REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

The biological evolution has resulted in the adaptation of both unicellular and multicellular organisms to the negative effects of extensive oxygen in reply to a gradual increase of free oxygen contents in the earth's atmosphere. During the evolution of organisms, survival in an aerobic environment came about by adaptive responses, both to the endogenous oxidative metabolism within the cells of organism, as well as the chemicals and low level radical to which they are exposed. This leads to oxidative stress, which is linked to various diseases (Genestra, 2007).

OXIDATIVE STRESS

Oxidative stress has been defined as a loss of balance between free radical or reactive oxygen species (ROS) production and the antioxidant systems, with negative effects on the metabolism of carbohydrates, lipids and proteins (Mates *et al.*, 2008). When oxidative stress is generated, causing selective DNA damage, it can be one of the most important factors on carcinogenesis, and also lead to apoptosis by activating the intrinsic apoptotic pathway (Reinehr *et al.*, 2008).

Oxidative stress has been linked to cardiovascular and infectious diseases, cancer, diabetes, and neurodegenerative pathologies (Powell *et al.*, 2005). It includes a cellular redox imbalance, which has been found to be present in various cancer cells compared with normal cells; the redox imbalance, thus, may be related to oncogenic stimulation (Valko *et al.*, 2006). It is generated by reactive oxygen species (ROS), such as hydroxy radicals and hydrogen peroxide has been linked to several cellular toxicity process (Tung *et al.*, 2008).

REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are highly dangerous byproducts of cellular metabolism that have a direct effect on the devolpment and growth of the cell and its survival (Janani *et al.*, 2008). ROS are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenis (Mates *et al.*, 2008).

ROS are well recognized for playing a dual role as both deleterious and beneficial species. The 'two-faced' character of ROS within cells act as second mesengers in intracellular signaling cascades, which induce and maintain the oncogenic phenotype of cancer cells (Valko *et al.*, 2000).

TYPES OF ROS

Oxygen derived species such as superoxide radical, hydrogen peroxide, singlet oxygen and hydroxy radical are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases, including cancer (Waris and Ahsan, 2006)

Superoxide anion radical ($O_2^{\bullet -}$)

The first oxygen radical occurring in aerobic organisms is the superoxide radical ($O_2^{\bullet -}$), which can be considered as the parent radical from which other oxygen radicals are derived. Some of the oxygen reducing enzymes forms stable complexes with oxygen while subsequently transferring two or even four electrons unless they release their reduction products (Nohl, 2004). Superoxide radicals ($O_2^{\bullet -}$) are one of the major products of radiolysis of cellular constituents in the presence of oxygen (Agarwal *et al.*, 2001).

Hydroxy radical ($OH^{\bullet}$)

$OH^{\bullet}$ is normally generated via two primary mechanisms, i.e., either by hemolytic scission of the water molecule during exposure to ionizing radiation and/or during the interaction of H_2O_2 with a transition metal (Hore, 2004). Among the oxygen derived species, $OH^{\bullet}$ is the most aggressive in damaging neighbouring macromolecules and causes more than the total damage substained as a consequence of free radicals (Pryor, 1995).

This is a highly reactive species has an estimated half-life with organisms in the order of 10^{-9}s. Oxidative stress could also be brought about by trace elements like iron (Fe) and copper (Cu) through glyoxidation and production of superoxide anion ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) free radical which can oxidize membranes proteins and lipids of lens (Sulochana, 2002).

Nitric oxide (NO)

Nitric oxide (NO), a ubiquitous free radical moiety was first discovered in the vascular endothelium (Gulati *et al.*, 2006). The role of nitric oxide in several pathophysiological states have been suggested and NO modulators are effectively used as therapeutic agents in medicine (Monocada and Higgs, 2002).

Nitric oxide (NO) is formed from guanidine nitrogen of L-arginine and during the process it consumes five electrons. The reaction is catalysed by the enzyme nitric oxide synthetase, which exists in several forms (Hore, 2004).

Hydrogen peroxide

H_2O_2 is formed during the dismutation of $O_2^{\bullet-}$ by a family of enzymes called superoxide dismutases (SOD), as well as via several other enzymatic reactions. The non-radical peroxide is generated as a consequence of the redox cycling of catecholamines, from mitochondrial respiration, during

respiratory burst of phagocytes and from microsomal cytochrome p450. Intracellular concentration of H_2O_2 is maintained by its enzymatic degradation by the action of catalase (CAT) and several glutathione peroxidases (Sivalokanathan *et al.*, 2004).

Hydrogen peroxide (H_2O_2) a non-radical ROS is generated *in vivo* by several enzyme systems and additionally it is produced intracellularly by the dismutation of the superoxide anion radical ($O_2^{\bullet -}$). *In vivo*, H_2O_2 is a weak oxidizing and reducing agent, also no electric charge allows H_2O_2 to traverse cell membranes and is therefore accessible to sites significantly removed from its point of generation (Reiter and Tan, 2003).

Antioxidant defense system

The antioxidant systems are major cell defenses, which protect membranes and cytosolic components against damage induced by free radicals under diseased conditions. Natural antioxidants are capable of inhibiting ROS production, thereby reducing the intracellular oxidative stress (Feng *et al.*, 2001).

The cell possesses two distinct antioxidant defense systems to counteract damaging ROS. They are

(i) Enzymatic antioxidants

(ii) Non-enzymatic antioxidants

(i) Enzymatic antioxidants

Enzymatic antioxidants are intracellular proteins. They undergo rapid degradation in blood plasma, lymph and other intracellular fluids. The instability of the enzymes in the extracellular medium significantly hampers their clinical use. Therefore, method of modification of enzyme antioxidants intended to increase the time of their circulation in blood without decreasing their enzymatic activity are extensively studied (Eremin, 2001).

The enzymatic defense mechanism includes activities of enzymes regenerating the reduced forms of antioxidants such as superoxide dismutase, catalase, peroxidase, glutathione reductase and glutathione peroxidase (Jovanovic *et al.*, 2006).

(ii) Non-enzymic antioxidants

Antioxidants such as vitamin C, tocopherols, carotenoids and polyphenols are able to quench free radicals and together with endogenous systems of defense, they limit oxidative stress and reduce the risk of associated degenerative diseases (Nicolle *et al.*, 2003).

SILYMARIN

Silymarin is a naturally derived polyphenolic antioxidant, which also acts as a potent anticancer compound on several cancers (Ramakrishnan *et al.*, 2008). It is composed mainly of silibinin with smaller amounts of other stereoisomers (isosilybin, dihydrosilybin, silydianin and silychristin) (Kohno *et al.*, 2005).

Silibinin is the major component found in silymarin and is thought to be the most biologically active compound. Experimental evidence suggests that there is no significant difference between silymarin and silibinin in terms of chemopreventive or biological activities in several *in vitro* and *in vivo* cancer models (Katiyar, 2005).

APOPTOSIS

Apoptosis or programmed cell death is an evolutionarily conserved and genetically regulated process, which helps an essential role in the development and homeostasis of higher organisms (Chen *et al.*, 2003). It is a natural process by which cells undergo cell death to control cell number and proliferation (Daniel and Korsmeyer, 2004). It is characterized by certain unique morphological features such as cell shrinkage, DNA condensation

nucleus membrane, budding of the cell soma, single cell death without inflammatory reactions (Abend *et al.*, 2004).

Mitochondria is the major site for the generation of cellular oxidative stress and play a key role in mediating programmed cell death (apoptosis) (Birch-Machin, 2006)

Mitochondria are affected early in the apoptotic process and are thought to act as central regulators of apoptosis (Van Loo *et al.*, 2002).

MECHANISM OF APOPTOSIS

A cell undergoes apoptosis when certain intracellular or extracellular stimuli persuade it to activate its own demise. Every cell is wired up for apoptosis, and requires a single appropriate stimulus to initiate the process, via death receptors (extrinsic) or mitochondria (intrinsic) pathways. The apoptotic pathways are extensively networked to other important cellular signaling pathways, e.g., insulin receptor, RAS-PI_3K/AKT and Rb/E_2F pathways, thus expanding the cell's potential apoptosis inducing and regulatory capacity.

(i) Extrinsic pathway

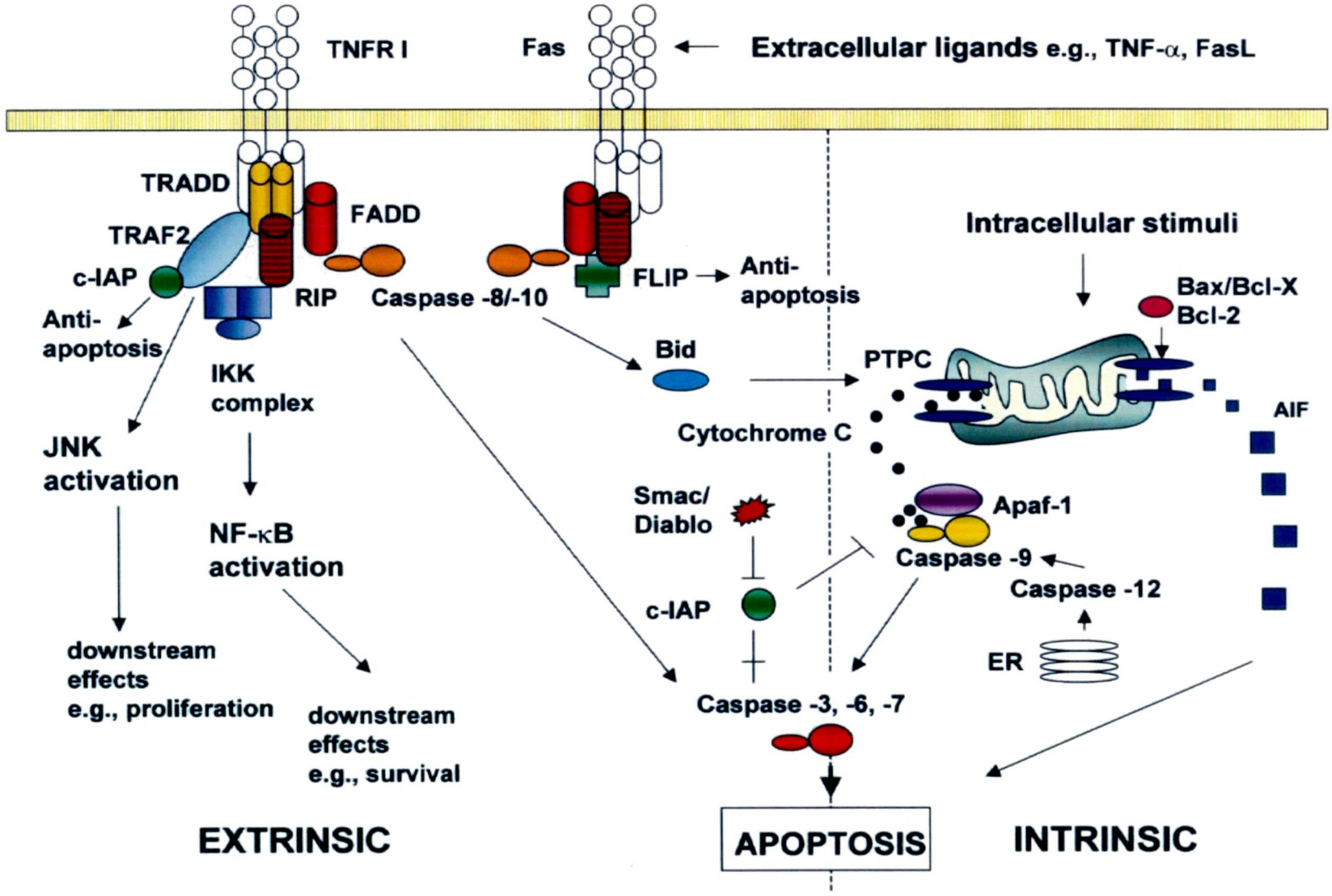
The extrinsic or death receptor mediated pathway is activated in response to the extracellular pro-apoptotic signals and integrated to apoptotic machinery and is mediated by ligand bound membrane receptors, such as CD95/ FAS/APO-1, DR 3-6 and TNF-RI-II and oligomerize some adaptor proteins and procaspase-8, followed by processing to caspase-8 (Thornburn, 2004). It is characterized by the presence-protein interaction modules known as the death domain (DO) in their cytoplasmic portions. Binding of specific ligands induces the receptor multimerization and the formation of a signaling complex known as DISC (death inducing signaling complex), which consists of various adaptor proteins including TRADD, FADD, DAXX, RID, RAIDD and FLIP. FADD acts as a bridge between DISC and caspase-8, which is

critical for the recruitment and oligomerization of caspase-8 and the activation of death receptor mediated, programmed cell death (Bodmer *et al.*, 2000).

The direct activation of the effector caspase-3 and –7 by caspase-8 utilize a mitochondrial death amplification loop. It involves the caspase-8 mediated mediated cleavage of the cytosolic BH3- only pro-apoptotic BCl-2 family member, Bid, which represents an integration of two apoptotic pathways. The cytosolic cytochrome C induces the formation of the apoptosome complex, which is composed of seven Apaf-1 (Apoptotic protease activating factor-1) molecules, each bound to one molecule of cyt C and a dimmer of caspase-9. Formation of apoptosome results in the activation of caspase-9, which thereby activates the effector caspases-3 and –7 to initiate the execution of apoptosis (Soderstrom *et al.*, 2002).

(ii) Intrinsic pathway

The intrinsic apoptotic pathway involves direct and active contribution of mitochondria. This pathway is initiated by receptor-independent apoptotic stimuli, such as DNA damaging agents, UV and γ-radiation, hypoxia and by deprivation of growth factors. These stimuli target the intracellular signal to the main apoptotic machinery (Saelens *et al.*, 2004).


TNFR I
Fas
Extracellular ligands e.g., TNF-α, FasL
TRADD
FADD
TRAF2
c-IAP
RIP
Caspase -8/-10
FLIP
Anti-apoptosis
Anti-apoptosis
IKK complex
JNK activation
NF-κB activation
downstream effects e.g., proliferation
downstream effects e.g., survival
Intracellular stimuli
Bax/Bcl-X
Bcl-2
Bid
PTPC
AIF
Cytochrome C
Smac/ Diablo
Apaf-1
Caspase -9
Caspase -12
c-IAP
ER
Caspase -3, -6, -7
APOPTOSIS
EXTRINSIC
INTRINSIC

In mammalian cells, Bcl-2 family proteins are one of the main "apoptotic sensors", which act primarily on the mitochondria, where they regulate the survival or death signal in a preventive or provocative fashion. Upon exposure to apoptotic insults many apoptosis regulator proteins such as cytochrome C, SMAC [second mitochondria derived activation of caspases/DIABLO (direct inhibitor of apoptosis-binding protein with low P^I) and Om'/Htr A_2/ high temperature requirement protein] are released from the mitochondria (Chen *et al.*, 2004). Additionally, proteins responsible for the caspase independent DNA fragmentation and apoptosis like nuclear morphology (apoptosis inducing factor (AIF) and endonuclease G) are also released from the mitochondria following apoptotic stimuli. Mitochondrial integrity is critical for maintaining the cellular homeostasis and proper compartmentalization of the apoptotic mediators (Van Loo *et al.*, 2002).

ROLE OF CASPASES

Caspases are a class of cysteine-aspartyl proteases that are synthesized as inactive precursor enzymes or proenzymes. These proteases typically lie dormant in the healthy cell and in response to cell death stimuli are converted, either by proteolytic cleavage or by recruitment into large complexes, into active enzymes. Once activated, caspases cleave their substrates typically after conserved aspartate residues and are responsible for most of the biochemical and morphological features of apoptotic cell death. To date, 14 distinct mammalian caspase have been identified, 7 of which are central to the regulation of the apoptotic process (Hunter *et al.*, 2007).

REGULATION OF APOPTOSIS

Members of Bcl-2 family play a crucial role in the regulation of apoptosis. For instance, the overexpression of anti-apoptotic Bcl-2 family prevents the release of cytochrome C, while overexpression of pro-apoptotic Bcl-2 member, Bax, facilitates the formation of mitochondrial pores and the release of cyt C.

SIGNIFICANCE OF APOPTOSIS

Apoptosis plays a pivotal role in the cytotoxic activity of most chemotherapeutic drugs and defects in this pathway provide a basis for drug resistance in many cancers (Nguyen and James, 2003). In mammalian ovary, apoptosis is mainly involved in the removal of oocytes during fetal life and of granulosa cells of the growing follicles during adult life (Rolaki *et al.*, 2005).

Apoptosis accounts for the normal tissue scaffolding (e.g fingers) and the removal of unwanted lymphocytes (Malik *et al.*, 2007).

ROLE OF APOPTOSIS IN CANCER

Apoptosis is said to be defective in cancer and that tumor cells evade apoptosis. Apoptosis occurs in cells committed to proliferation, even stem cells, although these constitute a reservoir of tumor cell proliferation (Evan, 2001).

Dysregulation of apoptosis is the hallmark of cancer cells and agents that activate programmed cell death could be valuable anticancer therapeutics. Apoptosis has been widely used for the diagnosis and prognosis of cancer and is currently the subject of intense research because of its occurrence during neoplasm in response to cancer chemotheraphy and radiation (Hu and Kavanagh., 2003).

ETOPOSIDE

Etoposide [4'- Dimethyl epipodophyllantoxin-9- (4-6-o-ethylidene)-β-D-glucopyranoside] is used in the treatment of various malignancies such as small cell lung cancer, germ cell tumors and lymphomas (Toffoli *et al.*, 2004). It is an inhibitor of topoisomerase II, which ligates double strand breaks necessary to maintain DNA topology during replication (Simon *et al.*, 2007).

MEDICINAL PLANTS

The past decades have shown the increasing interest of medicine and the pharmaceutical industry in products of processing of plant materials, which contain rich complexes of biologically active substances. Plants have been considered as a valuable source of natural products and explored continuously for the therapeuticals for human well being. The use of plant products for pharmaceutical purposes has been gradually increasing, according to World Health Organization; medicinal plants would be the best source for obtaining a variety of drugs. About 80 per cent of individuals from developed countries use traditional medicines, derived from medicinal plants (Khanna and Kannabiran, 2008).

To promote the proper use of herbal medicine and to determine their potential as a source for new drugs, it is essential to study medicinal plants, which have folkfore reputation in a more intensified way (Parekh and Chanda, 2007). India is one of the richest countries in the world with regard to diversity of medicinal plants. Ayurveda, a form of Indian traditional medicine, mentions several plant species and outlines numerous medicinal uses for each. Extracts of these plants have served as medicine for thousands of years. Plants have been used for centuries as a remedy for human diseases because they contain components of therapeutic values (Kaushik and Goyal, 2008).

The plant selected for the present study is *Triticum aestivum*, which is commonly called wheat grass. It is the most important food crop of the world and is cultivated under a wide range of agro- ecological conditions. It is adaptable to a wide range of soil and climatic conditions and can be grown extensively throughout the world (Ahmad *et al.*, 2009).

Earlier studies conducted in our laboratory revealed the antioxidant activity of *Triticum aestivum* wherein the 4^{th} day plant has shown the maximum antioxidant activity (Vidya, 2007). Studies have also shown that the extent of the leaves of *Triticum aestivum* can influence the extent of apoptosis induced by oxidative stress (Malathy, 2008; Mathew, 2008).

In the present study, the effect of leaves of the 4^{th} day plants on the apoptotic events induced by etoposide was studied in chick embryo fibroblasts. The layout of the study, the methodology adopted and the procedures involved in estimating the various parameters are elaborated in the next chapter with the support of the appendices.