METHODOLOGY

3.0 METHODOLOGY

The present study was carried out to study the effect of *Triticum aestivum* (Plate 1) leaf extract on apoptosis induced in primary chick embryo cells by etoposide. This chapter describes the methodology that was used for assaying various parameters for analyzing the antiapoptotic activity of *Triticum aestivum* in the present study. The detailed procedures for the various assays are given in the appendices.

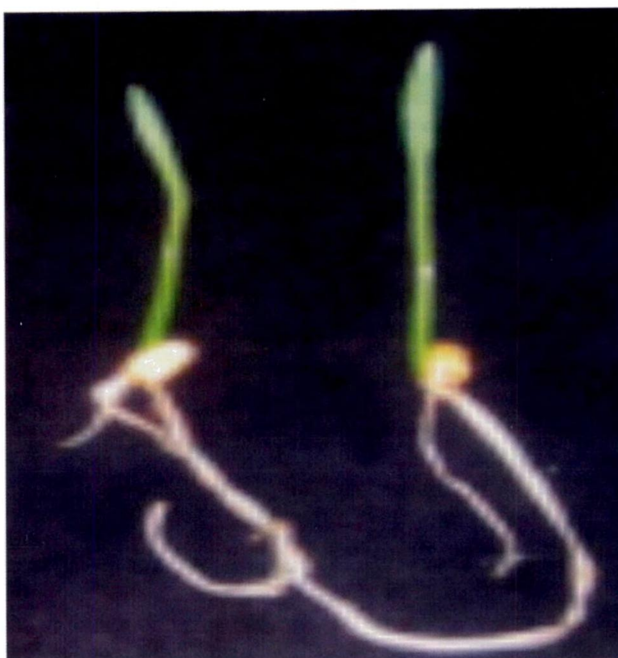
Earlier studies in our laboratory on wheat grass had shown that higher antioxidant content was observed in the leaves on the 4th day after sowing (Vidya, 2007). The seeds of *Triticum aestivum* were procured from a local market and were grown in the medicinal garden within the University premises as pot culture.

The leaves of the plantlets were collected on the fourth day after sowing for each analysis. The leaves were thoroughly washed in running tap water in order to remove any waste droplets. They were then weighed and extracted in methanol.

Preparation of methanolic extract of leaves

One gram of the leaf was homogenized in approximately 1ml of methanol. The extract was clarified by centrifugation. The supernatant was collected and dried at 60°C well protected from light. The residue obtained after drying the methanol extract was weighed and dissolved in a known amount of dimethyl sulfoxide (DMSO) to yield a concentration of 20mg/5μl. DMSO was maintained at a minimum level to avoid DMSO induced events, if any.

PLATE 1



Triticum aestivum

Establishing the primary cell culture

The chick embryo fibroblasts isolated from 8-11 day old chick embryo were cultured in DMEM (PAA) medium in 25cm^2 tissue culture flasks (Appendix I) and incubated at 37°C. The medium was supplemented with 10 per cent FBS (PAA). Penicillin and streptomycin were also added to the medium to 1X final concentration from a 100X stock.

Once a confluent monolayer was obtained (Plate 2), the cells were removed by trypsinisation and seeded in 6 well plate and 96 well plates. The cells were allowed to grow by incubating in 5 per cent CO_2 and 95 per cent humidity to monolayer and then subjected to treatments. After treatment with oxidative stress and /or leaf extract, the cells were washed with PBS and then used for the analysis of various parameters.

Treatment groups

The following treatment groups were set up

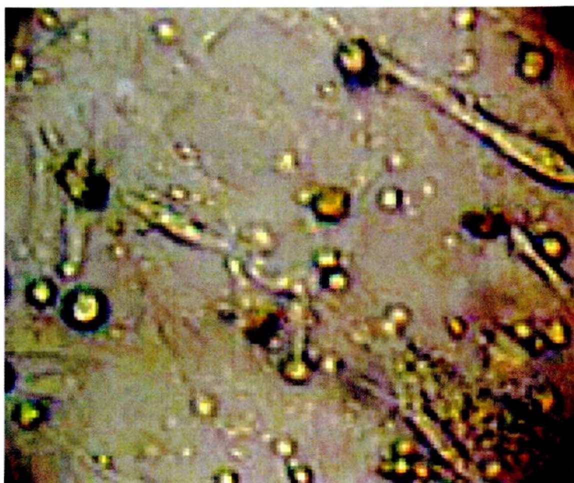
1) Untreated (negative) control
2) Etoposide treated (positive) control
3) Methanolic extract of *Triticum aestivum* leaf treated group
4) Etoposide+ methanol extract of *Triticum aestivum* leaf treated group

The concentration of etoposide used for all the assays was 200µm, while the extract was 20mg/ml.

Parameters analysed

The extent of cell survival was quantified using the SRB assay and the MTT assay. SRB assay provides a sensitive measure of drug-induced cytotoxicity. The protocol developed by Skehan *et al.* (1990) was used (Appendix II). The MTT assay, which quantifies the viability of cells, was done by procedure of Igarish and Miyazawa (2001), which is elaborated in Appendix III.

PLATE 2



MICROGRAPH OF CHICK EMBRYO FIBROBLASTS

Morphological changes during apoptosis

The various morphological changes characteristic of apoptosis were observed in the presence and the absence of etoposide and the leaf extract. Giemsa staining was done for better visualization under phase contrast microscpe (Motic, HongKong). The protocol employed by Chih *et al.* (2001) was used and the details are explained in Appendix IV.

Nuclear changes during apoptosis

Apoptotic nuclei undergo typical changes including chromatin condensation, peripheral marginalization, nuclear shrinkage and subsequent fragmentation (Cohen *et al.*, 2003). These changes were investigated in the cells in the presence and absence of leaf extract and / or etoposide, by ethidium bromide staining as described by Mercille and Massie (1994) with minor modifications, and propidium iodide staining as described by Sarker *et al.* (2000). The details of these procedures have been given in Appendices V and VI respectively.

Apoptotic index

Nuclear condensation and fragmentation in apoptotic cells can be visualized by staining the nucleus and its fragments with a fluorescent dye such as DAPI that binds the DNA. The procedure developed by Rashmi *et al.* (2003) was employed and is explained in Appendix VII.

DNA fragmentation

DNA fragmentation occurs as a late phase of apoptosis. The DNA isolated from etoposide treated as well as etoposide and extract treated cells were subjected to agarose gel electrophoresis and observed for the electrophoretic pattern during apoptosis. This was done by the method proposed by Yin *et al.* (1994) with slight modifications. The details of the procedure have been documented in Appendix VIII.

The result obtained for the various parameters analysed and the inferences that can be drawn from them are presented and discussed in the next chapter, with relevant literature support.