented and quantified using a digital gel documentation system and its software (AlphaEaseFC Alpha

DigiDoc 1201). The IDV values are presented in table I.

The assay indicated that etoposide induced DNA fragmentation and latex milk reverted the DNA damage as can be inferred from the IDV values shown in table I and Fig. 5.

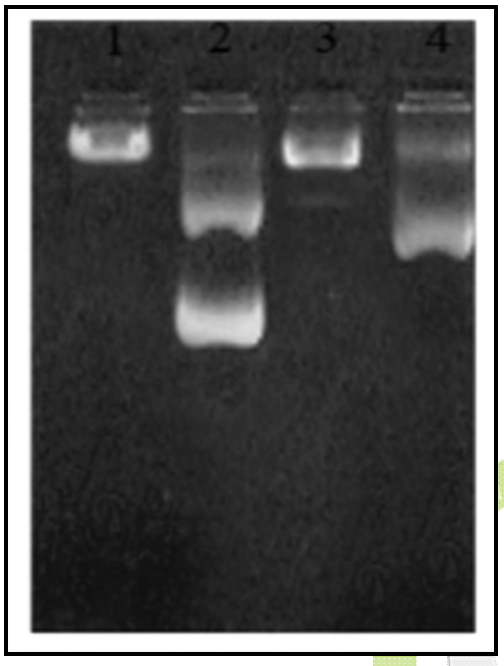

Figure 5: DNA fragmentation by Agarose gel electrophoresis; Lane 1-Control; Lane 2- Cells and latex milk; Lane 3- cells and Etoposide; Lane 4- Cells, latex milk and Etoposide.

Table 1: IDV Values by Agarose Gel Electrophoresis

SAMPLE	IDV VALUES	
	CONTROL	ETOPOSIDE
No extract	8903200	7595912 5331115
Euphorbia antiquorum	8860710	8340019 3050215

The apoptosis proportion of cells was increased by treatment of etoposide in spleenocytes as deliberated by DNA fragmentation assay similar findings reported by other researchers. HL-60 cells treated with aqueous ethanol seed extract of *Ziziphus mauritiana* showed apoptosis in a concentration-dependent manner as evidenced by the formation of internucleosomal DNA fragments [18] Copper nanoparticles (CuNPs) treated U937 cancer cells displayed a ladder pattern of inter nucleosomal DNA fragmentation on TBE-agarose gel electrophoresis in DNA ladder assay which is also another hallmark of apoptosis.[19]

CONCLUSION

Methanol extract of latex milk of *Euphorbia antiquorum* was tested for the apoptotic activity using mice spleenocytes as model for untransformed cells and it showed latex milk is safe to normal spleen cells in lower concentration. The need of the present study is to develop drugs that can potentially target cancer cells by means of their inherent difference to normal cells. The development of such drugs with differential action will be very valuable in cancer chemotherapy without the observed side effects. These findings potentiate the use of latex milk of *Euphorbia antiquorum* in combination therapy when this extract can overcome the toxic side effect of chemotherapeutic drug, because it is safe to normal cells.

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