Chapter 2

Review of literature

2.1. INTRODUCTION

From time immemorial, human beings were confronted with various diseases of which, some proved to be critical. Advancement in science and technology helped in controlling many of the dreaded diseases from recurring and inflicting havoc in the population. Nevertheless, the struggle against such diseases (for e.g. cancer), seems eternal. With more and more revelations on the nature and types of various cancers, the battle against cancer is still going on. Scientists and doctors in this area are working hard to contain them, with great success against some types and less success against others. The quest for new potent drugs is continuing as researches worldwide have exposed the potency of plants in controlling many of the dreaded diseases. This is the rationale behind the selection of mulberry as the plant of interest in the present study.

2.2. MULBERRY

Mulberry belongs to family Moraceae. It is grown extensively for leaves that are the only source of food for silkworms and forms an integral part of sericulture industry in many silk producing countries. Growing mulberry is therefore a way of life for many people in rural India, China, etc. It was also grown for medicinal purposes in the past. It is used extensively in the traditional medicine in many countries as such or in combination with other herbs (Andallu et al., 2001). Soni et al. (2009) have thrown light on the medicinal properties of different parts of the plant that can be used in treatment of various ailments. Leaves are antibacterial, astringent and can be taken internally for cold, influenza, eye infections and elephantiasis. Stems are reported to be having antirheumatic, diuretic and hypotensive effects. Fruits can be used for relieving constipation in elders and also against anemia, hypertension and diabetes. It can also be used to prevent premature graying of hair. A tincture from bark relieves toothache. Root bark is used as diuretic, hypotensive, expectorant and sedative and can be used in
treatment of asthma, cough, bronchitis, hypertension and diabetes. Antimicrobial and antiinflammatory effects of various parts of the plant are also reported (Chatterjee et al., 1983; Sastri, 1962). Presence of such vast variety of compounds makes mulberry one of the medicinally important plants in Asian countries. In China, mulberry leaves are used in the treatment of fever and also to protect against liver damage (Zhishen et al., 1999). They are used as antihyperglycemic food by people having diabetes in Japan and Korea (Kim et al., 2003).

More than a thousand varieties of mulberry are available in Japan whereas in India, there are about 400 mulberry varieties of which, 85 are indigenous and 114 are exotic in origin (Soni et al., 2009). Morus alba L., the most common and widely seen species among all mulberry varieties, is the plant selected for the present study.

2.2.1. *Morus alba* L. - PLANT PROFILE AND TAXONOMY

*Morus alba* L. (*M. alba* L., commonly called white mulberry) has a long history in the medical world as it has wide applications in conventional and contemporary medicines (Grover et al., 2002). It is a fast growing shrub/tree grown in subtropical/mild temperate regions. The following is the taxonomical classification of *M. alba* L.

Kingdom: Plantae

Sub-kingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Hamamelididae

Order: Urticales

Family: Moraceae

Genus: Morus L.

Species: *Morus alba* L.
2.2.2. CHEMICAL CONSTITUENTS OF MULBERRY

Mulberry is a plant with rich medicinal properties (Priya, 2012). Gulubova and Boiadzhiev (1975) reported that the subcutaneous injection of mulberry leaf extracts led to the de-granulation of beta cells of islets of langerhans in rabbits. Alkaloids from the plant possess glycosidase inhibitory activity (Asano et al., 2001). The extract of mulberry is antidiabetic, reduces atherosclerosis and can be used for the treatment of Alzheimer’s disease (Lin et al., 2009). Zhishen and his co-workers have reported the presence of rutin, quercetin, isoquercetin and other flavonoids in mulberry leaves (Zhishen et al., 1999). The various other components from mulberry that are found to be medicinally important are alkaloids, essential fatty acids like oleic and linoleic acids, prenylated flavonoids (kuwanons, morusin, sanggenon F, etc.), terpenoids (like betunilic acid), arylbenzofurans, deoxynojirimycin (DNJ), flavones like moracin M, steppogenin-4’-O-β-D-glucosiade and mulberroside A, phenolic compounds, etc.

Moran 20K and 1-DNJ from mulberry are used against diabetes mellitus (Kimura et al., 2007). Certain flavanones (kuwanon G, C, leachianone, mulberrofuran G, albanol B, etc.) act as antimicrobial agents (Kim et al., 1999). Investigations on the neuroprotective effect of cyanidin and its derivative (C3G-cyanidin3-O-b-D-glucopyranoside) from mulberry showed protective effect against cerebral ischemia (Kang et al., 2006). Sanggenol P, a new isoprenylated flavonoid, was isolated from M. alba L. along with nine other known ones. The already known flavonoids isolated from mulberry were cyclomorusin, morusin, mulberrofuran G, sanggenol A, sanggenol L, sanggenol N, cyclomulberrin, cyclocommunol and ursolic acid (Geng et al., 2010). A pair of isomeric diels-alder-type adducts, mulberrofuran G and isomulberrofuran G, were isolated from the root bark of M. alba L. The inhibiting activity of mulberrofuran G on the DNA replication of hepatitis B virus (HBV) showed moderate activity, with an IC\textsubscript{50} value of 3.99 \textmu M, according to the \textit{in vitro} antiHBV assay on the HepG 2.2.15 cell line (Geng et al., 2012).

Yang et al. (2010) isolated two new flavanes - (2R,4S)-2’,4’-dihydroxy-2H-furan-(3”,4” : 8,7)-flavan-4-ol and (2S)-2’,4’-dihydroxy-7-methoxyl-8-butyricflavane along with four known flavonoids from the leaves of M. alba L. The isolation of a few known
triterpenoidal phytoconstituents (α-amyricin acetate, β-amyricin-β-D-glucopyranoside and betulinic acid) from the stem bark of *M. alba* L. by Ali and Ali (2013) resulted in their structure elucidation. They also isolated and characterized two lupeol-type pentacyclic triterpenoids namely, lup-20(29)-en-3β-ol-27-oic acid (moruslupenoic acid A) and lup-12, 20(29)-dien-3β-ol-26-oic acid (moruslupenoic acid B). The structures of these isolated phytoconstituents were established by spectral and chemical analysis.

Morachalcones B and C are the two new chalcone derivatives isolated from the leaves of *M. alba* L. Both represent chalcones with unusual furan rings formed by the cyclization between C-alpha-OH and C-2-OH. Moderate cytotoxic activity was observed when these derivatives were tested against HCT-8 and BGC823 human cancer cell lines (Yang *et al.*, 2010). Weber *et al.* (2012) demonstrated the antioxidant and free-radical scavenging effect of the toxic principle (oxyresveratrol) from the mulberry wood which showed protective effects against cerebral ischemia. The neuroprotective ability of oxyresveratrol was investigated using an *in vitro* model. Oxyresveratrol significantly inhibited the neuronal death induced by trauma. The ethanol extract from the leaves of *M. alba* L. yielded four diels-alder type adducts mulberrofuran F1, mulberrofuran F, chalcomoracin and kuwanon J along with two chalcones (morachalcone A and isobavachalcone) and three flavones (norartocarpetin, kuwanon C and 6-eranylapigenin). Few of these compounds exhibited cytotoxicity against A549, Be17402, BGC823, HCT-8 and A2780 cell lines *in vitro* (Yang *et al.*, 2010).

Oh *et al.* (2010) isolated two methyl ester compounds identified as pheophorbide and 13\(^2\)(S)-hydroxy-pheophorbide from the methanol extract of *M. alba* L. leaves. These pheophorbides inhibited the MCH (melanin-concentrating hormone) mediated extracellular signal-regulated kinase (ERK) phosphorylation in chinese hamster ovary (CHO) cells expressing human MCH-1 receptor giving proof for their role as modulators of MCH-1 receptor and MCH-mediated ERK signaling. Mulberroside A was reported to be one of the major bioactive components isolated from mulberry. Investigations revealed that when mulberroside A was incubated with intestinal bacteria, it underwent rapid deglycosylation and generated two monoglucosides and its aglycone sequentially in oral route in humans and rats (Mei *et al.*, 2012).
The antioxidant property of various extracts of mulberry leaves was reported previously (Kim et al., 1999; Ohsugi et al., 1999; Arabshahi-Delouee and Urooj, 2007; Katsube et al., 2006). Doi et al. (2000) reported that the extracts from mulberry leaves inhibited the oxidative modification of low density lipoprotein of both rabbits and human. DPPH radical scavenging activity of the extract was also checked.

2.2.3. REPORTS ON LECTIN FROM MULBERRY AND RELATED FAMILIES

Lectin is present in different parts as well as different varieties of mulberry. In 1998, a team led by Sunanta Ratanapo isolated two lectins from the leaves of *M. alba* L. by a multistep chromatographic procedure (Ratanapo et al., 1998). The lectins were found to be specific towards N-glycolylneuraminic acid, lactose and galactose. Purification and characterization of three galactose specific lectins from mulberry seeds were reported by Yeasmin et al. (2001). Apart from galactose, the lectins were found to be specific towards methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, lactose and raffinose. The lectins exhibited strong cytotoxic effect in brine shrimp lethality bioassay.

Crude extracts from the bark of the black mulberry (*M. nigra*) tree contained a mixture of gal-specific and man-specific lectins (mornigaG and mornigaM), which were identified and isolated by consecutive affinity chromatography on immobilized galactose and mannose, respectively. Of these two lectins, mornigaG resembled jacalin in terms of its molecular structure, specificity, and co- and post-translational processing. From this, it was concluded that it followed the secretory pathway, thus accumulating in the vacuolar compartment. MornigaM showed complete disparity in being a novel type of highly active man-specific jacalin-related lectin that is synthesized without signal peptide or other vacuolar targeting sequences. It was found that, it finally accumulated in the cytoplasm (vanDamme et al., 2002).

Isolation of mornigaM, the mannose specific lectin from mulberry (*M. nigra*) bark by affinity chromatography on Man-Sepharose 4B and three successive rounds of affinity chromatography (Rabijns et al., 2005) revealed that the carbohydrate binding cavity readily accepted mannose without any major structural reorganization. A lectin with
specificity towards mannose and glucose was isolated and purified from mulberry seeds by affinity chromatography on ConA-Sepharose (Absar et al., 2005). The lectin showed a high degree of sequence similarity with some of the previously isolated jacalin related lectin.

Two structurally identical, mannose-specific, jacalin-related lectins (mornigaM and artocarpin) were studied for their differential effects on human T lymphocyte activation and cell death (Benoist et al., 2009). Though having the same nominal sugar specificity and three-dimensional structures, they differently activated lymphocytes and displayed different cytotoxic effects. Both lectins triggered lymphocyte activation, but only mornigaM was capable of inducing cell death. This was perhaps because only mornigaM was competent enough to interact with the carbohydrate moieties on cell surface and thus induce cell death. It also suggested that fine adjustments in N-glycans helps in distinguishing activation and cell death at the lymphocyte surface.

2.3. CANCER – AN OVERVIEW

Cancer is the uncontrolled growth of any cell which, otherwise was growing normally. The basic difference between a normal and cancer cell may lie in the genetic make-up or in the alterations happening to the genetic material upon exposure to agents causing a change in that make-up. As a result, the cell is no more capable of carrying out the functions that it ought to do. Certain signals that regulate the cells’ homeostasis is misunderstood or bypassed by the system resulting in immortality of cells with sustained angiogenesis, finally resulting in invasion and metastasis. Loss of cell-cell interaction is another typical feature of cancer cells.

Cancer may occur as a result of mutation of certain genes that control the process regulating the balance of cell growth and death. As an evolutionary conserved process, this balance between life and death is controlled by a tightly regulated mechanism called apoptosis. Apoptosis, or programmed cell death as it is called, is an active and well defined process that regulates cells in tissues of multi-cellular organisms (Meier et al., 2000).
2.3.1. CARCINOGENS AND CARCINOGENESIS

Substances that are known to promote or aggravate cancer by increasing the rate of cell division, acting on DNA resulting in mutations, etc. are said to be carcinogens. Basically, three types of carcinogens are identified – ionizing radiations (e.g. radon gas), chemicals (e.g. dioxins) and biological molecules (e.g. aflatoxin B1). Most of the carcinogens act singly or in combination with other agents, thereby interacting with DNA, thus hampering normal cellular functions. This, in turn, will result in tumor formation that will spread to and invade other tissues/organs resulting in the final breakdown of the organ, eventually culminating in death.

Carcinogenesis (Figure 2.1) or the formation of tumors is a process initiated as a result of the failure of cells to obey the cellular processes effecting subsequent functional, structural and behavioral defects in cells. The identity of the newly formed cells is different from the parent cells once they start to metastasize. They assume new properties, grow and multiply faster than the normal cells and finally results in losing some functional/behavioral characteristics which are reflected in cell cycle also.

Figure 2.1. Steps involved in carcinogenesis

Multistep carcinogenesis model (Adapted from Soria et al., 2003, with slight modification)
2.3.2. DIFFERENT CANCERS/OCCURRENCE/STATISTICS (WORLD SCENARIO)

Worldwide, cancer is seen as a menace affecting the normal life of patients and the people associated with them. Figure 2.2 represents the different types of cancers (and also their prevalence and incidence) that are affecting both males and females.

Figure 2.2. Different cancers with their prevalence and incidence

Thousand of people are affected with cancer every year. A representative figure showing the estimated numbers of new cancer cases (incidence) and prevalent cases (Five-year Survival) in 2002. Courtesy: Parkin et al., 2002

The International Agency for Research on Cancer (IARC), a WHO initiative, came up with the following statistics (Figure 2.3) on the different types of cancers and the mortality due to them.
Following is the key facts and findings about cancer released by WHO media centre as on February, 2012. (http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900)

- According to the WHO reports, cancer accounts for the major deaths worldwide – this is almost around 13% of all deaths in 2008
- Most of the affected people die from lung, stomach, liver, colon and breast cancer each year
- The frequency and the types of cancers differ between men and women
- About 30% of all cancer deaths occur because of dietary and behavioral problems. People who lead sedentary life, who are alcohol and tobacco addicts, those who take less of vegetable and fruits, etc. are at risk of contacting cancer
- Use of tobacco is the most leading reason for contacting cancer. Globally, around 22% of cancer deaths are due to the use of tobacco. 71% of global lung cancer deaths are due to the tobacco chewing habit of people
- Viral infections such as HBV/HCV and HPV are also responsible for certain percentage of cancer deaths, especially in low- and middle-income countries
The scenario is not different for other cancers: Total deaths due to cancer are projected to continue rising, with an estimated 13.1 million deaths in 2030, worldwide.

2.3.3. PRESENT APPROACH TO COMBAT CANCER

Currently, cancer is treated by conventional methods like surgery, radiation, chemotherapy, etc. Once identified with cancer, surgery is the first mode of treatment. The underlying strategy for the treatment modalities is to minimize the imbalances caused by cancer and thus bringing the mechanism back to normal. Generally, this involves the controlling of signaling pathways by administering drugs. Many of these drugs target the proteins involved in apoptotic pathway and induce cancer cell death or helps in enhancing the sensitivity of cancer cells to drugs and radiation (Liu et al., 2011).

2.3.4. LIMITATIONS OF CURRENT TREATMENT MODALITIES

Normally, it is observed that the efficacy of the chemical/medicine is compromised by the side effects of the treatment in the conventional therapy (radiation, surgery, chemotherapy, etc.). Cells respond differently to the chemical and it may result in reduced dosage effect, delay in bringing about the effect or even termination of the treatment. Other treatment modalities like transplantation, angiogenesis inhibitors and biological therapies are few of the alternate solutions to this predicament (Tsao et al., 2004). Most of these lines of treatments fail to address the major concerns of the patients - that of the pain and other problems like water accumulation, nausea, insomnia, etc. associated with it right from the onset of the disease.

2.3.5. NATURAL PRODUCTS AS THE ALTERNATE APPROACH

Documents approximately as old as 6000 years have described the rich heritage that the Greek, Roman, Arabic, Chinese, Egyptian and Indian systems followed and reveals a lot about the knowledge that these ancient civilizations had on the use of plants for various medicinal purposes (Bernhoft, 2010). Recent researches also aim at finding out novel therapeutics or bioactive compounds from natural source so that counter effects of chemicals and synthetic drugs can be avoided. These compounds are produced as
secondary metabolites which educe certain important pharmacological or toxicological
effects in man and animals. Taxol, vinblastin, vincristine, irinotecan, etoposide,
teniposide, topotecan, etc. are a few of the plant derived anticancer drugs that have been
approved by FDA in recent past (El-Menshawi et al., 2010).

2.3.5.1. MAJOR PLANT GROUPS CONTAINING ANTICANCER COMPOUNDS

Almost 75% of the world population still depends on plants and plant products for
curing ailments and diseases (Abelson, 1990). Though it cannot be said for sure that one
group of plant is more important than the other, all major families have medicinal
properties and are rich source of one or the other type of medicinally important
compounds. Many of the traditional, complementary and alternate systems of treatments
rely on the vast amount of bioactive compounds present in plants with high potentials to
cure a vast variety of diseases including cancer. More than half of the currently available
drugs are derived from plants or are compounds synthesized/designed with a natural
compound as a model/lead molecule. Molecular modifications of this type generate
structural analogues with greater pharmacological activity and fewer side effects. Thus,
more affordable drugs, which are far more efficient than the present ones, are obtained
(Gordaliza, 2007). Probably, this is the motivation for the current interest among
scientists and pharmaceutical companies for investigating the active principles from plant
source for their curative effects as they have diverse effect on different types of diseases,
especially cancer. Soladoye et al. (2010) exclusively studied 73 plant species and the
results from their study revealed that 33% of leaves, 27% of barks, 19% of roots and 10%
of seeds showed efficiency in cancer management. Tuber (3%), fruit (1%), bulb (3%) and
juice (4%) also were found to be effective but in lesser ratios compared to the other parts.
Apocyanaceae, Berberidaceae, Rubiaceae, Acanthaceae, Euphorbeceae, Rutaceae,
Leguminosae, Combretaceae, Cucurbitaceae, Zingiberaceae - all these are families of
plants with rich anticancer compounds. Some of these have had early entries in anticancer
studies and are still valuable contributors to cancer therapeutic field (Cretu et al., 2012;
Dhiman et al., 2012). Thirty six synthetic or semi-synthetic analogs of many of these
plant derivatives are currently used in cancer therapy on various cancer cell lines with
many more to add to this list (Nirmala et al., 2011). Major among these bioactive compounds are phenolics and polyphenols, flavones and flavonols, alkaloids, polypeptides and lectins (Tsuchiya et al., 1996; Sher, 2009).

2.3.5.1.1. POLYPHENOLS

According to Stéphane Quideau (2011) the term “polyphenol” should be used to define compounds exclusively derived from the shikimate/phenylpropanoidand/or the polyketide pathway, featuring more than one phenolic unit and deprived of nitrogen-based functions. Enzyme inhibition by the oxidized compounds through a reaction with the sulfhydryl groups or through certain non-specific interactions with molecules like proteins is thought to be the mode of action of these set of molecules (e.g. catechol, pyrogallol, etc.).

2.3.5.1.2. FLAVONES AND FLAVONOLS

Flavones are a class of flavonoids based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) whereas flavonols are a class of flavonoids that have the 3-hydroxyflavone backbone. They are present in a wide variety of fruits and vegetables. They exert their activity by forming complexes with extracellular and soluble proteins and also with bacterial cell walls. When they are more lipophilic, they may also disrupt the microbial membranes. Catechin, galangin, etc. belong to this group.

2.3.5.1.3. ALKALOIDS

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. These heterocyclic nitrogenous compounds are highly aromatic in nature. They act by intercalating with the DNA. Morphine is one of the first alkaloids isolated with good medical properties. Codeine and heroin, both derivatives of morphine, are also important compounds. Diterpene alkaloids are yet another class of molecules isolated from plants with excellent antimicrobial (Omulokoli et al., 1997), antiHIV (McMohan et al., 1995) and anticancer activities (Marmont and Damasio, 1967).
2.3.5.1.4. POLYPEPTIDES AND LECTINS

Positively charged peptides are generally inhibitory to microorganisms; these peptides act upon microbes by forming ion channels in the microbial membrane (Zhang and Lewis, 1997) or by competitive inhibition of the adhesion of microbial proteins to the polysaccharide receptors on the host membrane (Sharon and Ofek, 1986). Lectins are glycoproteins of non-immune origin capable of agglutinating cells (Goldstein et al., 1980). Lectins are inhibitory to microbial proliferation; they probably interact by inhibiting the critical host cell components (Favero et al., 1993) and attaching the protein components. Literature reveals the efficiency of lectins to slowdown or inhibit various human disease conditions such as inflammation (Assreuy et al., 1997), diabetes (Gray and Flatt, 1999), ulcer (Saito et al., 1989; Teradaira et al., 1993), cancer (Zuo et al., 2012; Chan et al., 2012), etc.

2.4. PLANT BASED MOLECULES IN CANCER THERAPEUTICS

2.4.1. DATA ON THE COMPOUNDS FROM PLANTS IN CLINICAL PRACTICE/TRIALS IN CANCER TREATMENT

* Catharanthus roseus*, the Madagascar periwinkle, is traditionally used for treating diabetes. But, two of the most successful and prominent anticancer drug compounds (the vinca alkaloids, vinblastin and vincristin) used in cancer treatment are obtained from this plant. Vinblastin is used against Hodgkin’s disease and vincristin for curing leukaemia, lymphomas and small cells lung cancer. Podophyllotoxin, a resinous compound isolated from the roots of *Podophyllum peltatum* (May apple), is the lead molecule from which teniposide and etoposide were developed. Both are used against several types of cancer. Paclitaxel is another compound isolated from the bark of the Pacific yew tree, *Taxus brevifolia* (Salim et al., 2008). The unique mode of action of this anticancer compound has evoked much interest in the clinical development of further derivatives of paclitaxel in larger amounts from related species by different methods (chemical, semi synthetic and plant cell culture methods).

In a review on the natural bioactive compounds with anticancer potential, Patel et al. (2010) reported that the overall chemo-preventive effect of a plant may be due to the
presence of various compounds in the extract. This overall effect contributes to the
disruption of normal mechanisms of cancer cell which results in apoptosis/cell death
(mediated by the disruption of mitochondrial membrane potential, cytochrome c release,
activation of caspase 3, cell cycle arrest, etc.).

A thorough data on the various other compounds in different stages of clinical
trials is available in *Bioactive compounds in plants – benefits and risks for man and
animals* edited by Aksel Bernhoft (Bernhoft, 2010). All these researches point to the fact
that natural products offer a vast variety of compounds to be utilized for treating an
equally huge number of varied diseases.

2.4.2. ADVANTAGES AND DISADVANTAGES OF PLANT BASED THERAPY

Plants are excellent sources of different compounds with relatively good
anticancer effect (alone or in combination with other drugs). Going through the history
and records from researches, it is also clear that most of the semi-synthetic/synthetic
compounds owe their origin to natural products. Modern treatment techniques like
surgery, chemotherapy, radiation, etc. can be avoided to an extent by the proper
administration of herbal drugs. Laboratory trials have revealed that there are hundreds of
plants with anticancer effects and therefore, there is no dearth of source or supply.

The major disadvantage of using plant products as anticancer agents is the cost of
production of the compound when they are to be used as a drug. Besides, the compounds
may be highly effective in their crude form but when they are processed, they may lose
activity or may be less potent. Moreover, the herbal drugs are generally not administered
along with conventional therapies like surgery as sometimes they may interfere with
blood coagulation, complicating the whole process. However, these shortcomings are
being dealt with much care and more experiments are underway to isolate compounds by
various methods and produce them in bulk (by recombinant techniques) so that they are
available to the common mass without much cost/delay.
2.5. MECHANISM OF CELL DEATH BY COMPOUNDS OF PLANT ORIGIN

The range of action of plant products is beyond comparison. They wield their anticancer effect by different mechanisms like reduction of reactive oxygen species (ROS), down regulation of certain proteins, reduction in telomerase activity, bringing about apoptosis, etc. Among this, apoptosis is the most extensively studied and well regulated control mechanism of cell death.

2.5.1. APOPTOSIS

The Greek word ‘apoptosis’ means “falling off or dropping off”- highlighting death as an integral part of the life cycle of any organism. This is a well defined, highly regulated process, actively involved in the development, regulation and maintenance of cell populations of all multi-cellular organisms. Apoptosis is important in both physiological and pathological conditions (Holcik et al., 2005).

Figure 2.4. Mechanism of apoptosis

Various stages in the apoptotic machinery where the cell components are fragmented and phagocytized

Apoptosis is characterized by a precise disassembly of cells as revealed by DNA fragmentation, degradation of cytoskeletal and nuclear proteins, shrunken cytoplasm and
the characteristic blebbing of the membrane (Figure 2.4). Cross-linking of proteins, formation of apoptotic bodies and expression of ligands for phagocytic cell receptors are also observed during the process. The signal for destruction proceeds from cytoplasm to the nucleus resulting in the above said typical features. The final outcome of the whole apoptotic cascade is the uptake by phagocytic cells. The cell collapses and apoptotic bodies formed are engulfed by surrounding cells thus, very quietly eliminating the cell (Martinvalet et al., 2005).

2.5.1.1. SIGNIFICANCE OF APOPTOSIS – IN NORMAL AND DISEASED CONDITIONS

Apoptosis is biologically very significant, being involved in a variety of conditions like development, differentiation, proliferation/homeostasis, regulatory functions of immune system, etc. Removal of defective and harmful cells is mediated through apoptosis. Various pathological conditions incriminate the dysfunction or disregulation of apoptosis. Flaws in apoptosis results in cancer, auto immune disorders, spreading of viral infections, etc. whereas redundant apoptosis enhances neuro degenerative disorders, AIDS, ischemic diseases, etc. (Fadeel et al., 1999).

Any defect in apoptosis will lead to the survival of neoplastic and genetically unstable cells which contributes to tumor pathogenesis. In addition, deregulation of apoptosis takes the cells to the verge of cell death and metastasis by effecting resistance to chemicals and radiations (Fridman and Lowe, 2003; Kaufmann and Vaux, 2003). Means to kill cells by apoptosis can include the direct induction of pro-apoptotic molecules, control of anti-apoptotic proteins, re-activation of the functions of tumor suppressor genes, etc. Compounds derived from plants are found to have very promising in vitro antitumor activity and are being engaged as potential anticancer agents (Fleischer et al., 2006; Makin and Hickman, 2000; Frisch and Screaton, 2001).

2.5.1.2. MAJOR PATHWAYS LEADING TO APOPTOSIS

Apoptosis occurs as a result of the involvement of many major and minor components distributed in the cell system. Though these components are found to be
conserved throughout evolution, many more components have been discovered in the evolutionary process. Broadly, two pathways culminate in the execution of apoptosis: one that is characterized by the engagement of cell surface death receptors - the extrinsic pathway and the other which involves crucial mitochondrial events - the intrinsic pathway (Kasibhatla and Tseng, 2003).

2.5.1.2.1. EXTRINSIC OR RECEPTOR MEDIATED PATHWAY

The extrinsic pathway, which initiates apoptosis with the active involvement of trans-membrane receptor-mediated interactions, engages death receptors as signaling molecules. Ligand binding forms an integral part of extrinsic pathway (Figure 2.5) of apoptosis mediated by external receptors and begins when a stimulus triggered by external stress decides that the cell should die. The stimulus activates death ligands through trans-membrane death receptors that belong to the tumor necrosis factor (TNF) super-family of cell surface receptors. They contain cytoplasmic protein motifs called death domains that enable death receptors to engage the cells’ apoptotic machinery. There are different death receptors that are generally activated upon binding of their corresponding ligands - FasR/FasL, TNFR1/TNF-α, DR3/Apo3L, DR4/Apo2L and DR5/Apo2L.

Upon ligation of death receptors by their ligands, the receptors undergo dimerization resulting in clustering of the receptors’ death domain. For e.g., with the binding of Fas ligand (FasL) to the Fas receptor (FasR), adapter molecules like FAS associated death ligand (FADD) are recruited at the site to bind with the receptor. Now, FADD associates with caspase 8 through the dimerization of death effector domain which, in turn, recruits caspase 8 to the activated death receptor CD95 (also called Apo-1 or Fas) to form the death inducing signaling complex (DISC). DISC will activate the auto-catalytic process of caspase resulting in the execution of apoptotic signaling cascade. Oligomerization of caspase 8 after DISC formation leads to cleavage and activation of caspase 8. Cleaved caspase 8 is released into the cytoplasm; it then guides the cleavage of effector caspase 3 downstream which cleaves several other caspases leading to the typical morphological changes that accompany apoptosis (Fulda and
Debatin, 2004; Schulze-Osthoff et al., 1998; Czerski and Nunez, 2004; Pop and Salvesen, 2009). Thus, the whole process forms the death receptor mediated apoptosis of extrinsic pathway (Elmore, 2007).

**Figure 2.5. The extrinsic pathway of apoptosis**

![Extrinsic Pathway Diagram](https://www.biooncology.com)

*The extrinsic pathway involves various ligands and their receptors. Binding of ligands like FasL, TNF-α, Apo3L and Apo2L form an integral part of extrinsic pathway (photo courtesy www.biooncology.com)*

2.5.1.2.2. INTRINSIC OR MITOCHONDRIA MEDIATED PATHWAY

Intrinsic pathway (Figure 2.6) is initiated when non-receptor mediated stimuli (such as absence of growth factors, hormones and cytokines) invoke intracellular signals that trigger apoptosis. These signals act through mitochondria, leading to the failure in suppressing death programs and are directed to the cell, thus instigating apoptosis. Radiations, toxins, hypoxia, viral infections, free radicals, etc. are other signals that prompt apoptosis (Elmore, 2007). The cellular stresses activate the tumor suppressor protein p53 to initiate the intrinsic pathway. The pro-apoptotic proteins, Puma and Noxa are up-regulated and this in turn, activates other pro-apoptotic proteins - Bax and Bak- of the BCL2 family. There, they undergo conformational changes. Oligomerization of Bax
and Bak leads to their translocation to the outer mitochondrial membrane and the formation of a permeability transition pore, resulting in the loss of mitochondrial transmembrane potential and the release of cytochrome C. Cytochrome C is essentially a cofactor needed for caspase 9 activation by the adapter molecule, Apaf-1. Further, cytochrome C binds and activates Apaf-1 and procaspases 9 to form apoptosome (Figure 2.7). This will lead to activation of caspase 9. Thus, the whole process involves the intrinsic pathway of apoptosis, which can also be activated by damage to cellular DNA.

Apart from cytochrome C, certain other pro-apoptotic factors like SMAC/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low pI), AIF (apoptosis-inducing factor), and CIDE-B (cell death-inducing DFF45-like effector protein B) are also released by mitochondria (Gottlieb and Granville, 2002; Wang, 2001).

Thus, if the cell is committed to death by apoptosis, the mitochondrial contents are released and SMAC/DIABLO will sequester the inhibitors of apoptosis proteins (IAP) to insure that they do not obstruct the process on track. There are buffer zones too, which control accidental release of the mitochondrial contents.
Figure 2.6. The intrinsic pathway of apoptosis

[Diagram of the intrinsic pathway of apoptosis]

The intrinsic pathway of apoptosis involves changes associated with mitochondria and activation of various proteins leading to cell damage and apoptosis.

(photo courtesy www.biooncology.com)

Figure 2.7. Various stages in the formation of apoptosome

[Diagram of various stages in the formation of apoptosome]

Cytochrome C, adapter molecule Apaf-1 and procaspases 9 forms apoptosome
2.5.1.3. OTHER MOLECULAR EVENTS INVOLVED IN APOPTOSIS

Apart from the two pathways, other morphological and biochemical changes are also involved in the apoptotic processes that are worth mentioning. These events are found to be associated with apoptosis irrespective of the pathways. Caspases are found to be actively involved in both pathways of apoptosis. Changes associated with plasma membrane and DNA fragmentation are prominent outcomes of apoptosis, observed in both cases.

2.5.1.3.1. CHANGES IN PLASMA MEMBRANE

Most of the activities involved in extrinsic and intrinsic pathways bring about some major changes in the plasma membrane. It is observed that, during the initial stages of apoptosis, the cytoplasmic levels of Ca\textsuperscript{2+} changes and the cells start shrinking. There is a loss of contact between the extracellular matrix and the cells’ immediate neighbors. Consequently, the plasma membrane also starts to shrivel and sialic acid residues are lost from the cell surface glycoproteins. Cell-cell junctions are no more functional and microvilli are lost. Plasma membrane becomes leaky. Phosphatidyl serine (PS), which is present on the inner surface, is now translocated to the outer surface. In due course, budding appears on the cell surface resulting in the formation of apoptotic bodies. Finally, the cell is phagocytized by macrophages (vanEngeland et al., 1997).

2.5.1.3.2. CASPASE ACTIVATION

The basic machinery that brings about apoptosis is found to be quite similar in all animal cells. The players here are a family of proteases called caspases (Figure 2.8) that have a cysteine at their active site and cleave their target proteins at specific aspartic acid residues. They are closely related to mammalian interleukin-1β-converting enzyme (ICE) (Zeiss, 2003). Caspases are classified into initiators (caspases 2, 8, 9 and 10), effectors or executioners (caspases 3, 6 and 7) and inflammatory (caspases 1, 4 and 5) caspases (Cohen, 1997; Boatright and Salvesan, 2003). They exist as pro-enzymes (zymogens) which, when activated in response to apoptotic stimuli, cleave a multitude of other
cellular substrates at specific aspartate residues to bring about a hierarchial cascade taking the cell to the mouth of death irreversibly.

Figure 2.8. Caspase organization

A). The prodomain precedes the catalytic domain, composed of two covalently linked subunits. Sites for (auto) proteolysis at Asp residues are indicated. B). Activation mechanisms. Initiators are monomers that activate by prodomain-mediated dimerization. Executioners are dimers that activate by cleavage of intersubunit linkers. Following activation, additional proteolytic events mature the caspases to more stable forms, prone to regulation (Taken from Pop and Salvesen, 2009)

Once the initiator caspases are activated by means of association with an adaptor molecule, they cleave and activate the downstream effector.executioner caspases. These then cleave their substrates to co-ordinate the dismantling of the cell components, finally resulting in cell death (Kasibhatla and Tseng, 2003).

2.5.1.3.3. DNA FRAGMENTATION

An injury to DNA can contribute to the functional disability of a cell and so, will be efficiently eliminated by the cells’ repair mechanism by the activation of cell cycle check points. This is the primary response of any cell to DNA damage. However, when
the damage is beyond repair, it results in harmful effects like chromosomal changes, gene mutations and malignant transformations (Roos and Kaina, 2006). In such cases, it will resort to apoptosis with the prime goal of protecting a multicellular organism against a damaged cell (Wang, 2001). DNA damage is considered as one of the important hallmarks of cancer and is induced as part of apoptotic machinery. Wang et al. (1997) assayed a biochemical system and purified a protein called DNA fragmentation factor (DFF) that, in presence of an activated caspase 3, is capable of causing chromosomal DNA fragmentation. It was found that DFF has a DNase activity and cleaves DNA into oligonucleosomal fragments.

2.6. HYPOTHESIS OF THE STUDY

- Antioxidants present in mulberry extract can induce toxicity in cancer cells
- Different chromatographic techniques can be employed for the purification of lectin from mulberry leaves
- Mulberry leaf lectin can induce antiproliferative effects in cancer cells by apoptotic machinery

2.7. OBJECTIVES

Following are the objectives aimed at proving/establishing the hypothesis of the present study

- To purify the lectin present in mulberry leaf using different chromatographic approaches
- To test whether the purified lectin can induce apoptosis in cancer cells
- To determine the antioxidant activity of mulberry extract by various standard assays
- To test whether the antioxidant capacity of mulberry extract helps in protecting UV-induced DNA damage
- To test whether the antioxidants present in the extract can kill the cancer cells by inducing apoptosis