1. INTRODUCTION

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1. INTRODUCTION

1.1 Cancer: basic perspective

Cell is the basic structural, functional and biological unit of all living organisms. A cell arises from pre-existing cell through the process of cell division which is one of the most fundamental processes of all living organisms. Prokaryotes divide by binary fission. Eukaryotes have much more complex and well defined regulation of cell division.

In eukaryotes, cell multiplication is tightly controlled through cell cycle. The cell cycle or cell division cycle is a cyclical and directional process which involves a series of events that take place in a sequential way (Schafer, 1998; Schnerch et al., 2012). The cell cycle is divided into four phases i.e. G₁, S, G₂ and M. G₁ is the first gap phase during which cells prepare for DNA replication. It is during the G₁ phase that the cell integrates mitogenic and growth inhibitory signals and makes the decision to proceed, pause, or exit the cell cycle. S phase is defined as the phase in which DNA synthesis occurs. G₂ is the second gap phase during which the cell prepares for the process of division. In M phase or mitotic phase, the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. The term G₀ is used to describe the cells that have exited the cell cycle and have become quiescent (Figure 1) (Johnson and Walker, 1999).

The progression of the cell cycle is regulated by protein kinase complexes, each of which consists of a cyclin and a cyclin-dependent kinase (CDK) (Sherr and Roberts, 1999). CDKs are expressed constitutively throughout the cell cycle, whereas levels of cyclin are restricted by transcriptional regulation of cyclin-encoding genes.
and by ubiquitin-mediated degradation (Koepp et al., 1999). Activation of CDK requires the binding of a cyclin in addition to site-specific phosphorylation. The progression from G₁ phase to S phase is regulated by cyclin complexes such as cyclin-D–CDK4, cyclin-D–CDK6, cyclin-E–CDK2 and cyclin-A–CDK2 (Sherr and Roberts, 1999).

![Four phases of cell cycle](image)

**Figure 1: Four phases of cell cycle**

The progression from G₂ to M phase is regulated by the cyclin-B–CDK1 complex. Inactive cyclin-B–CDK1 complexes accumulate during the G₂ phase of the cell cycle because phosphorylation of CDK1 by Weel and Myt1 inhibit CDK1 activity. Entry into mitosis requires that cyclin-B–CDK1 complexes are activated by CDC25C phosphatase, which removes the inhibitory phosphorylation of CDK1 (Smits and Medema, 2001).
Mitotic exit occurs after ubiquitination and proteolytic degradation of cyclin B by the anaphase-promoting complex (APC), which inactivates CDK1 (Figure 2). Among other functions, this control system of the cell ensures that cells divide when it is needed, so as to maintain the correct shape and size of organs and tissues. Any failure in any of the regulatory mechanism may lead a cell to grow and divide in an uncontrolled manner and contribute to tumor development (Maya-Mendoza et al., 2009).

The term tumor is derived from Latin word “tumere” meaning swelling or lump (Vincent, 1985). A tumor, also known as a neoplasm, is an abnormal mass of tissue which may be solid or fluid-filled. A tumor can be benign (non-cancerous) or malignant (cancerous).
Malignant tumors are commonly referred to as ‘cancer’ suggesting its tendency to cling and reach out to adjacent tissues (Abercrombie and Ambrose, 1962; Tarin, 2013). The terms neoplasm/neoplasia – Latin, “new growth”, and malignant tumor are commonly used interchangeably (Friedberg, 1986). The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 BC) who is considered as the “Father of medicine.” In Greek, this word refers to a crab most likely applied to the disease because of the finger-like spreading projections which resemble the shape of a crab. The Roman physician, Celsus (28-50 BC) later translated the Greek term into cancer, the Latin word for crab.

Cancer is one of the leading causes of death worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 (Stewart and Kleihues, 2003). Cancer causing agents or carcinogens may be broadly categorized as physical, chemical and biological (viral) agents. Physical agents such as radiation (UV rays, γ-rays, X-rays, electromagnetic radiation) can penetrate the nucleus and affect the DNA to cause mutation in the genes associated with cell multiplication and survival. Most of the cancers may be caused by several chemical agents such as benzopyrene, acetyl aminoflourene (AAT), dimethyl amino-azo-benzene (DAB), carbon tetrachloride, asbestos, aflatoxin B1 etc. Biological agents include certain viruses such as Simian virus 40, Rous sarcoma virus, Polyoma virus etc. Cancer causing viruses include the Human papillomavirus (HPV). Hepatitis B and C viruses cause almost 80 % of liver cancer in the world (Perz et al., 2006).

For a cell to become cancerous, multiple mutations in different set of genes is required which together affect the cell to escape normal regulation of control
mechanisms of cell division (Croce, 2008) and therefore, it is usually considered as a multihit process (Hanahan and Weinberg, 2000).

The one group of genes involved in the development of malignancy are referred to as oncogenes (Greek; oncos, a tumor). Oncogenes encode proteins that control cell proliferation, apoptosis, or both. They can be activated by alterations resulting from mutation or gene fusion (Konopka et al., 1985) by juxtaposition to enhancer elements (Tsujimoto et al., 1985) or by amplification. Translocations and mutations can occur as initiating events (Finger et al., 1986) during tumor progression, whereas amplification usually occurs during progression. The effect of oncogenes through oncoproteins facilitating attainment of cancerous properties could involve six ways: one group of oncogenes i.e. erbB and fms which are growth factors involved in cellular communication such as proliferation, growth, differentiation and survival. Their overproduction is associated with cancer and has proven to be a key to the process of angiogenesis. Examples include vascular endothelial factor in breast and colorectal cancers and fibroblast growth factor in hemangiomas. A second category of growth factor receptors i.e. myc and fos which relay the signals conducted by growth factors to the target cell. Their overproduction is also associated with cancer as they continue to release proliferative signals in the absence or derease of growth factors. Their over expression is especially found in non-small-cell lung cancer, breast, ovarian and colorectal cancers. Category three consists of non-receptor tyrosine kinases i.e. Abl1 that initiate tyrosine kinase activity (phosphorylation of tyrosine residues) in the absence of receptors as their name suggests. They are also found to be increased in cancers such as neuroblastoma and small-cell lung cancer (Loescher and Whitesell, 2003). The fourth category i.e c-H-
ras, c-K-ras which are membrane-associated G proteins, or guanine nucleotide-binding proteins. These proteins act as on-off switches (signal transducers) for growth factor receptors on the cell surface. Their increase is implicated in cancer by altering the cell membrane in malignant transformation as well as transmitting stimulatory signals without prompt. These oncogenes are implicated in two-thirds of malignant tumors, a fraction containing virtually all types of human cancer. The fifth category of oncogene i.e. braf which are serine threonine kinases, are components of intracellular signal transduction. They begin a cascade leading to cell division and are over-stimulated in cancer states. Transcription factors i.e. myb are the sixth category of oncogenes i.e. they bind to DNA (deoxyribonucleic acid) and initiate changes in gene expression, either regulatory or transformational.

Tumor suppressor genes (TSGs) work as antioncogenes whose protein products regulate cell growth by blocking the action of growth promoting proteins (Loescher and Whitesell, 2003). Some have been seen to directly antagonize the action of proto-oncogenes in growth regulation (Fearon, 1998). Some of these genes are normally active transcription factors within the cell nucleus. Abnormal repression of tumor suppressor genes results in deregulation of the cell cycle (excess cellular proliferation by prolonging proliferation signals) or cellular disorganization (Loescher and Whitesell, 2003). Tumor suppressor genes function in the cell cycle by inducing checkpoints, pauses, or arrests in certain stages of the cycle. These checkpoints allow for DNA repair and act to ensure the integrity of the cell’s genome (Rudin and Thompson, 1998). TSGs mutations differ from other potential carcinogenic mutations because the alteration in TSGs produces a modification in the protein products that directly contributes to the cancerous transformation. More than
12 such genes have been identified (Fearon, 1998). $p53$ gene is a tumor suppressor gene which codes for the protein called $p53$. It is the most commonly mutated gene in human malignancy. Even heterozygous mutation of $p53$ is associated with high rates of carcinogenesis in many tissues (Rudin and Thompson, 1998). Another tumor suppressor gene, known as the retinoblastoma gene ($Rb$), is also inactivated in many tumors (Loescher and Whitesell, 2003) and it is so called because it was first studied in the inheritance of retinoblastoma. $Rb$ gene functions as a cell cycle regulator. It interacts with a family of transcription factors to inhibit their activity and prevent entrance into the synthesis (S) phase of the cell cycle (Rudin and Thompson, 1998).

Cancers are classified by the type of cell that was initially altered. The main categories include carcinomas, sarcomas and lymphomas/leukaemia’s (Cairns, 1986).

i) **Carcinomas**- They usually originate from the epithelial layer of cells that form the lining of external parts of the body or the internal linings of organs within the body. Carcinomas account for about 80-90 percent of all cancer cases since epithelial tissues are most abundantly found in the body from being present in the skin to the covering and lining of organs and internal passage ways, such as the gastrointestinal tract. Carcinomas usually affect organs or glands capable of secretion including breast, lungs, bladder, colon and prostate. Carcinomas are of two types – adenocarcinoma and squamous cell carcinoma. Adenocarcinoma develops in an organ or gland and squamous cell carcinoma originates in squamous epithelium. Adenocarcinomas may affect mucous membranes and are first seen as a thickened plaque-like white mucosa. These are rapidly spreading cancers.

ii) **Sarcomas**- These cancers originate in connective and supportive tissues like muscles, bones, cartilage and fat. Bone cancer is one of the sarcomas termed
osteosarcoma. It affects the young ones most commonly. Sarcomas appear like the tissue in which they grow. Other examples include chondrosarcoma (cartilage), leiomyosarcoma (smooth muscles), rhabdomyosarcoma (skeletal muscles), mesothelial sarcoma or mesothelioma (membranous lining of body cavities), fibrosarcoma (fibrous tissue), angiosarcoma or hemangioendothelioma (blood vessels), liposarcoma (adipose or fatty tissue), glioma or astrocytoma (neurogenic connective tissue found in the brain), myxosarcoma (primitive embryonic connective tissue) and mesenchymous or mixed mesodermal tumor (mixed connective tissue types).

iii) **Lymphomas/Leukemias**- Lymphomas are cancers in which there is excessive production of lymphocytes by the lymph nodes and spleen. Hodgkin’s disease is an example of lymphoma. Lymphomas constitute about 5 percent of human cancers. Leukemia’s are neoplastic growth of leukocytes (white blood cells) and are characterized by excessive production of the cells. They constitute about 4-5 percent of human cancers.

Hanahan and Weinberg (2000) pointed out that malignant growth is a manifestation of six essential alterations in cell physiology (Figure 3) which are as follows:

i. **Self-sufficiency in growth signals**- Normal cells require mitogenic growth signals (epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, etc) before they can move from a quiescent state into an active proliferative state while cancer cells can grow and divide without external growth signals. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signalling molecules i.e. diffusible growth factors, extracellular matrix
components and cell-to-cell interaction molecules. Cancer cells develop numerous ways to generate their own growth signal like some oncogenes products that act by mimicking normal growth signalling in one way or another and thereby disrupt the homeostatic mechanism of the normal cell.

ii. Insensitivity to anti-growth signals- Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signalling circuits. Cancer cells evade these antiproliferative signals by becoming insensitive to this antigrowth signal. For example, the growth inhibitor signals are funnelled through the downstream pRb, the product of Rb tumor suppressor gene, which prevent the inappropriate transition from G1 to S phase. If pRb is damaged or disrupted through a mutation in its gene, the cell can divide uncontrollably and may lead to cancer formation (Weinberg, 1995; Nevins, 2001).

iii. Evading apoptosis- The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death- apoptosis- represents a major source of this attrition. Cell death pathway can be blocked at different levels of the signalling cascade by up-regulation of antiapoptotic proteins (Bcl-2, Bcl-XL and Mcl-1) and/or by down regulation or dysfunction of pro-apoptotic molecules such as Bax, Bak and BH3 domain (Adams and Cory, 2007). Evasion of apoptosis contributes to both tumorigenesis and treatment resistance (Fulda, 2009; Fernald and Kurokawa, 2013).
iv. Limitless replicative potential- The resulting deregulated proliferation programme suffices to enable the generation of the vast cell populations that constitute macroscopic tumors. Telomere maintenance is evident in virtually all types of malignant cells (Shay and Bacchetti, 1997); 85%–90% of them succeed in doing so by upregulating expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA (Bryan and Cech, 1999), while the remainder have invented a way of activating a mechanism, termed ALT, which appears to maintain telomeres through recombination-based interchromosomal exchanges of sequence information (Bryan et al., 1995). Although the mechanism is not completely understood, in tumor cells, the telomerase enzymes prevents the formation of critically short telomeres, adding GGTTAG repeats to the end of the chromosomes immortalizing the cells (Kellard, 2007; Tarkanyi and Aradi, 2008).

v. Sustained angiogenesis- The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel. Tumors appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (Hanahan and Folkman, 1996). By the process of angiogenesis, tumors make growth factor which induces formation of new capillary blood vessels to supply the cells with nutrient and oxygen. One common strategy for shifting the balance involves altered gene transcription. Many tumors evidence increased expression of VEGF and/or FGFs compared to their normal tissue counterparts.

vi. Tissue invasion and metastasis- During the development of most types of cancers, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new
colonies. These distant settlements of tumor cells metastases are the cause of 90% of human cancer deaths (Sporn, 1996). Several classes of protein involved in the tethering of cells to their surrounding in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell-cell adhesion molecules such as cadherins and integrins (Aplin et al., 1998) and matrix degrading proteases (Chambers and Matrisian, 1997).

1.2 Cancer treatment

Cancer treatment aims at curing the disease or prolonging the patient’s life considerably. Cancer can be commonly treated by surgery, chemotherapy, radiation therapy, hormonal therapy and targeted therapy (including immunotherapy such as monoclonal antibody therapy) etc. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient.

Figure 3: Six hallmarks of cancer (Source: Hanahan and Weinberg, 2000).
**Surgery**- In surgery, the surgeon operates on the patients and removes the cancerous tissues, provided the cancer has not metastasized. Some cancers can be completely removed with surgery alone, but undetected malignant cells may metastasize to other organ. The type of surgical procedure(s) recommended to a patient with different types of cancer (breast, cervical, colorectal, etc.) is determined by various factors including age, current health, previous illnesses, tumor size and cancer stage.

**Chemotherapy**- It is the use of drugs to kill cancer cells, usually by stopping the cancer cells ability to grow and divide and is used either singly as monotherapy or in combination with surgery and/or radiotherapy (Takimoto and Calvo, 2008). In chemotherapy hundreds of drugs have been approved for use against various types of cancers (Black and Livingston, 1990 a, b). Some clinically used common cancer chemotherapeutic drugs include cisplatin, doxorubicin, chlorambucil, cyclophosphamide, 5-fluorouracil etc. However, the full effectiveness of a chemotherapy drug may be limited due to the development of various side effects such as nephrotoxicity by cisplatin (Yao et al., 2007), myelodysplastic syndrome (MDS) and gonadal toxicity after cyclophosphamide administration (Haubitz, 2007) and doxorubicin induced cardiac myopathy (Batist et al., 2001) and also due to the development of drug resistance by cancer cells (Kartalou and Essigmann, 2001; Sengupta et al., 2011).

**Radiotherapy**- Radiotherapy uses radiation such as X-rays, gamma rays, electron beams or protons, to kill or damage cancer cells and stop them from growing and multiplying. It is a localised treatment, which means it generally only affects the part of the body where the radiation is directed, but some nearby normal cells may also be affected. It may also be used as part of adjuvant therapy to prevent tumor recurrence.
after surgery to remove a primary malignant tumor (for example, early stages of breast cancer).

**Hormone therapy** - Hormone therapy is most often used after surgery (as adjuvant therapy) to help reduce the risk of the cancer coming back, but it can be started before surgery (as neo adjuvant treatment) as well. It is usually used for at least 5 years. Tumors that are hormone-insensitive do not respond to hormone therapy. For example, the production of testosterone is reduced in men with prostate cancer. Hormone therapy is commonly used to treat prostate cancer, breast cancer and ovarian cancer (Miura et al., 2011). Tamoxifen, an antiestrogen drug, has been used in hormone therapy for the treatment of breast cancer.

**Immunotherapy** - Immunotherapy, or the concept of boosting the immune system to target and destroy cancer cells, has been a goal of cancer treatment for many years. However, limited success has been achieved with traditional immunotherapy, as cancer cells tend to evolve mechanisms that evade immune detection.

**Gene therapy** - Cancer gene therapy is the transfer of nucleic acids into tumor or normal cells to eliminate or reduce tumor burden by direct cell-killing. The term gene therapy encompasses a wide range of treatment types that all use genetic material to modify cells (either *in vitro* or *in vivo*) to help effect a cure (Mulligan, 1993).
1.2.1 Anticancer drugs and their possible mechanism(s) of action

Different anticancer drugs have distinctly diverse mechanisms of action (Figure 4).

Methotrexate (MTX) is an anti-folate that inhibits de novo purine and thymidine nucleotide synthesis. MTX induces death in rapidly replicating cells and is used in the treatment of multiple cancers. MTX inhibits thymidine synthesis by targeting dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS) (Rushworth et al., 2015).

5-Fluorouracil (5-FU) is a widely used anticancer drug. Since 1957, it has played an important role in the treatment of colon cancer and is used for patients with breast and other cancers, like those of the head and neck (Grem, 2000). 5-FU is a
pyrimidine analogue that can be misincorporated into RNA and DNA in place of uracil or thymine. 5-FU can be misincorporated into DNA of drug treated cells, and accumulation of 5-FU in the genome, rather than uracil excision, is correlated with 5-FU cytotoxicity in mammalian cells (An et al., 2007).

Hydroxyurea (HU), a clinically established inhibitor of DNA, plays an important role in the treatment for sickle cell disease and cancer chemotherapy (Yarbro, 1992; Sorg et al., 2005). It inhibits ribonucleotide diphosphate reductase, the enzyme that converts ribonucleotides into deoxyribonucleotides, which is essential for the DNA synthesis and repair (Danenberg et al., 1999).

6-mercaptopurine (6-MP), an inhibitor of purine metabolism, is an antineoplastic and immunosuppressive drug, which is increasingly used in a wide spectrum of immune related disorders (Estlin, 2001; Nielsen et al., 2001).

6-thioguanine is enzymatically converted by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) into cytotoxic nucleotides. These are incorporated into DNA as fraudulent bases, an important step that results in damage and arrest of replication by crosslinkage, single-strand breaks, interstrand cross-links, and sister chromatid exchange (Coulthard and Hogarth, 2005).

Alkylation agents are electrophiles that covalently transfer alkyl-groups onto the DNA bases (Helleday et al., 2008). There are different groups of alkylating agents i.e. the nitrogen mustards, nitrosoureas, aziridine compounds, alkyl sulphonates and the triazine compounds. The nitrogen mustards and nitrosoureas were the first DNA damaging drugs used to treat cancer patients (Hurley, 2002; Woods and Turchi, 2013). The nitrogen mustards are bifunctional alkylating agents that damage DNA by forming guanine-guanine and guanine-adenine interstrand
crosslinks. Mechlorethamine, bendamustine, melphalan, chlorambucil, ifosfamide and cyclophosphamide are the nitrogen mustards most commonly used today (Holland et al., 2003). Closely related to the nitrogen mustards are the aziridine compounds, such as mitomycin C and thiotepa, which also form guanine-guanine and guanine-adenine interstrand crosslinks (Holland et al., 2003; Fu et al., 2012). The majority of nitrosoureas are monofunctional chloroethymethylating agents that add chloroethyl groups to the N7 and O6 of guanine (Fu et al., 2012). Examples of chloroethymethylating agents are carmustine, lomustine and nimustine (ACNU) (Holland et al., 2003). Finally, the triazine compounds such as dacarbazine, procarbazine and temozolomide are monofunctional methylating agents whose cytotoxicity is linked to their production of O-6-methylguanine, despite this accounting for only approximately 5% of DNA adducts generated by these compounds (Newlands et al., 1997; Fu et al., 2012).

Antibiotics such as bleomycin (blenoxane) preferentially intercalates DNA at guanine-cytosine and guanine-thymine sequences, resulting in spontaneous oxidation and formation of free oxygen radicals that cause strand breakage. Doxorubicin (DOX, Adriamycin), a planar anthracycline glycoside antibiotic which intercalates between DNA bases and DNA topoisomerases inhibitor, is a first-line drug for many cancer types, but is primarily used in breast carcinoma, pediatric solid tumors, ovarian cancer and Hodgkin’s disease. Etoposide (Etopophos) and teniposide (Vumon) are both DNA topoisomerase II inhibitors for the utilization in leukemia treatment. Irinotecan (Camptosar) and topotecan (Hycamtin) belong to the camptothecin family. Both of these DNA topoisomerase I inhibitors are FDA-
approved for the treatment of refractory metastatic colon cancer and relapsed ovarian carcinoma, respectively.

Drugs that block mitosis seem to suppress the dynamic of microtubules and kill tumor cells. Paclitaxel (taxol) and Vinca alkaloids are the first class of antimitotic agents to be discovered and inhibit cancer cell proliferation. Most of these compounds are antimitotic agents that inhibit cell proliferation by acting on the polymerization dynamics of spindles, which are essential to proper spindle function of microtubules (Jordan and Wilson, 2004).

Based on their target specificity, these could be broadly classified as i) microtubule interfering agents, ii) inhibiting DNA replication, iii) Topoisomerase poisoning and iv) DNA alkalyting agents (Ancuceanu and Istdudor, 2004; Ravindran et al., 2009).

Currently, a lot of research is going on to design novel anticancer drugs targeting specifically to malignant cells with no or less side effects (Verma et al., 2010). The various anticancer drugs grouped under different classes depending on their molecular and cellular targets are shown in Table 1 below:

**Table 1: Cancer chemotherapeutic drugs that affect DNA as their main mechanism of action**

<table>
<thead>
<tr>
<th>Type</th>
<th>Examples of Drugs</th>
<th>Mechanism of Action</th>
<th>Type of DNA Damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Nitrogen mustards Nitrosoureas Temozolomide S23906</td>
<td>Base alkylation-Monofunctional DNA adducts Inter, intra and DNA-protein crosslinks</td>
<td>Block the replication machinery leading to strand breaks</td>
</tr>
<tr>
<td>Platinum drugs</td>
<td>Cisplatin Carboplatin</td>
<td>Monofunctional DNA adducts</td>
<td>Block the replication machinery leading to strand breaks</td>
</tr>
</tbody>
</table>
Oxaliplatin
Inter, intra and DNA-protein crosslinks
Block the replication machinery leading to strand breaks

Antimetabolites
5-Fluorouracil
Misincorporates into DNA
Depletes dNTPs
Blocks the replication machinery leading to strand breaks

Topoisomerase poisons
Camptothecin
Etoposide
Inhibit topoisomerase enzymes in complex with DNA
SSBs and DSBs

1.3 Plants as source of anticancer agents

Natural products, especially plants, have been used for the treatment of various diseases for thousands of years. Sumerians and Akkaidians first recorded the medicinal uses of plants in about 2600 BC (Samuelson, 1999). The Chinese book Materia Medica, in which there is description of more than 600 medicinal plants has been well documented with the first record dating from about 1100 BC (Cragg et al., 1997). The Greeks had also made substantial contribution to the rational development of the herbal drugs. Phytochemicals have been known to offer protection against a variety of chronic ailments which include cardiovascular diseases, obesity, diabetes, and cancer. In case of cancer protection, it has been estimated that the diets which are rich in phytochemicals can reduce cancer risk by 20% (WCRF/AICR, 1997). The secondary metabolites are responsible for the medicinal properties of various drugs. Recently, it has been reported in the literature that approximately 49% of 877 small molecules that were introduced as new pharmaceuticals between 1981 and 2002 by New Chemicals Entities were either...
natural products or semi-synthetic analogs or synthetic products based on natural product models. The first anticancer agents from plant sources were obtained in 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. These discoveries prompted the United States National Cancer Institute (NCI) to initiate an extensive plant collection program in 1960. This led to the discovery of many novel chemotypes showing a range of cytotoxic activities, including the paclitaxel, etoposide, topotecan and camptothecins (Cragg and Newmann, 2005). Vinblastine and vincristine are being used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi’s sarcoma.

Another evidence of success in natural product based drug development is the discovery of paclitaxel from the bark of the Pacific Yew, *Taxus brevifolia Nutt.* (Taxaceae). Paclitaxel is used against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer. Indian Ayurvedic medicine reports the use of the plant *Taxus baccata* for the treatment of cancers (Cragg and Newmann, 2005).

Camptothecin, isolated from the Chinese ornamental tree *Camptotheca acuminata* (Nyssaceae) was advanced to clinical trials by NCI in the 1970s but was dropped because of severe bladder toxicity. The semi-synthetic derivatives of camptothecin i.e. topotecan and irinotecan are used for the treatment of ovarian and small cell lung cancers, and colorectal cancers, respectively (Creemer et al., 1996; Bertino; 1997).

The roots of Podophyllum species, *Podophyllum peltatum* and *Podophyllum emodi* contains an active antitumor agent epipodophyllotoxin, an isomer of
podophyllotoxin (Berberidaceae) which is used in the treatment of skin cancer and warts. For the treatment of lymphomas and bronchial and testicular cancers, two semi-synthetic derivatives of epipodophyllotoxin i.e. etoposide and teniposide are being used (Harvey, 1999).

Another plant derived agent in clinical use is Homoharringtonine isolated from the Chinese tree *Cephalotaxus harringtonia* (Cephalotaxaceae) (Itokawa and Wang, 2005). Combretastatins isolated from the bark of the South African tree *Combretum caffrum* (Combretaceae) are active against colon, lung and leukemia cancers and it is expected that this molecule is the most cytotoxic phytomolecule (Petit et al., 1987; Ohsumi et al., 1998).

Silvestrol isolated from the fruits of *Aglaila sylvestre* (Meliaceae) exhibited cytotoxicity against lung and breast cancer cell lines (Cragg and Newman, 2005).

Elliptinium, a derivative of ellipticine isolated from species of several genera of the Apocynaceae family including *Bleekeria vitensis*, a Fijian medicinal plant is marketed in France for the treatment of breast cancer (Cragg and Newman, 2005).

Dragon's blood is the popular name for a dark red viscous sap produced by *Croton lechleri*. This herb is used in folk medicine as an anti-inflammatory, antimicrobial and anticancer agent (Hartwell, 1969; Pieters et al., 1993; Lopes, 2004).

Crude extracts from plants like *Colubrina macrocarpa*, *Hemiangium excelsum* and *Acacia pennatula* have been shown to possess a selective cytotoxic activity against human tumor cells (Popoca, 1998).

In order to treat cancer, extracts of *Teucrium polium* and *Pistacia lentiscus* are being used in the Palestinian and Israeli territories (Al-Howiriny et al., 2005).
Saudi Arabia, aerial parts of *Commiphora opobalsamum* are commonly used to treat cancer.

Plants of *Astragalus species* are used to treat leukemia and promote wound healing (Calis et al., 1997).

A potent anticancer agent has been identified in Chinese medicinal herb *Paris polyphylla* that has been used to treat liver cancer in China for many years and has been reported as an agent that can overcome drug resistance.


*Lithospermum erythrorhizon* produces a herbal medicine Shikonin which has been reported to inhibit tumor growth in mice (Lee et al., 2008). Phytochemicals like genestein, Indole-3-Carbinol (I3C), 3,3’-diindolemethane, curcumin (-)-epigallocatechin-3-gallate, resveratrol and lycopene are known to prevent growth of malignant cells by modulating various cellular signalling pathways and inducing apoptosis of cancer cells selectively without affecting normal cells (Sarkar et al., 2010). The chemopreventive and therapeutic potential of green tea polyphenols Catechin, Epigallocatechin-3-Gallate (EGCG) are well documented. They inhibit proliferation of cancer cells, possess antioxidant properties, induce apoptosis of cancer cells and affect the epigenome as well (Darvesh and Bishayee, 2013). The active component present in *Nigella sativa* known as thymoquinone targets various...
signalling pathways involved in the process of carcinogenesis, thus suggesting its possible role in cancer therapy (Ghosheh et al., 1999). *Andrographis paniculata* contains andrographolide, which is an anticancer compound that inhibits interleukin-6 (IL-6) mediated signalling, and induces programmed cell death (Rajagopal et al., 2003). Acetyl-11-Keto-B-Boswellic Acid (AKBA), the active component of *Boswellia serrata*, acts as anti-angiogenic agents by inhibiting Vascular Endothelial Growth Factor (VEGF) signalling (Zhang et al., 2013). Asiatic acid, a pentacyclic triterpene present in *Centella asiatica* decreases viability of cancer cells by increasing expression of p53 (Lee et al., 2002). A plethora of antitumor properties are found in Curcumin, active component obtained from *Curcuma longa* and is effective in prevention of multiple steps involved in the process of development of cancer (Bar-Sela et al., 2010). *Panax ginseng* contains ginsenosides which shows anti proliferative, anti invasive and anti angiogenic properties (Kang et al., 2011).

Another synthetic agent based on a natural product model is roscovitine which is derived from olomucine, originally isolated from the cotyledons of the radish, *Raphanus sativus L.* (Brassicaceae) (Meijer and Raymond, 2003). Olomucine was shown to inhibit cyclin-dependent kinases (Cdk), proteins which play a major role in cell cycle progression, and chemical modification resulted in the more potent inhibitor, roscovitine, which currently is in Phase II clinical trials in Europe.

Another case of considerable interest is that of thapsigargin (TG), isolated from the umbelliferous plant, *Thapsia garganica L.* (Apiaceae), collected on the Mediterranean island of Ibiza (Denmeade et al., 2003). Thapsigargin induces apoptosis (cell death) in quiescent and proliferating prostate cancer cells.
Flavopiridol is totally synthetic, but the basis for its novel flavonoid structure is a natural product, rohitukine, isolated as the constituent responsible for antiinflammatory and immunomodulatory activity from *Dysoxylum binectariferum Hook. f.* (Meliaceae), which is phylogenetically related to the Ayurvedic plant, *Dysoxylum malabaricum Bedd.*, used for rheumatoid arthritis. Flavopiridol was one of the over 100 analogs synthesized during structure–activity studies, and was found to possess tyrosine kinase activity and potent growth inhibitory activity against a series of breast and lung carcinoma cell lines (Senderowicz and Sausville, 2000).

In an attempt to explore the flora and harness their anticancer potential in the development of newer drugs through preclinical and clinical trials another plant-derived drug betulinic acid has shown good promise.

1.4 Betulinic acid: distribution and isolation

Triterpenes represent a varied and important class of natural compounds. Among the various triterpenes present in plant kingdom, pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several biological and medicinal properties. These lupane-derived triterpenoid plant extracts show many antitumor properties which have been demonstrated for the past 25 years for their cytostatic activity on various *in vivo* cancer model systems (Sandberg et al., 1987).

Betulinic acid (3b-hydroxy-lup-20(29)-en-28-oic acid) (BA) (Figure 5) is one such naturally occurring pentacyclic lupane type triterpene, distributed widely throughout the plant kingdom. It was originally isolated from the bark of the white birch, *Betula pubescens*, from which it got its name (Tan et al., 2003). Hundreds of
published reports have described that betulinic acid occurs across a multitude of taxonomically diverse genera. Since the structurally related precursor betulin has got a widespread occurrence among plants, it is conceivable that the distribution of betulinic acid is even much greater (Hayek et al., 1989).

Other known sources of betulinic acid include *Ziziphus* spp. (Rhamnaceae), (Pisha et al., 1995) *Syzygium* spp. (Myrtaceae) (Kashiwada et al., 1998), *Diospyros* spp. (Ebenaceae) (Recio et al., 1995) and *Paeonia* spp. (Paeoniaceae) (Ikuta et al., 1995).

A multitude of extraction and isolation schemes have been used for the procurement of betulinic acid. Typically, dry plant material is extracted with CHCl₃ (for aglycons) (Kinoshita et al., 1998), MeOH (for both aglycons and glycosylated derivatives) (Kinoshita et al., 1998) or even H₂O (Macias et al., 1995). The plant may be defatted with hexane prior to extraction to remove non-polar materials (Sun et al., 1998; Higa et al., 1998). The resultant extracts can be dried and further extracted with other solvents (Ryu et al., 1992; Enwerem et al., 2001) or directly subjected to column chromatography (Jagadeesh et al., 1998).

![Figure 5: Chemical structure of betulinic acid (3b-hydroxy-lup-20(29)-en-28-oic acid)](image-url)
1.4.1 Physicochemical properties of betulinic acid

The pure compound of betulinic acid appears as a white crystalline solid, melts at 295-297°C. It was isolated from *Melaleuca cajuput* and was chromatographed on a silica gel column using chloroform as eluent (Faujian et al., 2010). It exhibits limited solubility in organic alcohols such as methanol, ethanol, chloroform, and ether and has low solubility in water, petroleum ether, dimethyl formamide and benzene (Yogeswari and Sriram, 2005). However, it is highly soluble in pyridine, DMSO and acetic acid. It is not readily visible on thin layer chromatography plates under UV (254 and 365 nm) but is easily detected following exposure to iodine vapours, anisaldehyde, anisaldehyde – sulphuric acid, or vanillin – sulphuric acid spray reagents (Cichewicz and Kouzi, 2004). The pharmacokinetics and tissue distribution of betulinic acid was studied in CD-1 mice. The results showed that after i.p 250 and 500 mg/kg dose, the serum concentrations reached peaks at 0.15 and 0.23 h, respectively (Guthrie, 2008). The 250 and 500 mg/kg betulinic acid i.p. doses were found to have elimination half-lives of 11.5 and 11.8 h and total clearances of 13.6 and 13.5 l/Kg/h, respectively (Yang et al., 2012). Recently, a robust assay based on liquid chromatography/mass spectrometry was developed to conduct a quantitative analysis of betulinic acid in mouse, rat and dog plasma. At 15 and 25 mg/ml in mouse, rat or dog plasma, betulinic acid was 99.99% bound to serum proteins, and at 5 mg/ml betulinic acid was > or =99.97% bound following i.p. or intravenous administration *in vivo* (Guthrie, 2008).
1.4.2 Synthesis and derivatization of betulinic acid

Although betulinic acid is widely available from numerous botanical sources, these sources are not sufficient to meet the growing demand for betulinic acid among members of the scientific community for biological and clinical testing. Fortunately, the betulinic acid precursor betulin is available from many plant species in significant yields of >20% (Hayek et al., 1989). Furthermore, methods for the multi-ton production of betulin from birch-kraft pulp have been proposed (Hayek et al., 1989). Therefore, semi-synthetic methods for the production of betulinic acid from betulin are necessary to provide sufficient quantities of this bioactive triterpene. Two simple methods have been reported for the scale-up production of betulinic acid from betulin (Kim et al., 1997). The first method is outlined. The triterpene betulin is subjected to Jones oxidation to give betulonic acid in 75% yield. Reduction of betulonic acid by NaBH_4 in THF provides a mixture of 3a- and 3b-hydroxyl products (5:95). Crystallization of the product mixture from MeOH affords the 3b-hydroxyl product (betulinic acid) in 75% yield. The remaining product mixture can in turn be re-oxidized and reduced as indicated to give a 71% overall yield of betulinic acid. In an alternative method (Kim et al., 1997) the primary C-28 hydroxyl group in betulin can be protected followed by acetylation of the secondary alcohol at C-3. The protecting group of the C-28 hydroxyl is then removed and the primary alcohol is oxidized to the carboxylic acid. Subsequently, the acetyl moiety is removed yielding betulinic acid in 88% yield. Although this method is efficient, it unfortunately suffers from an increased expenditure of time and materials as compared to the simplicity of the two-step scheme.
There exists a great deal of interest in probing the structural features responsible for the pharmacological effects of betulinic acid and to further optimize its activity profile. As a result, numerous derivatization studies have been performed on betulinic acid leading to the production of an array of betulinic acid analogs. Betulinic acid possesses three sites that are highly amenable to derivatization, including the C-3 hydroxyl, C-20 alkene, and C-28 carboxylic acid positions. In addition, betulinic acid has been subjected to solid-phase synthetic modifications for the preparation of combinatorial libraries (Pathak et al., 2002). This method holds the potential for the production and subsequent pharmacological evaluation of large sets of betulinic acid analogs. A number of interesting rearranged betulinic acid analogs has also been prepared. Furthermore, microorganisms have been utilized as biocatalysts for the preparation of betulinic acid derivatives (Chatterjee et al., 1999; Kouzi et al., 2000).

1.4.3 Biological activities of betulinic acid

A. Betulinic acid as an anti-cancer agent

In 1995, betulinic acid was reported as a highly selective growth inhibitor of human melanoma, neuroectodermal and malignant tumor cells and was reported to induce apoptosis in these cells (Pisha et al., 1995).

The antitumor activity of betulinic acid has been extensively studied against a panel of cancer cell lines, primary tumor samples and xenograft mouse models. While initial reports suggested that betulinic acid is selectively cytotoxic against melanoma cell lines, anticancer activity was subsequently also reported against other types of human cancers including neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia as well as several carcinoma, i.e. head and neck, colon,
breast, hepatocellular, lung, prostate, renal cell, ovarian or cervix carcinoma. Also, betulinic acid was cytotoxic in different models of drug resistance, for example primary pediatric acute leukemia samples that were refractory to standard chemotherapeutic agents (Qian et al., 2011). Further, there is evidence that betulinic acid is preferentially cytotoxic against metastatic over non-metastatic melanoma cell lines. Moreover, betulinic acid is cooperated with different cytotoxic stimuli to suppress tumor growth, including ionizing radiation, chemotherapeutic drugs or the death receptor ligand TRAIL. This suggests that betulinic acid may be used as sensitizer in combination regimens to enhance the efficacy of anticancer therapy. By comparison, normal cells of different origin have been reported to be much more resistant to betulinic acid than cancer cells pointing to some tumor selectivity (Fulda, 2008).

However, unlike betulin, the oxidized derivative betulinic acid possesses a number of intriguing pharmacological effects (Yang et al., 2012). Betulinic acid has been reported to inhibit growth of cancer cells, without affecting normal cells and its lack of cytotoxic activity has been demonstrated in human astrocytes, human dermal fibroblasts, peripheral blood lymphoblasts and animal studies. The cytotoxicity and mode of cell death of betulinic acid were determined using MTT assay and DNA fragmentation analysis respectively. Cells treated with high concentrations of betulinic acid exhibited features characteristic of apoptosis such as blebbing, shrinkage and a number of small cytoplasm body masses when viewed under an inverted light microscope after 24 h (Yazan et al., 2009). The in vitro sensitivity of betulinic acid was for broad cell line panels derived from lung, colorectal, breast, prostate and cervical cancer, which are the prevalent cancer types characterized with
highest mortalities in woman and men. Multiple assays were used in order to allow a reliable assessment of anticancer efficacy of betulinic acid. After 48 h of treatment with betulinic acid, cell viability as assessed with MTT and cell death as measured with propidium iodide exclusion showed clear differences in sensitivity between cell lines. However, in all cell lines tested colony formation was completely halted at remarkably equal concentrations that are likely attainable in vivo. The results substantiate the possible applications of betulinic acid as a chemotherapeutic agent for the most prevalent human cancer types (Kessler et al., 2007).

**B. Anti-HIV Activity**

Betulinic acid isolated from the MeOH extract of the leaves of *Syzgium claviflorum* was tested on HIV-1 replication in H9 lymphocyte cells (Fujioka et al., 1994). It was found that betulinic acid shows an inhibitory activity against HIV-1 replication with an EC50 value of 1.4 μM and inhibited uninfected H9 cells growth with an IC50 value of 13 μM.

Theo et al. (2009) isolated betulinic acid from the stem bark of *Peltophorum africanum*, a traditional South African medicinal plant. They evaluated it for the inhibitory activities against HIV-1NL4-3 (X4-HIV-1) and HIV-1JRCSF (R5-HIV-1). Betulinic acid inhibited against HIV-1NL4-3 and HIV-1JRCSF with IC50 values of 0.04 and 0.002 μg/ml, respectively. They suggested that betulinic acid could be used as potential therapeutics for HIV-1. Betulinic acid was isolated from the leaves and twigs of *Cratoxylum arborescens* and was tested in the HIV-1 RT assay and syncytium assay (Reutrakul et al., 2006). Betulinic acid showed anti-HIV-1 activity in the syncytium assay with IC50 value of 9.8 μg/ml and in the RT assay with IC50
of 10.8 μg/ml. The results showed that betulinic acid is the most active of isolated compounds in the syncytium assay.

C. Antibacterial activity

Betulinic acid extracted from the leaves of Vitex negundo demonstrated antibacterial activity against Bacillus subtilis at a concentration of 1000 mg/disc with a zone of inhibition of 18.8 mm² (Chandramu et al., 2003).

D. Antimalarial activity

The in vitro antiplasmodial activity (IC50) of betulinic acid isolated from the root bark of the Tanzanian tree against Chloroquine-resistant (K1) and sensitive (T9-96) Plasmodium falciparum were found to be 19.6 mg/ml and 25.9 mg/ml respectively. When betulinic acid was tested for in vivo activity in a murine malaria model (Plasmodium berghei), the top dosage employed (250 mg/kg/day) was ineffective at reducing parasitemia and exhibited some toxicity (Steele et al., 1999).

Betulinic acid isolated from Triphyophyllum peltatum and Ancistrocladus heyneanus also exhibited moderate to good in vitro antimalarial activity against asexual erythrocytic stages of the human malaria parasite Plasmodium falciparum (Bringmann et al., 1997).

E. Analgesic and anti-inflammatory activity

Betulinic acid isolated from Diospyros leucomelas showed anti-inflammatory activity in the Carrageenan and serotonin paw edema tests and TPA and EPP ear edema tests (Recio et al., 1995). Betulinic acid isolated form Ipomoea pes-caprae showed pronounced antinociceptive properties in the writhing test and formalin test in mice (Krogh et al., 1999).
F. Antihelmintic activity

Enwerem et al. (2001) examined the anthelmintic activity of methanol, hexane and ethyl acetate extracts of Berlina grandiflora, which contain betulinic acid as the major component. Caenorhabditis elegans, a free living soil nematode, was used as an in vitro model in the study. The results showed that the crude extracts (500 ppm) showed antihelmintic activity in the order ethyl acetate > methanol > hexane. Betulinic acid isolated from the ethyl acetate fraction showed strong anthelmintic activity at 100 ppm comparable to piperazine.

G. Toxicity of betulinic acid

Betulinic acid has been found to exhibit greatly reduced in vitro cytotoxicity against normal dermal fibroblasts and peripheral blood lymphocytes (Zuco et al., 2002). Administration of betulinic acid intraperitoneally to rats at doses of 200 and 400 mg/kg provided no evidence of toxicity (Sandberg et al., 1987). Likewise, Pisha and co-workers (Pisha et al., 1995) reported no toxicity has been found upon intraperitoneal administration of six doses (500 mg/kg each) of betulinic acid to mice on every fourth day. Further tests using a similar treatment regimen of six doses (250 mg/kg each) of betulinic acid on every third day to mice were also non-toxic. Based on these findings, it can be inferred that betulinic acid possesses no cytotoxicity at even relatively high therapeutic doses.

H. Other Biological Activities

Betulinic acid has been tested for a variety of other biological activities. Several studies have examined the potential antimicrobial application of betulinic acid (Schuhly et al., 1999). Betulinic acid has been shown to be inactive in vitro against Bacillus subtilis (Nick et al., 1995), Escherichia coli, (Nick et al., 1995;
Betulinic acid has also been tested against the essential fungal enzyme chitin synthase II (Jeong et al., 1999). In this experiment, betulinic acid exhibited an IC50 value of 98.7 mg/mL, whereas betulin was shown to be completely inactive. Other studies showed betulinic acid to be non-toxic to *Penicillium citrinum* (Higa et al., 1998). Collectively, these reports indicate that betulinic acid is not a promising antimicrobial agent; however, some of its more active derivatives may offer new leads toward the development of triterpene-based antimicrobial agents.

Betulinic acid was also examined for its spasmogenic activity (Bejar et al., 1995). It was tested *in vitro* against isolated rat fundus at 1x10^{-4} M. Betulinic acid demonstrated partial agonistic effects relative to serotonin and produced 46.5 and 60.5% contractile response, respectively.

Betulinic acid has been reported to exhibit a variety of other biological effects. Studies revealed that betulinic acid possess antinociceptive (Kinoshita et al., 1998) and anti-HSV-1 activities (Ryu et al., 1992). In addition, betulinic acid has been shown to exert inhibitory activity against cyclic AMP-dependent protein kinase (Wang and Polya, 1996) sulfonylureas (Zhu et al., 1996) and stromelysin and collagenase (Sun et al., 1998).
1.4.4 Mechanism of action of anti-cancer activity

Numerous studies over the last years have been done to elucidate the molecular mechanisms of betulinic acid-mediated antitumor activity. Betulinic acid is an experimental antineoplastic agent that induces apoptosis in melanoma cells in vitro and in vivo, as well as in neuroectodermal tumor cell lines in vitro (Wick et al., 1999). The mechanism of betulinic acid-mediated anticancer activity is by induction of apoptosis through the activation of caspases independent of the p53 gene status and CD95 activation (Fulda et al., 1999). Betulinic acid induced apoptosis is mediated via direct effects on mitochondria (Fulda et al., 1999). One characteristic feature of betulinic acid’s cytotoxicity is its ability to trigger the mitochondrial pathway of apoptosis in cancer cells. Apoptosis pathways can be initiated at the level of the mitochondria by the release of apoptotic factors such as cytochrome C, Smac or AIF from the mitochondrial intermembrane space into the cytosol (mitochondrial or intrinsic pathway). Smac promotes apoptosis by neutralizing “Inhibitor of Apoptosis Proteins” (IAP)-mediated inhibition of caspase-3 and -9. The main mechanism of anticancer action of betulinic acid is known as the induction of apoptosis in cells independent of their p53 status. It is suggested that betulinic acid could induce mitochondria to undergo permeability transition (PT), which causes the release of cytochrome c into the cytosol, the activation of caspases (interleukin 1β-converting enzyme/Ced-3-like proteases), and DNA fragmentation. Another mechanism indicates betulinic acid acting as the inhibitor of aminopeptidaseN (APN), since APN is closely associated with extracellular matrix components; its inhibition is likely to prevent the melanoma invasion into basement membranes. By its lipophilic character, betulinic acid may directly or indirectly influence the
lysosomal membrane properties causing lysosomal membrane permeabilization or, alternatively, betulinic acid may act as a lysosomotropic agent like siramesine. Siramesine is a lipophilic compound that shares some interesting features with betulinic acid such as induction of p53, Bcl-2 and caspase independent cell death and induction of cytoprotective autophagosomes. Betulinic acid also induces proteasome-dependent degradation of Sp1, Sp3, and Sp4 proteins. Laboratory studies have shown that both basal and hormone-induced expression of VEGF in cancer cell lines is dependent on Sp protein expression, and regulation of survivin in some cells is also dependent on these transcription factors. Moreover, because Sp proteins are up-regulated in many tumors/cancer cells and are associated with proliferative, angiogenic, and antiapoptotic pathways, it is hypothesized that the anticarcinogenic activity of betulinic acid may be due, in part, to down-regulation of Sp proteins. Betulinic acid also decreased expression of Sp1, Sp3, and Sp4 proteins in SK-MEL2 melanoma cancer cells, and similar results were obtained in other cancer lines (Chintharlapalli et al., 2007). Betulinic acid inhibits VEGF and survivin promoter expression through proteasome-dependent degradation of Sp proteins. Expression of both VEGF and survivin in some cancer cell lines is regulated by Sp protein interactions with GC-rich promoter sites therefore, the effects of betulinic acid on decreased expression of VEGF and survivin through Sp protein degradation was further investigated. The studies show that betulinic acid decreased luciferase activity in LNCaP cells transfected with pVEGF1 and pVEGF2, and these effects were reversed by the proteasome inhibitor MG132; similar results were observed using the proteasome inhibitor lactacystin. The studies further confirm that betulinic acid–induced degradation of Sp proteins results in decreased VEGF expression in LNCaP.
cells, and this is consistent with RNA interference studies showing that Sp1, Sp3, and Sp4 regulate VEGF expression in cancer cell lines. Betulinic acid perturbs mitochondrial functions. It has been found to activate two human apoptotic pathways: the mitochondrial apoptotic pathway and NF-κB pathway. It induces apoptosis independently of CD95 (AP0-1/FAS) and p53. The other apoptosis pathway affected by betulinic acid is the NF-κB pathway. DNA damage activates NF-κB. NF-κB leads to inflammation, the synthesis of ROS, cytokines and chemokines including TNF, lymphotoxins, IL-6, and IL-8, and growth and angiogenic factors. NF-κB can lead to malignant proliferation, the prevention of apoptosis, and an increase in metastasis and angiogenesis (Qian et al., 2011).

1.4.5 Metabolism and disposition of betulinic acid

Absorption, Distribution, and Pharmacokinetics- Because of its potential clinical application for the treatment of cancer and HIV infection, studies aimed at determining the fate of betulinic acid in mammals have been initiated. The poor solubility of betulinic acid in aqueous systems generated a great deal of interest in investigating several formulation schemes (Son et al., 1998; Rusmawati et al., 2001). In one study (Udeani et al., 1999) a formulation of polyvinylpyrrolidone (PVP)–betulinic acid complex was administered to CD-1 mice as an intraperitoneal dose of 250 or 500 mg/kg. These high dosage levels were selected because they were previously found to be effective in vivo for the treatment of cancer in experimental mice (Pisha et al., 1995). The authors concluded that, under these experimental conditions, the pharmacokinetic data of betulinic acid were best described fitting a standard two compartment first-order model. Peak serum concentrations of betulinic acid were observed at 0.146 and 0.228 h for the 250 and 500 mg/kg doses,
respectively, by LC–MS analysis. At the 250 and 500 mg/kg doses, betulinic acid exhibited distribution volumes of 106 and 108 L/kg, respectively, and half-lives of 11.5 and 11.8 h, respectively.

The distribution of betulinic acid, administered at 500 mg/kg, was found to vary considerably among the various tissues over the course of 24 h. Additional LC–ESMS (negative ion mode) analyses were performed by Shin et al. (1999) to detect betulinic acid in nude mice bearing human melanoma following a single 500 mg/kg intraperitoneal dose. Concentrations of betulinic acid in the blood, liver, lung, kidney, and tumor were determined 24 h post-injection. Interestingly, the highest concentration of betulinic acid was found in the tumor with virtually none in the blood. A significant portion of betulinic acid was also found in the liver. In an effort to elucidate its metabolic fate in humans, microorganisms were utilized as in vitro model systems to predict and prepare the potential mammalian metabolites of betulinic acid. Microorganisms have long been recognized as appropriate tools for studying mammalian drug metabolism based on the extensive homology between microbial and mammalian metabolic pathways (Clark and Hufford, 1991). In a series of pertinent experiments (Chatterjee et al., 1999; Kouzi et al., 2000) betulinic acid was incubated with resting-cell suspensions of Cunninghamella spp. (NRRL 5695), Cunninghamella elegans (ATCC 9244), Bacillus megaterium (ATCC 13368 and 14581), and Mucor mucedo (UI-4605). From these studies, a series of oxidized and conjugated metabolites of betulinic acid were obtained. All biotransformation products of betulinic acid were evaluated for antimelanoma activity. In addition to the use of microorganisms as models of its mammalian metabolism, molecular modeling studies have been conducted to predict the sites of metabolism mediated by
human cytochrome P450 enzyme systems in betulinic acid. One such system is the human CYP2C9. Human CYP2C9 exhibits selectivity for substrates containing an ionizable carboxylic acid group or an analogous group/isostere, and the usual site of metabolism lies at a fairly well-defined distance from this structural feature. However, there are additional criteria associated with substrate selectivity for CYP2C9 which relate to various other contacts with active site residues, including hydrogen bonding, π–π stacking, and certain hydrophobic interactions. Many of the key regions (substrate recognition sites) governing substrate selectivity within the CYP2 family have been probed using site directed mutagenesis, and a number of particularly important contact points with potential substrates have been identified. Based on the accumulating evidence from mutagenesis and other experimental data, a homology model for human CYP2C9 has been constructed and shown to be consistent with the known substrate selectivity characteristics exhibited by CYP2C9. Betulinic acid, which contains a free carboxylic acid group, may act as a substrate for human CYP2C9. Hence, a modelling study was undertaken utilizing the constructed CYP2C9 homology model in an attempt to predict the sites of CYP2C9-mediated metabolism in betulinic acid (Lewis, unpublished results). The betulinic acid molecule was fitted with a previously constructed template of six typical CYP2C9 substrates (tolbutamide, tienilic acid, diclofenac, warfarin, ibuprofen, and naproxen) which had been docked within the CYP2C9 model in an orientation consistent with their known sites of metabolism. The need to minimize adverse steric interactions was a very important factor in selecting the most appropriate orientation for the betulinic acid molecule within the active site of the CYP2C9 model. Although the molecule of betulinic acid is somewhat larger than the majority of typical CYP2C9
substrates, the study revealed that the most likely sites of oxidative metabolism mediated by human CYP2C9 in betulinic acid are the C-23 and C-6a positions. These potential sites of metabolism are consistent with the distance ranges encountered for other substrates of this enzyme. In addition to CYP2C9, a homology model for human CYP3A4 was also utilized to predict the sites of CYP3A4-mediated metabolism in betulinic acid (Lewis, unpublished results). Similar to the CYP2C9 model predictions, results from the CYP3A4 modelling study indicated that positions C-6 and C-23 (or C-24) in betulinic acid are the most likely sites of metabolism mediated by human CYP3A4.

According to World Health Organization, 80 % of the people living in rural areas depend on natural medicinal products as primary healthcare system. A great deal of pharmaceutical research done in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the natural products used in the treatment of cancer. Some natural products protect the body from cancer by enhancing detoxification functions of the body. Some of them reduce the toxic side effects of chemotherapy and radiotherapy. Scientists all over the world are concentrating on the natural products to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of natural anticancer products, their formulations can be designed to attack the cancerous cells without harming normal cells of the body (Manju et al., 2012). To date, the majority of studies were devoted to the investigation of betulinic acid, as this derivative revealed better bioavailability and cytotoxic effects. Because of its relative reactive cytotoxicity against malignant cells compared to normal cells, betulinic acid is a promising new experimental anti-cancer agent for the treatment of
human cancer today (Fulda, 2009). Recently, studies showed that substituted BA analogs can be proteasome inhibitors, and the C-3 and C-30 positions of betulinic acid are the pharmacophores for improving the proteasome inhibition activity. Within the C-3 and C-30 substitutions, larger side chains with lipophilic or aromatic side chains are favoured for increased inhibition of the chymotrypsin-like activity of 20S proteasome. C-3 and C-30 modified BA compounds showed low toxicity in the previous anti-AIDS studies, and bevirimat, a C-3 substituted BA, has already succeeded in phase II b clinical trials.

Despite the wide importance of ethnobotanical studies on the therapeutic uses of betulinic acid, its exact role has not been fully explored. Most of the anticancer research on betulinic acid has been carried out using cancer cell lines in vitro and the effects of betulinic acid in vivo needs to be further researched. Moreover, the anticancer activity of betulinic acid against murine ascites Dalton’s lymphoma has not been studied.

Therefore, the present research study for the Ph.D. degree was undertaken to evaluate the antitumor effects of betulinic acid against murine ascites Dalton’s lymphoma, and to explore the possible mechanism of its antitumor activity with reference to apoptosis and biochemical change.