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Full Length Research Paper

Cytotoxic studies of latex of *Euphorbia antiquorum* in *in vitro* models

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The isolation and chemical characterization of plant derived compounds and its possible use for human well being requires solid and lengthy research. One of the determinants in this complex evaluation concerns the toxic potential of the studied agent. Our objective was to study the cytotoxicity of the methanolic extract latex of *Euphorbia antiquorum* using *in vitro* models namely *Saccharomyces cerevisiae* cells, brine shrimp and chick embryo fibroblasts. Hence, we analyzed the viability of normal cells exposed to varying concentrations of the latex in *S. cerevisiae*. Cytotoxicity was also tested using brine shrimp assay and LC_{50} was calculated. The cell viability was assessed by MTT assay, SRB assay and Neutral red assay using various concentrations of the latex. Our study was focused on the combined effect of etoposide, a standard chemotherapeutic drug and latex of *E. antiquorum* on normal cells namely chick embryo fibroblasts and apoptotic events were observed using DAPI, PI EtBr staining. Cytotoxicity assays using *S. cerevisiae* and Brine shrimps showed that the latex was safe at lower concentrations compared to higher dose. Latex effectively counteracted etoposide mediated cytotoxicity, implying that these extracts can be used to protect non-cancerous cells in the body against etoposide induced ill-effects during cancer treatment.

Key words: Cytotoxicity, *in vitro* models, LC_{50}, brineshrimp assay, *Euphorbia antiquorum.*

INTRODUCTION

The plant kingdom has been the best source of remedies for curing a variety of disease and pain (Salazar-Aranda et al., 2009). Phytotheraphy in Asia is particularly widespread (Mohanty and Cock, 2010). The substances that can either inhibit the growth of pathogens or kill them or have no or least cytotoxicity to host cells are considered candidates for developing new antimicrobial drugs (Rajendran and Ramakrishnan, 2010). However, the isolation and chemical characterization of plant derived compounds and its possible use for human well being requires solid and lengthy research and one of the determinants in this complex evaluation concerns the toxic potential of the studied agent (Perez, 2005).

Saccharomyces cerevisiae is commonly known as baker's yeast. Yeast is an easily manipulated model system (Costa et al., 2007). Hence, the candidate plant was subjected to cytotoxic testing to analyze its toxic potential and evaluate concentration at which it gives maximum protection to the survival of cells. Different chick embryo model systems allow for comprehensive analysis of specific stages and aspects of cancer cell dissemination (Deryugina and Quigley, 2008). Hence, the sacrificing vertebrate animals will be overcomed by using *S. cerevisiae* and primary cells as model system. The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of cytotoxicity (Manilal et al., 2009). Research using *in vitro* cell culture methods has a number of limitations to a complete understanding of biological systems *in vivo*. The primary somatic cells, however, are valuable tools to enable the study of a variety of cellular and biochemical functions under tightly controlled experimental conditions which provides the internal environment of the system (Oh et al., 2007).

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METHODOLOGY

Collection of latex

The 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 was collected from plant by breaking up the stem of *Euphorbia antiquorum*. The latex was collected in the morning hours between 8 to 9 am in a glass container and maintained in an ice-cold condition till the use of latex for extraction.

Preparation of sample

The latex collected was extracted with methanol. The methanolic extract was prepared by dissolving 1.0 ml of latex in 5.0 ml of methanol and allowed to evaporate at 60°C in a water bath. The remaining residue was dissolved in minimum amount of DMSO (5 µl/20 mg) stored and used for the assays. This concentration is safe to cells. In fact, DMSO is being used as a cryoprotectant to freeze the cells. We have also tested for its cyototoxicity and optimized the dose.

Cytotoxic assays

The latex of *E. antiquorum* was reported to possess toxic properties. Despite the plant's high toxicity, appropriate doses of its extract have been found to have medicinal effects recognized in traditional medicine around the world (Kaushik and Goyal, 2008). Hence, cytotoxic studies are adopted to assess the appropriate concentration at which the latex extract gives maximum protection and less toxic to the cells. Cytotoxic effect of latex of *E. antiquorum* was analyzed by the following methods. Viable cells and dead cells present after treatment with different concentrations of latex of *E. antiquorum* namely 10, 20, 30, 40, 50 µg in the presence of *S. cerevisiae* cells, tested using the following cytotoxic assays.

The extent of viability of yeast cells with different concentration of latex was analyzed by MTT assay (Igarashi and Miyazawa, 2001). The extent of viable cells present in the treatment group based on the uptake of neutral red dye by viable cells which was then measured photometrically by neutral red assay (Borenfreund et al., 1990). The extent of cell death was also analyzed after treatment with different concentrations of latex by the SRB assay (Skehan et al., 1990).

Brine shrimp lethality test

The toxicity towards brine shrimp with different concentration of latex will be a measure of cytotoxic properties of the test materials. The brine shrimp eggs were procured from Xavier's College of Arts and Science, Thirunelvelli, which was maintained at room temperature (Meyer et al., 1982; Solis et al., 1993).

Apoptotic assays

Chemotherapeutic drugs are efficient in treating diseases, when combined with chemotherapy herbal medicine would rise the efficacy and lower toxic reaction these facts have raise the feasibility of the combination of herbal medicine and chemotherapy (Wen-Jing et al., 2006). The primary cells were cultured from the 8th to 10th day egg of chick embryo. The etoposide and/or plant extract exposure was given for 24 h to cultured primary cells. Concentration of etoposide used for the entire assay was 200 µM. The nuclear changes that are characteristic of apoptotic events resulted after the treatment due to the effect of etoposide and *E. antiquroum* was validated by the following assays, namely EtBr staining, PI and DAPI staining. Ethidium bromide staining technique is used to differentiate the final nuclear apoptotic morphology as explained by Mercille and Massie (1994). The cleavage of nuclear scaffold and chromatin condensation after treatment was investigated by PI staining (Sarker, 2000). Nuclear condensation and fragmentation in dead cells can be visualized by staining the nucleus and its fragments with fluorescent dye, DAPI (Rashmi et al., 2003).

RESULTS

The combination of several cytotoxicity endpoints in a single assay increases the chance that potential bioactive cytotoxic compounds are discovered during the screening of a mixture of natural compounds (Ivanva and Uhilig, 2008). Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relaying mainly on traditional medicines for primary health care (Owalabi and Omogbai, 2007).

Cytotoxicity assays

The extent of cytotoxicity can be measured by MTT, SRB and NR assays which assesses the maximum viability of cells. The percent viability was analyzed by MTT assay after treatment with different concentration of latex of E. *antiquorum.* The results obtained for the cell survival as determined by MTT assay, SRB and Neutral red assay showed that the viability of cells sharply decreased with increase in concentration which indicates that the latex of *E. antiquorum* exhibited minimum cytotoxic effects to *S. cerevisiae* cells in a concentration dependant manner. The results obtained for the cytotoxicity assays are tabulated in Table 1. The results obtained for the cytotoxicity assays imply that the percentage of cell viability decreased with increase in latex concentration, which supports the indication that the toxicity of latex increases with the increase in concentration.

Brine shrimp lethality test

The Brine shrimps cytotoxicity was considered as convenient probe for preliminary assessment of toxicity test samples. The small aquatic organisms are nowadays used for cytotoxic studies to analyze the toxic effects of plant extract. The lethality of the methanolic extract of latex of *E. antiquorum* was determined using brine shrimp. The lethal concentration LC_{50} of the test sample after 24 h was obtained by a plot of percent of the

Table 1. Effect of varying concentrations of methanolic extract of latex of *E. antiquorum* on viability of *Saccharomyces cerevisiae* cells as determined by MTT, SRB and neutral red assay.

S/N	Sample (µg)	Cell survival (%)		
		MTT (µg)	SRB (µg)	NR (µg)
1	Control	100	100	100
2	10	60	91	84
	20	63	81	80
	30	77	80	76
	40	80	80	71
	50	86	70	69

Values are mean of duplicates. Control groups were fixed as 100% viability; the other groups were calculated relative to this.

shrimps killed against the logarithm of the sample concentration and the best-fit line was obtained from the data. The LC_{50} value of methanolic extract of latex was found to be 6.76 µg ml^{-1}. LC_{50} was obtained from best fit-line slope of the graph. The test was done in triplicates (n=3).

The present study revealed that the latex has cytotoxic effects at higher concentration. At lower concentration, the mortality rate of the shrimps found to be less which indicates that at low concentration the survival rate of shrimps are increased and protected from the effects of test sample. We next attempted to see the effect of the latex extract on the apoptotic events induced by chemotherapeutic drug etoposide using untransformed cells normal cells namely primary chick embryo fibroblasts. This was done in order to ascertain whether the cytotoxicity of latex can be exploited for anticancer therapy/combination therapy along with standard chemotherapeutic drug.

Ethidium bromide staining (EtBr)

The nuclear morphology that characterize apoptosis are chromatin condensation, nuclear fragmentation and cornering of the nuclear contents were analyzed using Ethidium bromide staining (EtBr). The nuclear changes were observed and quantified in the chick embryo fibroblast cells exposed to etoposide in the presence and the absence of methanolic extract of latex of *E. antiquorum*. The number of cells showing nuclear apoptotic morphology was counted in each experiment group, and the results are presented in Figure 1. The results of above experiment proved that the latex imparts complete protection to the primary cells which was exposed to etoposide and from the results it was also observed that latex does not induce apoptosis in normal cells but it modulates the apoptotic effects produced by etoposide. The apoptotic index was calculated and found that the groups treated with the latex showed less number of apoptosing cells compared to those treated with etoposide as indicated in Figure1.The results of the earlier discussed experiment proved that the latex imparts complete protection to the primary cells which was exposed to etoposide and from the results it was also observed that latex does not induce apoptosis in normal cells but it modulates the apoptotic effects produced by etoposide. Similar results were observed in propidium iodide staining (Plate 1) and DAPI staining as shown in Plate 2 and Figure 2. From the apoptotic assays, it was concluded that the latex extract of *E. antiqourum* was not or least cytotoxic to the normal cells and it gives maximum protection and reduced the apoptotic cell death imparted by etoposide. From the outcome of our study it was clear that the latex of *E. antiquorum* possessed cytoprotective effects in *S. cerevisiae* cells and in brine shrimps. The LC 50 value was found to be 6.76 µg ml-1. The studies also revealed that the latex gave maximum protection to the chick embryo fibroblast cells subjected to oxidative stress by Etoposide. All these findings confirm the cytoprotective effects of latex of *E. antiquorum*.

DISCUSSION

The cytotoxic assays have been carried out in order to assess the optimal concentration at which, the latex gives maximum protection to the cells. Cytotoxic assays MTT, SRB and Neutral red were used to analyze the cytotoxic effects of latex in *S. cerevisiae* cells. *S. cerevisiae* cells were used as a model organism for normal cells. The latex extract was added in 5 different concentrations namely 10, 20, 30, 40, 50 µg. The results revealed that the latex at lower concentration showed better cell survival compared to higher concentration. Najaran et al. (2009) evaluated the cytotoxicity activity of total methanol root extract of *Scutellaria litwinowii* and its different fractions by MTT assay using malignant cell lines HeLa, MCF-7 and Pcl2. His results showed this extract

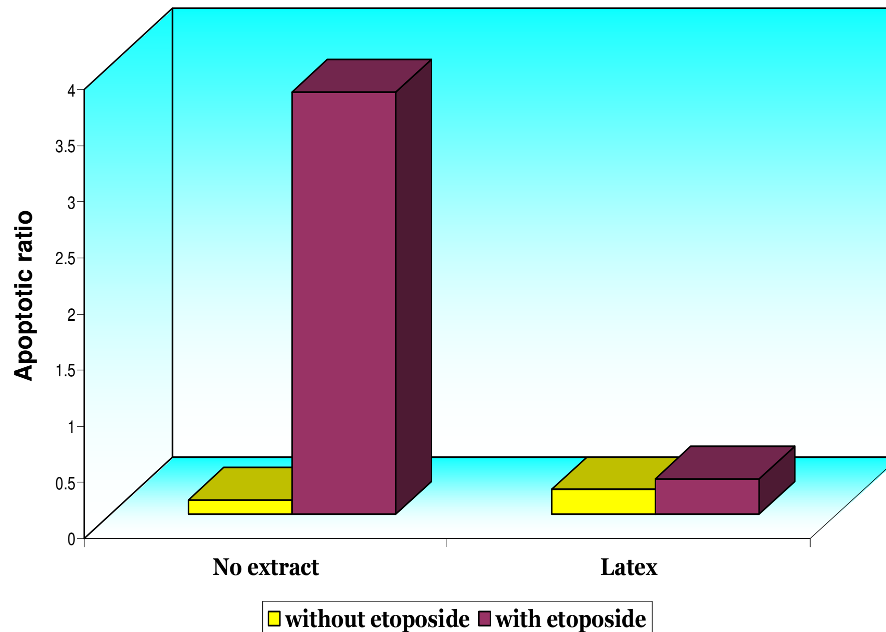

Figure 1. Effects of *Euphorbia antiquorum* latex on etoposide induced apoptosis in chick embryo fibroblasts (EtBr staining).

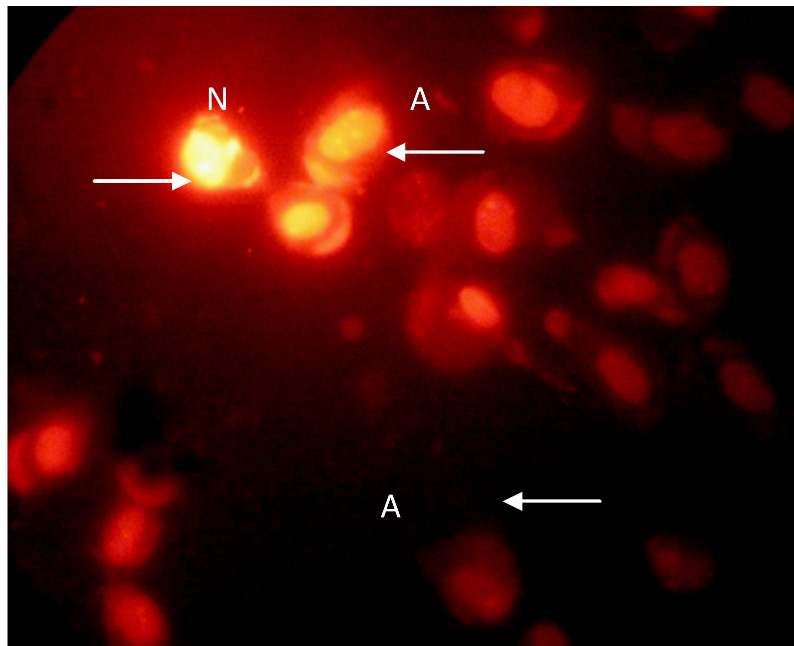

Plate 1. Ethidium bromide staining showing normal and apoptotic cells in chick embryo fibroblast cells.

decreased cell viability of cells in a concentration-dependent manner which is in agreement with our results.

Riagano et al. (2009) assessed the cytotoxic activity of different extracts from rhizomes and flowers of *Iris pseudopemila* and showed that the chloroform extract of rhizome possess good cytotoxic activity against a melonotic melanomma cancer cell line (C32) using SRB assay. Akroum et al. (2009) showed that the methanolic extracts of *Camellia sinesis*, *Cichorium intybusl*, *Lippie citriodora* and *Pucina granatum* exhibited noticeable cytotoxicity activities against FL cells which support our

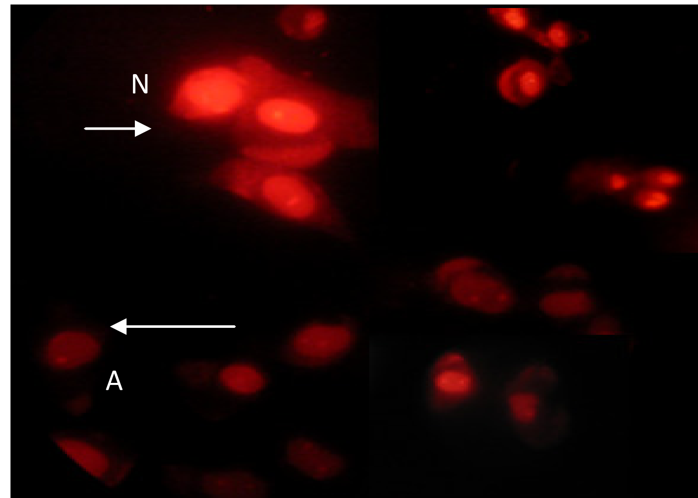

Plate 2. Nuclear morphology of normal and apoptotic cells by propidium iodide staining in chick embryo fibroblast cells.

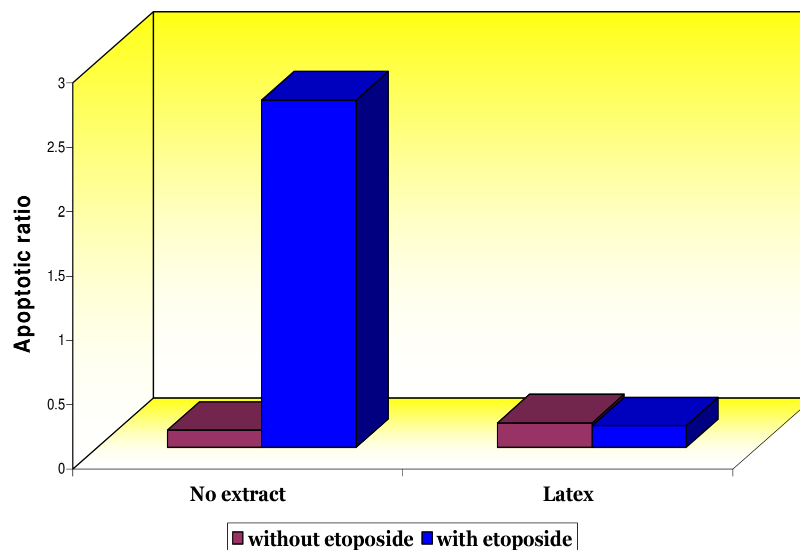

Figure 2. Effects of *Euphorbia antiquorum* latex on etoposide induced apoptosis in chick embryo fibroblasts (PI staining).

findings. Another model organism used to test cytotoxicity was brine shrimp. The cytotoxicity studies using brine shrimp showed that the latex was safe at lower concentration compared to higher dose. The methanolic extract of *Glycyrrhiza glabra* possessed potent cytotoxic activity having LC_{50} value of 0.771 µg/ml (Sultana et al., 2010). The results of studies using *S. cerevisiae* and brineshrimp helped us to optimize the dose at which the latex is safe. The latex was able to render protection to normal chick embryo fibroblast cells treated with anticancer drug etoposide. The results EtBr, PI and DAPI staining revealed the apoptotic nuclear morphology was seen in all the staining. Affrin et al. (2009) reported that the ethanolic leaf extract of *Piper sarmentosum* induced anticarcinogenic activity in HepG2 cells *in vitro* as confirmed by ethidium bromide staining which showed typical apoptotic morphological changes. Isoflavones including genistain from flower of lupin at high concentration induces apoptosis in Chinese hamster ovary cells was assessed in PI staining of cells by Rucinska et al. (2007). Diethyl ether extract of wood from sukan (*Artocarpus altilis*) decreases cell viability in a dose dependant manner and cells exhibited apoptotic morphology after treatment (Arung et al., 2009). The data

from the staining method revealed that the latex extract gave maximum protection to the cells undergoing apoptosis. The latex itself did not induce any apoptosis in normal cells but it modulates the apoptotic effect induced by etoposide. The results revealed a steep increase in the number of apoptotic primary cells in etoposide treated group. Latex effectively counteracted etoposide mediated cytotoxicity implying that these extracts can be used to protect non-cancerous cells in the body against etoposide induced ill-effects during cancer treatment. The results also substantiate the use of latex along with anticancer drug because it reduces the side effects of chemotherapy in normal cells. The outcome of the study suggests that the latex can be effectively used along with chemotherapeutic drugs for cancer treatment, because the latex by itself is not cytotoxic and when given along with the standard chemotherapeutic drug etoposide, it was able to counteract the toxicity of the drug in normal chick embryo fibroblasts. The results also suggest its use in combination therapy along with chemotherapy to fight cancer.

Conclusion

From the research work, it can be concluded that the latex of *E. antiquorum* was found to be safe to normal cells namely *S. cerevisiae*, brineshrimps and chick embryo fibroblast cells. Hence, the latex of candidate plant is safe to normal cells it can be recommended for medicinal uses. Further investigations can be extended to find its anticancer property using cell lines.

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