RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

Apoptosis is a highly regulated and fundamental biological process governing cell survival. During apoptosis, the integrity of the cell membrane is maintained, thereby preventing release of the histotoxic cell contents. Because apoptotic cells are instantly recognized by phagocytes and removed from the inflammatory site, successful apoptosis is now recognized to be crucial to the resolution of inflammation. Failure of inflammatory cells to undergo apoptosis or failure of subsequent phagocytic removal of apoptotic cells is believed to result in incomplete resolution and an exacerbation of the inflammatory response (Shaw *et al.*, 2009).

Apoptosis is characterized morphologically by cell shrinkage, disruption of cell-cell contacts, chromatin condensation, membrane blebbing, formation of apoptotic bodies, and finally, phagocytosis and degradation. Biochemically, apoptosis is detected by the presence of endonucleolytical DNA cleavage and cleavage of caspases. Both morphological and biochemical characteristics help in recognizing the presence of apoptotic cells (Erman *et al.*, 2009).

Medicinal plants are plants that provide people with medicine to prevent disease, maintain health or cure ailments. The use of the whole plant or plant part(s) as a herbal remedy is an important approach in providing new therapeutic products. The development of standardized phytomedicine products with proven efficacy, safety and quality is an important strategy in providing access to new medicinal plants. Because herbal medicines usually cost less than synthetic drugs, standardization and quality control are feasible, and the occurrence of undesirable side effects seems to be less frequent when compared to synthetic drugs (Stasi, 2005). Epidemiological

evidence increasingly suggests that consumption of a diet rich in plant foods has a protective effect against cardiovascular disease and certain forms of cancer (Neuhouser, 2004).

One such plant is *Triticum aestivum*, commonly called wheat grass. The present study focused on the ability of the methanolic extract of the leaves of *Triticum aestivum* in influencing the process of apoptosis induced by etoposide in chick embryo fibroblasts. The results obtained are presented and discussed below.

Sulphorhodamine B (SRB) assay

This assay provides a measure of drug-induced cytotoxicity and a sensitive method for measuring the viability of cells. It is well suited to high volumes of automated drug screening. In the present work, Sulphorhodamine B assay was used to quantify the extent of cell survival and proliferation efficiency of chick embryo fibroblasts exposed to etoposide in the presence and the absence of *Triticum aestivum* leaf extract. The data obtained are presented in Table I.

TABLE I

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON THE VIABILITY OF CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY SRB ASSAY

SAMPLE	CELL SURVIVAL (PER CENT)	
	CONTROL	ETOPOSIDE
No extract	100	26
Triticum aestivum	98	87

The values are means of triplicates. The control was fixed as having 100% survival and the per cent survival in the other groups were calculated relative to this.

The results obtained for the cytotoxicity as determined by the SRB assay showed that the viability drastically reduced in the etoposide treated group of cells. But the cell viability significantly increased in the *Triticum aestivum* leaf extract treated cells even in the presence of apoptosis-inducing stress. So the leaf extract, when administrated along with etoposide, was able to reverse the toxicity to the normal chick embryo fibroblast cells.

Several reports in the literature have validated the SRB assay as a relevant tool in quantifying the extent of survival. Chen *et al.* (2008) reported that Denbinobin exhibits cytotoxicity by inducing AIF in human colorectal cancer HCT-116 cells, which was identified by the SRB assay. The methanolic extract of *Tulborghia violacea* was screened for cytotoxic activity in HT-29, HeLa and MCF-7 carcinoma cell lines by using SRB assay (Bungu *et al.*, 2006).

Choi *et al.* (2007) examined the effect of anthocyanidins and anthocyanins against Dox-induced cytotoxicity, which was evaluated using the SRB assay in H9C2 cells. The protective effect was higher in cyanidins and delphinidin than other anthocyanidinins.

The per cent survival extent in the present study validated that the *Triticum aestivum* leaf extract has very good antiapoptotic property and protects the cells against death caused by etoposide.

MTT assay

It is known that MTT assay is an established technique to estimate the *in vitro* cellular viability in the disease-oriented screening protocols in cancer research. Besides measuring viability, activation, and proliferation, the MTT assay has also been recently adopted to measure the clonogenecity of cancer cells in culture. The MTT assay depends both on the number of cells present and on the mitochondrial activity per cell (Dharmu *et al.*, 2007).

In the present study, the per cent viability was analyzed by MTT assay in the different treatment groups. The values obtained are presented in Table II.

TABLE II

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON THE VIABILITY OF PRIMARY CHICK EMBRYO FIBROBLASTS TREATED WITH ETOPOSIDE AS DETERMINED BY MTT ASSAY

SAMPLE	CELL SURVIVAL (PER CENT)	
	CONTROL	ETOPOSIDE
No extract	100	48
Triticum aestivum	92	89

The values are means of triplicates. The control was fixed as having 100% survival and the per cent survival in the other groups were calculated relative to this.

Chick embryo fibroblasts treated with etoposide caused a marked decrease in the viability of the cells. The co-administration of leaf extract of *Triticum aestivum* caused an increased in cell viability. The extent of cell survival in the leaf extract group was comparable to that of untreated control suggesting that the plant extract was not cytotoxic to the cells.

Several studies reported in the literature have used MTT assay as a valuable tool. The cytotoxicity of five compounds isolated from the Chinese herb *Fructus schizandrae* to the cell lines such as KB, KBV 200, MCF-7/DOX and Bcl 7402 explained the reversal of p-glycoprotein mediated

multidrug resistance of cancer cells by using MTT assay (Huang *et al.*, 2008).

Li *et al.* (2009) investigated the effects of *Chrysanthemum indicum* extract on inhibition of proliferation and on apoptosis in a human hepatocellular carcinoma (HCC) MHCC97H cell line by using MTT assay. Rengyolone exhibited non-cytotoxic effects by inhibiting apoptosis via etoposide-induced caspase downregulation when it was administrated to human promyeloid leukemia U937 cells, as identified by MTT assay (Kim *et al.*, 2009). The cell viability estimated by the MTT assay showed that the diallyl disulphide (DADS) induced cytotoxicity in PC-3 (human prostate caner cell line) by expressing the apoptosis-associated proteins in androgen independent PC-3, which exhibited decreased cell survival with increased DADS concentration (Gayathri *et al.*, 2009).

Yuan *et al.* (2008) investigated the effect of various anticancer drugs on the PA 317/Tct-on/TRE-BCKP cells by using BCRP as a biomarker predicting resistance to 5-fluorouracil and viability of cells was measured by using MTT assay. MTT assay was designed to investigate the prevalence of leptin and Ob-R in middle eastern epithelial ovarian cancer (EOC) and to analyse the role of leptin and the mechanisms under its action in EOC tissue sample and cell lines (Uddin *et al.*, 2009).

The per cent viability obtained in the present study clearly demonstrated the extent to which the cells are protected by *Triticum aestivum* leaf extract in the presence of oxidative stress inducing agent etoposide. The increase in the per cent viability in the etoposide and plant extract treated cells confirm that the plant extract exhibits very good antiapoptotic effect.

Morphological changes

Giemsa staining

Morphological changes including membrane blebbing, cell shrinkage, disruption of cell-cell contacts, chromatin condensation and formation of apoptotic bodies characterize apoptosis (Erman *et al.*, 2009). In the present study, the morphological changes related to apoptosis in the chick embryo fibroblasts in both the presence and the absence of leaf extract and/or etoposide were observed by phase contrast microscope. The results obtained are presented in table III.

TABLE III

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON MORPHOLOGICAL CHANGES IN ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS (GIEMSA STAINING)

Sample	Normal cells (per cent)		Apoptotic cells (per cent)	
	Control	Etoposide	Control	Etoposide
No extract	95	27	5	73
Triticum aestivum	86	81	14	19

The values are means of triplicates.

Fibroblasts treated with etoposide caused a steep increase in the number of cells showing apoptotic morphological changes (Plate 3). The administration of *Triticum aestivum* leaf extract alone or with etoposide increased the number of live cells and a significant decrease was observed in the number of apoptotic cells. These results indicate that *Triticum aestivum* leaves can render protection against etoposide-induced cell death.

PLATE 3

ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS SHOWING APOPOTIC MORPHOLOGY (GIEMSA STAINING)

The apoptotic ratio was calculated as the ratio of apoptosing cells to the normal cells.The data obtained is represented in Figure 1.

Several reports in the literature have followed the typical morphological features as markers of apoptosis. Morphological changes were observed in urothelial cells during postnatal development in rat (Erman *et al.*, 2009).

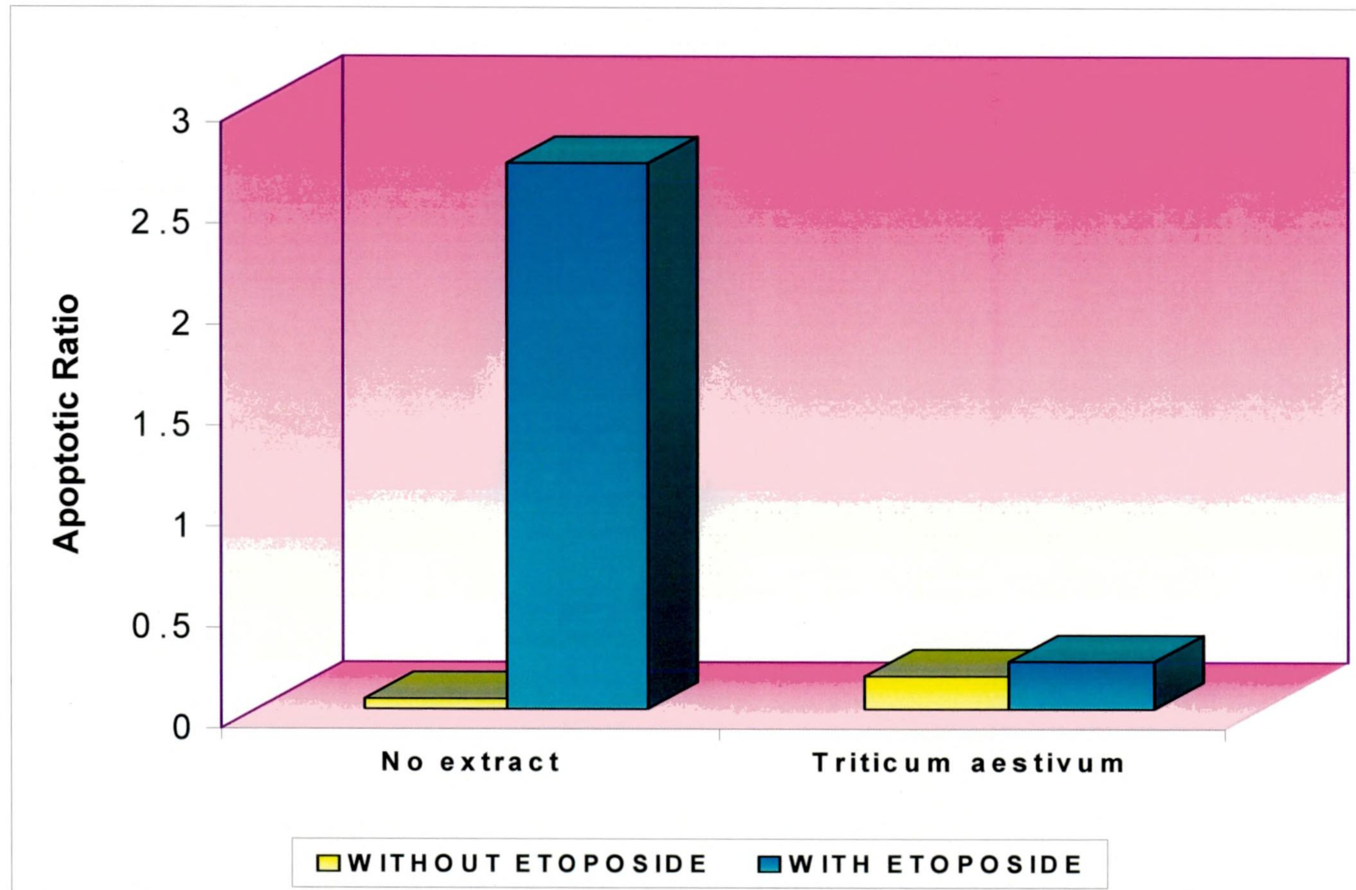
Ethanolic extracts from *P.sarmentosum* could induce apoptosis in Hep G2 cells, as proven using May-Grunwald Giemsa staining in MCF-7 breast cancer cells (Singh *et al.*, 2008). Zhou *et al.* (2007) reports that oridinin, a diterpenoid extracted from medicinal herb showed potent antitumor activity with low adverse effects on U937 cells. Hydrogenkwadaphnin (3-HK), a daphnane-type diterpene ester isolated from *Dendrostellera lessertii* (Thymelaeceae) induced apoptosis in chronic myelogenous leukemia cell K562, as shown by giemsa staining (Meshkini and Yazdanparast, 2007).

The biological stability of QY_1 pluripotential mesenchymal stem cell line from Sprague Dawley rat bone marrow was confirmed by giemsa staining (Xie *et al.*, 2006). Celecoxib treatment induced apoptosis was observed by giemsa staining associated with morphological changes in K562 cells (human chronic myeloid leukemia cell line) (Subhashini *et al.*, 2005). Small hairpin RNA (shRNA) targeting at Bcl-2 mRNA could enhance cytarabine (Ara-c) induced apoptosis in Raji cells was proved by He and Fang (2009) using giemsa staining.

In the present study, etoposide caused morphological changes such as membrane blebbing in chick embryo fibroblasts and the methanolic extract of *Triticum aestivum* leaves was very effective in decreasing the etoposide-induced apoptosis in chick embryo fibroblasts.

Figure 1

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON MORPHOLOGICAL CHANGES IN ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS (GIEMSA STAINING)



Nuclear changes

Nuclear changes such as nuclear fragmentation, chromatin condensation and marginalization, are the confirmatory signs of apoptosis. In the present study, nuclear changes were identified by ethidium bromide and propidium iodide staining. The apoptotic ratio was calculated by counting the cells using fluorescence microscope.

Ethidium bromide staining

Table IV represents the data obtained on the number of apoptosing chick embryo fibroblasts.

TABLE IV

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON PRIMARY CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (ETHIDIUM BROMIDE STAINING)

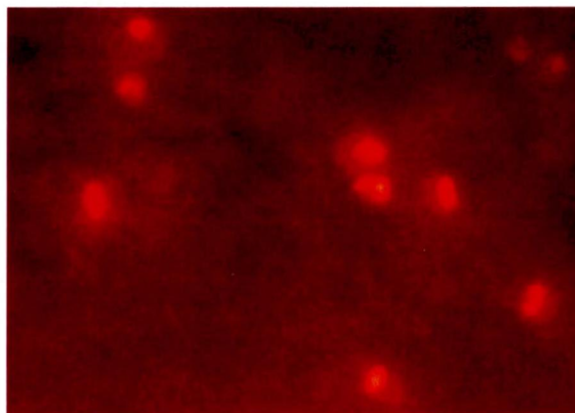
Sample	Normal cells (Per cent)		Apoptotic cells (Per cent)	
	Control	Etoposide	Control	Etoposide
No extract	90	21	10	79
Triticum aestivum	82	78	18	22

The values are means of triplicates

From the above data, the ratios of apoptosis to normal cells were calculated for each group, which is represented in Figure 2.

There was a steep increase in the number of cells undergoing apoptosis when subjected to etoposide treatment (Plate 4). When the methanolic extract of *Triticum aestivum* leaves was co-administrated to the cells, a significant decrease was observed in the number of apoptosing cells.

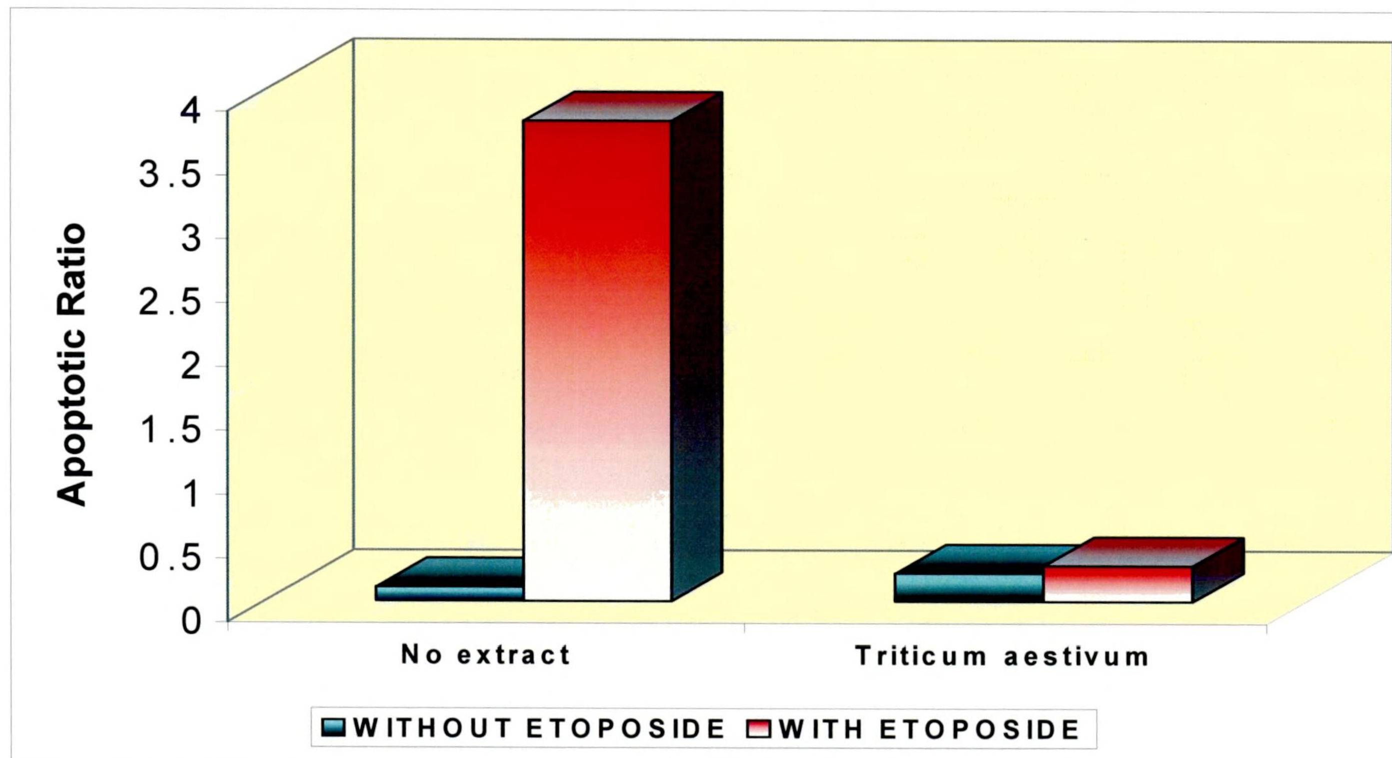
PLATE 4



ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS SHOWING APOPOTIC DEATH (ETHIDIUM BROMIDE STAINING)

Figure 2

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON PRIMARY CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (ETHIDIUM BROMIDE STAINING)



These results indicate that *Triticum aestivum* leaves can render protection against etoposide-induced cell death.

Ethidium bromide has been used as a staining agent to quantify the number of cells showing nuclear changes in several studies. Raquenez *et al.* (2009) reported that metastatic neuroblastoma exhibits chromatin condensation during apoptosis induced by Fenretinide.

Heptelidic acid, a sesquiterpene lactone, inhibited etoposide-induced apoptosis in human leukemia U937 cells as identified by ethidium bromide staining (Hee and Hwan, 2009). The apoptotic cells in K562, Hep G2, HL-60 and Jurkat cell lines were exhibited by ethidium bromide staining (Moosavi *et al.*, 2005).

EtBr staining was used for the evaluation of annexin V and calcein-AM as markers of mononuclear cell apoptosis during human immunodeficiency virus infection (Padma *et al.*, 2008). EtBr staining distinguishes between apoptotic cells by the morphological changes in the nucleus and was used to determine whether *Mycoplasma genitalium* LP induced apoptosis or necrosis in THP-1 cells (Wu *et al.*, 2008). Apoptosis induced by acrylamide was suppressed through caspase-3-independent pathway in mice testis, which was proved using EtBr staining (Zhang *et al.*, 2009).

Our results also show that staining with EtBr alone is a reliable method to quantitate the extent of apoptosis and its modulation by various agents. The results further affirm that the leaves of *Triticum aestivum* show anti-apoptotic activity in normal cells.

Propidium iodide staining

Propidium iodide staining is a routine parameter, since apoptotic cells become increasingly permeable to the molecule. The number of apoptotic

and normal cells in the various treatment groups was counted in a fluorescent microscope after PI staining and the data obtained are presented in table V.

TABLE V

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON NUCLEAR CHANGES IN ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS (PI STAINING)

Sample	Normal cells (Per cent)		Apoptotic cells (Per cent)	
	Control	Etoposide	Control	Etoposide
No extract	94	29	6	71
Triticum aestivum	84	77	16	23

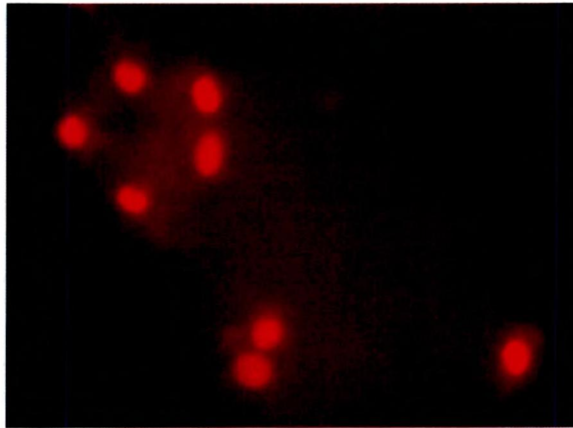
The values are means of triplicates.

From the above data, the ratios of apoptotic to normal cells were calculated for each group, which is represented in Figure 3.

Our results showed that upon etoposide exposure, a remarkable reduction was observed in the number of normal cells (Plate 5). This number increased sharply upon co-exposure to the methanolic extract of *Triticum aestivum* leaf. When the cells were treated with the plant extract alone, there was a slight decrease in the number of normal cells when compared with untreated control.

PI is a membrane impermeable fluorescent DNA-binding dye used to detect cell death. It has been used widely. In cortical neuronal cells, PI staining displayed the apoptotic cells when treated with glutamate and NMDA (Liu *et al.*, 2009).

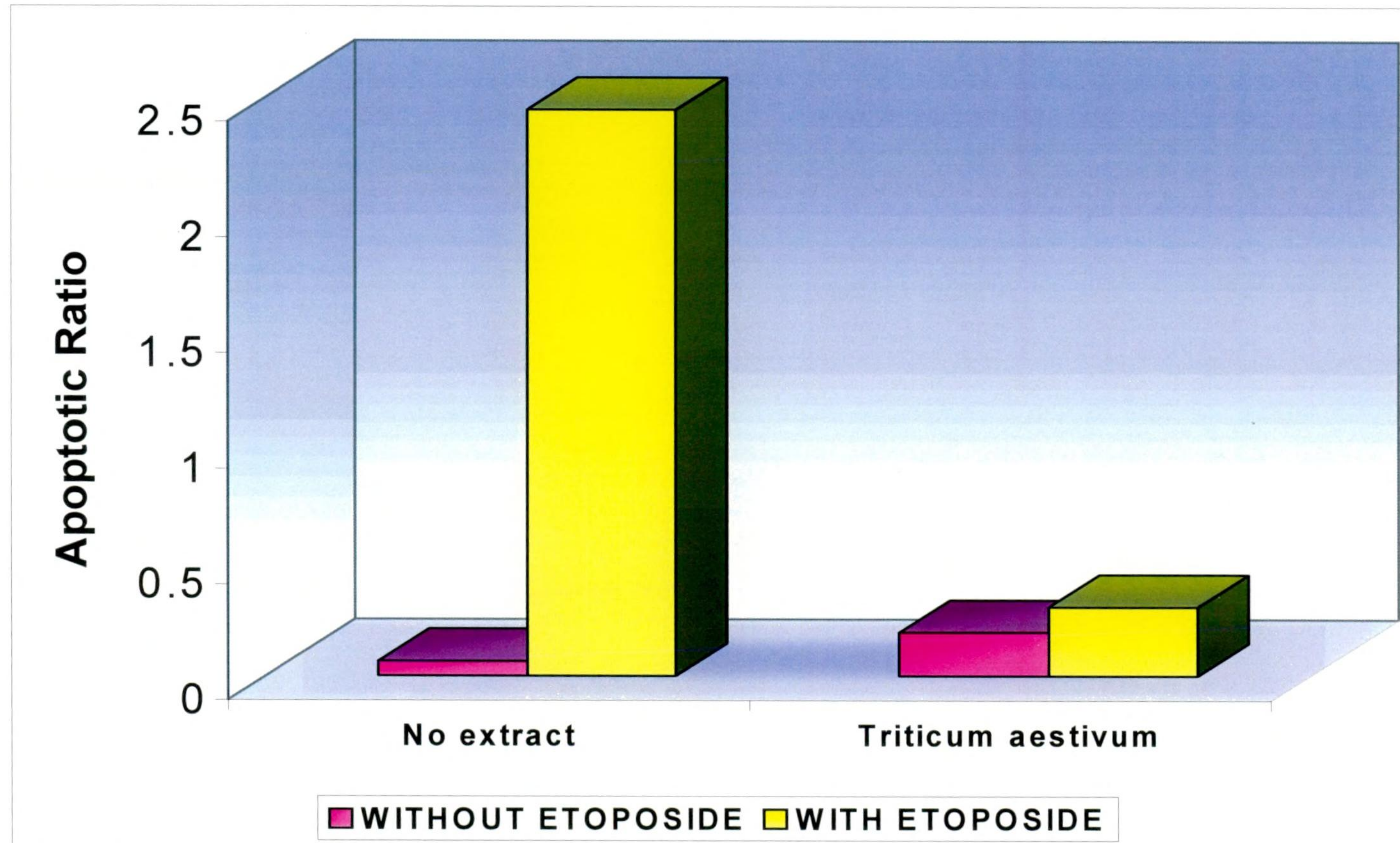
PLATE 5



ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS SHOWING APOPTOTIC DEATH (PROPIDIUM IODIDE STAINING)

Figure 3

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON NUCLEAR CHANGES IN ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS (PROPIDIUM IODIDE STAINING)



Gotz *et al.* (2005) observed that embryonic liver cells and forebrain cells underwent apoptosis in the absence of Bag-1 using PI staining. The human umbilical cord blood-derived stromal cells suppress xenogenic immune cell response *in vitro*, which was shown by Hao *et al.* (2009) using propidium iodide staining. Haddad *et al.* (2004) proved that the triterpene saponins obtained from *Albizia adianthifolia* (Mimosoceae) induces apoptosis on human leukemia T-cells (Jurkat cells) using propidium iodide staining.

Montecucco *et al.* (2009) suggested that oxaprozin-induced apoptosis in CD 40L-treated human monocytes is associated with cyclo-oxygenase (COX)-independent pathways, wherein apoptosis was evaluated by propidium iodide staining. The extent of FITC/propidium iodide staining has been used for studying the protection of cyclic GMP against peroxynitrite-induced apoptosis (Shaw *et al.*, 2009).

PI staining showed that catechol-induced cytotoxicity to neuroblastoma N2a cells was not directly a consequence of reactive oxygen species production; rather, it was due to GSH depletion followed by the induction of apoptosis (Lima *et al.*, 2008).

In the present study, PI staining was effectively used to strengthen the data on apoptosis induced by etoposide and its counteraction by *Triticum aestivum* leaf extract.

DAPI staining

DAPI is a fluorescent dye that binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity, which helps to highlight the nuclear changes during apoptosis. Cell number and density analyses can also be carried out using DAPI staining (Konduri *et al.*, 2007).

TABLE VI

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (DAPI STAINING)

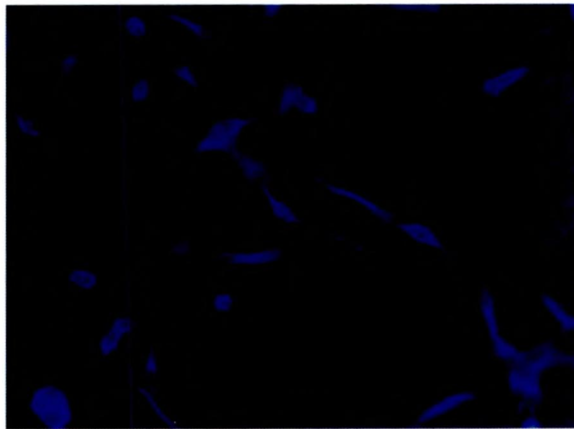
Sample	**Normal cells (Per cent)**		**Apoptotic cells (Per cent)**	
	Control	**Etoposide**	**Control**	**Etoposide**
No extract	93	24	7	76
Triticum aestivum	81	74	19	26

The values are means of triplicates.

From the above results, the ratios of apoptosing to normal cells were calculated for all the treatment groups and are presented in Figure 4.

The results of DAPI staining followed the same trend as for the other staining methods (Plate 6). DAPI staining has been reported in literature by many researchers. Hsu *et al.* (2007) used DAPI staining to detect apoptosis in human colonic carcinoma cells, in which berberine-induced apoptosis through the activation of JWK/p38 MAPK and Fas L. Hepatic injury induced by CCl_4 was recovered by curcumin and the apoptosis was studied by DAPI staining (Priya and Sudhakaran, 2008). TNF-alpha induced apoptosis in two prostatic tumoral cell lines (LNC a^P and PC3) as assayed by DAPI staining and also proved that p38 plays a role in prostatic tumor progression (Belinchon *et al.*, 2005).The extent of DAPI staining was used for proving that Bergapten, independent of its photoactivation, enhanced p53 gene expression and induced apoptosis in human breast cancer cells (Panno *et al.*, 2009). Our data demonstrate the protective effect of *Triticum aestivum* leaf extract against apoptotic cell death induced by etoposide, as determined by DAPI staining, following giemsa, EtBr and PI staining

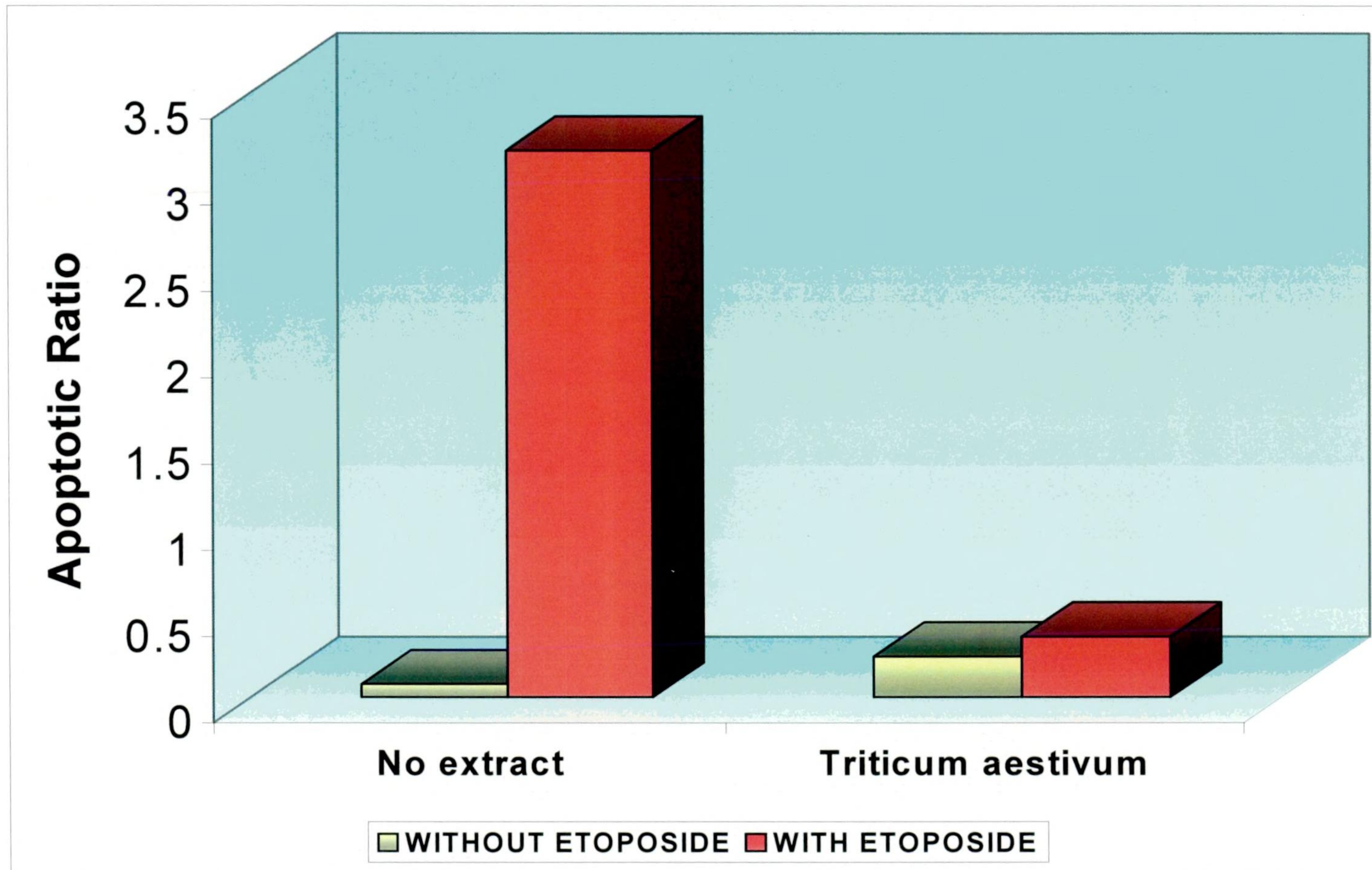
PLATE 6



ETOPOSIDE TREATED CHICK EMBRYO FIBROBLASTS SHOWING APOPTOTIC DEATH (DAPI STAINING)

Figure 4

EFFECTS OF *Triticum aestivum* LEAF EXTRACT ON CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (DAPI STAINING)



DNA fragmentation

Apoptosis is the predominant form of programmed cell death in which nucleosomal DNA fragmentation is the most prominent biological feature. DNA fragmentation is considered to be a hallmark of apoptosis (Cheah *et al.*, 2007).

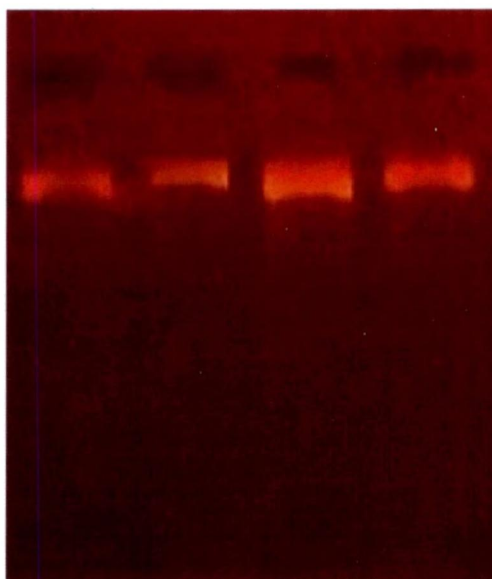
DNA fragmentation is a late event in apoptosis. In the present investigation, the extent of DNA fragmentation was quantified in the chick embryo fibroblasts exposed to etoposide with or without *Triticum aestivum* leaf extract (Plate 7). The extent of DNA damage was documented using a digital gel documentation system and its software (Alpha Ease Fc of Alpha Digidoc 1201).

The dysregulated apoptosis in neutrophils by NFKB expression in COPD subjects was identified by DNA fragmentation (Brown *et al.*, 2009). Tang *et al.* (2009) confirmed that from the assay of DNA fragmentation, mitochondrial death apoptosis cascade plays a very important role in asiatic acid-induced cancer apoptosis.

In the present study, etoposide caused DNA fragmentation in chick embryo fibroblasts, which are non-cancerous, untransformed cells. This toxic effect was effectively counteracted by the leaf extract of *Triticum aestivum*.

Thus, the present study clearly demonstrated that the methanolic extract of *Triticum aestivum* renders strong protection to primary chick embryo fibroblasts against etoposide-induced cell death. These observations support the use of the extract as a supportive therapy in etoposide-administered patients, to protect normal cells.

PLATE 7



DNA FRAGMENTATION IN THE DIFFERENT TREATMENT GROUPS

Lane 1- Untreated Control
Lane 2- Etoposide alone
Lane 3- *Triticum aestivum* Leaf Extract
Lane 4- Etoposide + *Triticum aestivum* Leaf Extract

The outcome of the present study also validates the use of chick embryo fibroblasts as an alternative model to study the apoptotic events evoked by agents like etoposide, and to analyse the effects of medicinal plants on these events.

The results obtained in the present investigation and the conclusions drawn from them are summarized in the next chapter.