EVALUATION & FORMULATION DEVELOPMENT OF SEMISYNTHETIC ANALOG (S) OF BOSWELLIC ACIDS AS ANTICANCER AGENTS

THESIS

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(COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH)

JAMMU-180001, INDIA

2011

Dedicated

To

My Parents….!!!

DECLARATION

I, **Gousia Chashoo**, hereby declare that this thesis entitled **'Evaluation and Formulation Development of Semisynthetic analog(s) of Boswellic acids as anticancer agents'** represents my own work except where due acknowledgement is made, commencing in the cancer pharmacology division, Indian Institute of Integrative medicines, Jammu. It has not been previously included in a thesis, dissertation or report submitted to this institute or to any other institutions for a degree, diploma or any other qualifications.

Dated: 14th July 2011

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Gousia Chashoo

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LIST OF ABBREVIATIONS

AKBA	Acetyl-11-keto-β-boswellic acid
AIF	Apoptosis Inducing Factor
Apaf-1	Apoptotic Proteases Activating Factor
ANG	Angiopoietin
ADME	Adsorption Distribution Metabolism and Excretion
ACN	Acetonitrile
AV	Average
α	Alpha
&	And
~	Approxy
BAs	Boswellic Acids
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
b.wt.	Body weight
β	Beta
DAPI	4'-6-Diamidino-2-phenylindole
DNase	Deoxyribonuclease
DTT	Dithiothreitol
DMSO	Di methyl Sulphoxide
DCM	Dichloromethane
DLS	Dynamic light scattering
º	Degree
EAC	Ehrlich Ascitic Carcinoma
EAT	Ehrlich Tumour
ELISA	Enzyme Linked Immunosorbent Assay
EDTA	Ethylene Diaminetetra acetic acid
FACS	Fluorescence Assisted Cell Cytometry
FSC	Forward Scatter
FITC	Flurscein Isothiocyanate
FCS	Fetal Calf Serum
γ	Gamma
H	Hours
HKBA	Hexanoyloxy derivative of 11-keto-β-boswellic acid

HPLC	High Performance Liquid Chromatagraphy
>	Greater than
IPA	Isopropyl Alcohol
KBA	11-keto-β-boswellic acid
MEM	Minimum Essential Medium
MMP	Mitochondria Membrane Potential
MMPs	Matrix Metalloproteinases
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
$\Delta\psi m$	Mitochondrial Membrane Potential Loss
M	Molar
m	Milli
mV	MilliVolt
mm	Millimeter
min	Minute
µ	Micro
µM	Miromolar
µl	Microliter
$MgCl_2$	Magnesium Chloride
NCCS	National Centre for Cell Sciences
NPs	Nanoparticles
NaCl	Sodium Chloride
n	Nano
nM	Nanomolar
PKBA	Propionyloxy derivative of 11-keto-β-boswellic acid
PBS	Phosphate buffered saline
PCD	Programmed cell death
PARP	Poly (ADP-ribose) Polymerase
PI	Propidium Iodide
PAGE	Polyacrylamide Gel Electrophoresis
PVDF	Poly (vinylidene fluoride)
PS	Phosphatidylserine
PLGA	Poly-Lactico-Glycolide

PEG	Polyethylene glycol
PVA	Polyvinyl Alcohol
%	Percent
RNase	Ribonuclease
Rpm	Rounds per minutes
RPMI	Rose Park Memorial Institute-1640
RT	Room Temperature
RPHPLC	Reverse Phase Isocratic Mode of High performance Liquid Chromatography
Rh-123	Rhodamine-123
SRB	Sulphorhodamine B
S-180	Sarcoma-180
SD	Standard Deviation
SEM	Scanning Electron Microscopic
SSC	Side Scatter
Sec	Second
SDS	Sodium Dodecyl Sulfate
<	Smaller than
TBST	Tris Buffered Saline and Tween-20
Tris buffer	Tris-Hydroxymethyl-Aminomethan
TCF	Tissue Culture Flask
TCA	Trichloro Acetic Acid
TEMED	Tetramethylethylenediamide
TEM	Transmission Electron Microscopy
TF	Transferrin
UV	Ultra voilet
λ	Wavelength

CHAPTER 1: INTRODUCTION

Cancer is a complex collection of diseases that can arise in almost any tissue in the body. A full blown cancer is characterised by the cells that have become decidedly antisocial and carry on their activities irrespective of the surrounding cells and tissues. Normal cells have a myriad of mechanisms that monitor their working in cooperation with other cells. Cancer cells disrupt this normal activity, they divide at their will regardless of how much they crowd their neighbours, move to the places they are not belonging to, attract blood vessels to themselves and stop obeying aging signals. It has therefore been stated that cancer cells misbehave and their mischief gives rise to tumor. Each cancer has its own unique pattern of behaviour which is determined by the tissue in which it is formed, the mutations cells have adopted and the chemistry in an individual's body. The development of cancer (carcinogenesis) is a multistage process and is initiated by an external agent i.e. a carcinogen e.g. a virus, an exogenous chemical compound, or ionizing radiation. Normally after entering the body these carcinogens are either excreted (chemicals), deactivated (chemicals or radiation), or inhibited (viruses), however, if any of the carcinogen is not neutralised by one of these mechanisms, it activates a normal cell and eventually induces a genetic mutation.

Cancer is the second largest health problem of the world after cardiovascular diseases in both developed and developing countries. According to the World Health Organization 7.6 million people died of cancer worldwide in the year 2007, which accounts for 13 percent of all the deaths, and the incidences, are expected to increase by 12 million deaths in 2030. As per the reports of Parkin et al., 2005 the most commonly diagnosed cancers worldwide are lung (1.35 million), breast (1.15 million) and colorectal (1million), and the most common causes of deaths due to cancers are lung cancer (1.18 million deaths), stomach cancer (700,000 deaths) and liver cancer (598,000 deaths). Breast cancer (4.4 million survivors up to 5 years following diagnosis) has been reported to be the most prevalent cancer in the world (Parkin et al., 2005). Throughout the world the sex ratio for cancer deaths is 1:3 (M: F), greater than the sex ratio of incidence (1:15) because fatality (lung, stomach, liver and oesophagus cancer) is more common among men than women (Parkin, 2001). According to World Health Organization 460,000 females died from breast cancer in

2008, while close to 610,000 males and females died from colorectal cancer. Compared to the western countries, cancer rates lower in India, but the incidences are increasing with increasing rural population to the cities, increase in life expectancy and changes in life style. Data from population based cancer registries in India have shown that the most commonly reported cancer sites in males are lung, stomach, oesophagus and larynx, however in case of females these include, cancer of cervix, breast, ovary and oesophagus (Sinha et al., 2003). Cancer has been the main focus of research from last three decades and still continues to be a challenge.

Depending on the type and stage of cancer, the most common treatments involve surgical removal of the tumor, radiation therapy, chemotherapy, immunotherapy and combinations thereof. Surgical resection is the primary procedure to remove cancers large enough to detect and manipulate. However, surgical resection alone in most cases cannot remove every cancer cell present; it leaves behind some microscopic tumor deposits that over time result in relapse and recurrent disease (Feng et al., 2004; Fisher, 1997). Radiation therapy on the other hand is the dosing of radiations to kill cancer cells and to stop or slow down their growth. It has been observed that radiation not only kills or slows down the growth of cancer cells; however it also affects the nearby healthy cells. Many reports have shown that surgery and radiation therapy can be used together (Mcleod and Thrall, 1989). Radiation shrinks the size of the cancer before surgery, or it may be used after surgery to kill cancer cells that remain in the body. Chemotherapy as distinct from the other forms of treatment uses certain antineoplastic agents to treat cancer cells locally and systemically. Broadly, most chemotherapeutic drugs work by impairing mitosis (cell division), thus effectively targeting fast dividing cells. Chemotherapy is delivered through a central line, giving more reliable access to the circulatory system while preventing phlebitis in peripheral veins. Years of testing and research have proved chemotherapy to be an effective cancer treatment. The majority of chemotherapeutic drugs can be divided into alkylating agents, anti-metabolites and plant alkaloids. Alkylating agents interfere with DNA integrity and function in rapidly proliferating tissues. The five major alkylating agents used in the chemotherapy are nitrogen mustard, ethylene amines, alkyl sulfonates, nitrosourea and triazenes. Mechlorethamine, Cyclophosphamide,

Ifosfamide, Melphalan and Chlorambucil are the most reactive drugs of nitrogen mustard family whereas Triethlenenemelamine, Thiotepa, Altretamine are ethyleneamines. Anti-metabolites like folic acid analogues (methotrextrate), pyrimidine analogues (5FU, cytarabine) and Purine analogues (mercaptopurine, azathioprine, thioguanine, fludarabine phosphate, pentostatin and cladribine) are the most active anti- neoplastic agents.

Most of the cancer chemotherapeutic drugs induce cancer cell to death by apoptotic pathways. Apoptosis is a tightly regulated multistep pathway that is responsible for cell death not only during development but also in adult multicellular organisms, in which it partly controls cell numbers. Defects in programmed cell death (apoptosis) plays an important role in tumor pathogenesis, by allowing neoplastic cells to survive beyond their normally intended lifespan, subverting the need for exogenous survival factors, providing protection from hypoxia and oxidative stress as tumor mass expands, and allowing time for accumulative genetic alterations that deregulate cell proliferation, interfere with differentiation, promote angiogenesis, and increase cell motility and invasiveness during tumor progression (Reed, 1999). A drug that activates apoptosis achieves a suitable therapeutic index in several ways. First, it activates a death cascade *via* a drug target that is uniquely expressed in a cancer cell, alternatively it is delivered to the target tissue in a manner that is selective for the cancer cell and finally it exploits a pathway that is activated by oncogenes, in order to provoke apoptosis selectively in cancer cells. It has become increasingly evident that apoptosis is an important mode of action for many antitumor agents, including ionizing radiation, alkylating agents such as cisplatin & 1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU), topoisomerase inhibitor such as etoposide, cytokine such as tumor necrosis factor (TNF) (Shih and Stutman, 1996), taxol (Gibb et al., 1997) & N-substituted benzamides such as metoclopramide and 3-chloroprocainamide (Pero et al., 1999).

The extensive chemical diversity of nature provides models and ideas for modern drug design (Strohl, 2000; Gullo et al., 2006). Natural Products, especially plants, have been used for the treatment of various diseases for thousands of years (Shoeb, 2006). The first written records on the medicinal uses of plants appeared in about

2600 BC from the Sumerians and Akkaidians (Samuelsson, 1999). World Health Organization has estimated that approximately 80% of the world's inhabitants rely on traditional system of medicine for their primary health care (Farnsworth et al., 1985). Plants have a long history in the treatment of cancer (Hartwell, 1982). In a survey, National Cancer Institute had collected about 35,000 plant samples from 20 different countries, of which around 114,000 extracts after screening were found to show anticancer activity (Shoeb, 2005). It has been estimated that prior to 1983 in US and among worldwide approved drugs between 1983 and 1994, 60% were of natural origin (Cragg et al., 1997). The isolation of the vinca alkaloids, vinblastine and vincristine from the *Madagascar periwinkle*, *Catharanthus roseus* (Apocynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into clinical use for the treatment of cancer (Cragg and Newman, 2005). The discovery of paclitaxel (Taxol) from the bark of the *Pacific Yew*, *Taxus brevifolia* (Taxaceae), is another evidence of the success in natural product drug discovery. *Taxus baccata* has also been reported to be used in the Indian Ayurvedic medicine for the treatment of cancer (Shoeb, 2006). The structure of paclitaxel was elucidated in 1971 and was clinically introduced to the US market in the early 1990s (Wani et al., 1971; Rowinsky et al., 1992). Paclitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer (Rowinsky et al., 1992). Both vinca alkaloids and taxanes e.g. vincristine and paclitaxel, block the polymerisation and depolymerisation, respectively of microtubules, thereby interfering with key steps in cell division, such as organisation of the mitotic spindle and thus the mitotic arrangement of the chromosomes. Camptothecin isolated from the Chinese ornamental tree *Camptotheca acuminate* (Nyssaceae) was advanced to clinical trials by NCI in the 1970s (Potmeisel, 1995). Camptothecin promotes DNA-strand breaks, thus disturbing DNA replication, whereas anthracyclines including doxorubicin and daunorubicin likely have multiple sites of action that include intercalation into DNA, inhibition of DNA topoisomerase II, production of free radicals and binding to the plasma membranes (Beck and Dalton 1997; Glimelius et al. 2001). Topotecan and irinotecan are semisynthetic derivatives of camptothecin and are used for the treatment of ovarian and small cell lung cancers,

and colorectal cancers, respectively (Creemers et al., 1996; Bertino, 1997). Epipodophyllotoxin is an isomer of podophyllotoxin which was isolated as the active antitumor agent from the roots of *Podophyllum* species, *Podophyllum peltatum* and *Podophyllum emodi* (Stahelin, 1973). Etoposide and teniposide are the two semisynthetic derivatives of *epipodophyllotoxin* and are used in the treatment of lymphomas, bronchial and testicular cancers (Cragg and Newman, 2005; Harvey, 1997). Homoharringtonine isolated from the Chinese tree *Cephalotaxus harringtonia* (Cephalotaxaceae) (Powell et al., 1970) is another plant derived agent in clinical use (Itokawa et al., 2005). A racemic mixture of harringtonine and homoharringtonine is used for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia (Cragg and Newman, 2005; Kantarjian et al., 1996). Elliptinium, a derivative of ellipticine, isolated from a Fijian medicinal plant *Bleekeria vitensis* is used for the treatment of breast cancer (Cragg and Newman, 2005). Flavopiridol a synthetic flavone, derived from the plant alkaloid rohitukine, isolated from *Dysoxylum binectariferum* (Meliaceae) (Kelland et al., 2000) is currently in phase I and II clinical trials against a broad range of tumors including leukemia, lymphomas and solid tumors (Smith et al., 2008). A synthetic agent roscovitine derived from natural product olomucine (Cragg and Newman, 2005; Meijer et al., 2003) which was originally isolated from *Raphanus sativus* (Brassicaceae) is in Phase II clinical trials (Dev et al., 2008). Combretastatins, the most cytotoxic phytomolecules isolated from the bark of *Combretum caffrum* (Combretaceae) (Pettit et al., 1987) were found to be active against colon, lung and blood cancers (Ohsumi et al., 1998; Pettit et al., 1995).

Although over the past 50 years, great strides have been made for treating cancer, it still continues to be a major health concern and therefore extensive efforts have been devoted for searching new therapeutic approaches. The major difficulty in the treatment is that cancerous and normal cells are remarkably similar. Even though cancer cells harbour mutated genes and resultant mutated proteins that affect cell division and/or contribute to oncogenesis, tumor cells and their normal counterparts share the same DNA and major metabolic pathways. Thus traditional chemotherapeutic compounds that attack DNA replication or cell division in a cancer cell can also attack a normal dividing cell, resulting in serious side effects such as

bone marrow and gastrointestinal toxicity. Therefore efforts are made to select only those candidate drug molecules which affect cancer cells without harming the normal ones. Within the sphere of cancer, a number of important new commercialised drugs have been obtained from natural sources, by either modifying them structurally or by synthesizing new compounds taking them as models. The etiology of major cancers is still largely unknown and there is a need for more effective and less toxic chemotherapeutic agents. The search for improved cytotoxic agents therefore continues to be an important line in the discovery of modern anticancer drugs.

The development of anticancer drug from plant resources depend on multiple target oriented approaches that will result in herbal medicine or an isolated active compound. However apart from this consideration the selection of a suitable plant for its pharmacological study is very important and decisive step. There are several ways by which this can be done, including traditional use, chemical content, toxicity, randomised selection or a combination of several criteria (Williamson et al., 1996). Additionally, semi synthesis processes of new compounds, obtained by molecular modification of the functional groups of lead compounds are able to generate structural analogues with greater pharmacological activity and with fewer side effects. Based on this strategy we have choosen semisynthetically modified analogues of boswellic acids from *boswellia serrata* for the present study. *Boswellia serrata* (Bruseraceae) commonly known as salai guggal or Indian Olibanum tree or Luban or Gund is a deciduous middle sized tree, mostly concentrated in tropical parts of Asia and Africa. In India it occurs in dry hilly forests of Rajasthan, Madhya Pradesh, Gujarat, Bihar, Assam, Orrisa as well as central penisular regions of Andhra Pradesh and Assam. In the traditional system of medicine *Boswellia serrata* has been used as an anti-inflammatory, anti-arthritic and anti-atherosclerotic agent. It has expectorant effects and improves immunity (Chopra et al., 1959). The gum resin obtained from the trunk of *Boswellia serrata* by tapping process has been used historically in the Indian System of Medicine for a variety of ailments. The commercial products from *Boswellia serrata* are available in Indian markets for treatment of various diseases from last fifteen years. The popularity and desire for natural and well tolerable herbal remedies (phytomedicine) allowed extracts of boswellia serrata to enter the markets of

the ‘western’ societies. The experimental data from animal models and studies with human subjects confirmed the potential of *Boswellia serrata* for the treatment of not only inflammation but also of cancer. The analysis of the ingredients of gum resin revealed that the pentacyclic triterpenic acids also called boswellic acids (BAs) such as 11-keto-*β*-boswellic acid (KBA), 3-O-acetyl-*β*-boswellic acid (ABA) and 3-O-acetyl-11-keto-*β*-boswellic acid (AKBA) are the major chemical constituents responsible for its anti-inflammatory, anti-proliferative and anticancer activities (Safayhi et al., 1992, Hoernlein et al., 1999). Structure activity relationship indicated that pentacyclic ring skeleton of boswellic acid is important for its anticancer activity (Hoernlein et al., 1999). The anticancer activities of BAs have been found to be in the order of acetyl-11-keto-*β*-boswellic acid (AKBA) as the most active, followed by 11-keto-*β*-boswellic acid (KBA), acety-*β*-boswellic acid (ABA) and *β*-boswellic acid (BA) respectively (Shah et al., 2009). AKBA was shown to induce apoptosis in HL-60 cells and inhibits topoisomerase I (Hoernlein et al., 1999). Since AKBA is showing more cytotoxic potential than KBA, attempt was made to synthesize preliminary propionyloxy derivative of KBA (PKBA) to increase the potency of the parent molecule and to decipher the role of acyl group at 3-α-hydroxy position of KBA. This study revealed that PKBA has a better anticancer potential than KBA and other boswellic acids including AKBA. In this regard, we synthesized further higher derivatives (with increasing alkoxy chain length at 3-α-hydroxy position) and based on the activity profile a hexonyloxy derivative of KBA (HKBA) was selected for comparative studies, to evaluate the role of increasing alkoxy chain length at 3-α-hydroxy position on the anticancer potential of KBA. Further, Boker and Wenking (1997) had reported that, high-doses of boswellic acids in tablet formulation (3x1200 mg/day) for a period of seven days when given to twenty-nine patients of malignant glioma (tumor of non-nervous cells of CNS) revealed peritumoral brain edema to get reduced by 33 % along with marked improvement in neurological symptoms and no effect on tumor proliferation. Keeping into consideration that boswellic acids offered in treatment of malignant glioma (Boker and Weinking, 1997) it was decided to design a nanoparticle based drug delivery system of the above mentioned analogues of boswellic acids for brain targeting.

Nanoparticles provide a range of new opportunities to increase the targeting of currently approved diagnostic and therapeutic agents to cancers. Nanoparticles carrying a chemotherapeutic can reduce the undesirable distribution of such agents. Certain tumors are located in difficult to reach sites such as the brain. Nanoparticles can access these sites by avoiding the systemic clearance by the RES and have the capacity to move across the blood brain barrier (BBB). Nanoparticles integrated with the diagonostic or therapeutic agents can either freely release these agents or undergo their own decomposition for the release to occur. Improvements in targeting can lead not only to increased efficiency of these agents but also to increased signal-to-noise ratios for diagnostics and better efficacy to toxicity ratios for therapeutics. The development of BBB targeting technologies is a very active field of research and development. The treatment of brain cancers is limited by the inadequacy in delivering therapeutic agents in such a way that drug molecules reach the desired targets. To overcome the limited access of antineoplastic agents to the brain one of the strategies is to take advantage of physiology which offers BBB nutrient carriers or specific receptors, mediating transport *via* these transporter systems, an example is to conjugate the therapeutic drug with a protein or a monoclonal antibody that gains access to the brain by either receptor or adsorptive-mediated transcytosis (e.g. transferrin receptor-mediated targeting). Transferrin receptors (Tf-R) are highly expressed on endothelial cells of the BBB. In the current study we had developed transferrin appended PLGA nanoparticles of PKBA and HKBA for brain targeting.

Objectives

The main objectives of the present work are:

1. To evaluate the *in vitro* cytotoxic potential of PKBA and HKBA against a panel of human cancer cell lines and to determine their IC_{50} values.
2. To study the *in vivo* anticancer activity of these analogues against Ehrlich ascitic carcinoma (EAC), Ehrlich tumor (Solid) and Sarcoma-180 (solid) tumor models in Swiss albino mice.
3. To investigate the mechanism of cell death induced by these analogues *via* Microscopic studies: fluorescence microscopy, scanned electron microscopy and also by DNA gel electrophoresis.

4. To study the extent of DNA damage by comet assay.
5. To investigate the annexin V/FITC binding, cell cycle progression and loss of mitochondrial membrane potential *via* flowcytometry.
6. To study the key events in PKBA and HKBA induced apoptosis pathway by investigating the caspase activation, cytochrome c and PARP expression.
7. To study the effect of test compounds on DNA Topoisomerses I &II.
8. To determine the effect of these analogues on pro-angiogenic factors: Ang-2 & MMP-2
9. To prepare a nanoparticle based drug delivery system of these analogues and to determine their IC_{50} values
10. To carry out the plasma and brain pharmacokinetics of both the analogues and their nanoparticles in Swiss albino mice.

Cancer is characterised by the cells with abnormal gene expression, unregulated growth, impaired differentiation, invasiveness and metastatic potential (Coleman et al., 2002; Yance et al., 1999). Different types of cancers are classified according to the tissues they belong to e.g. colorectal cancer affects large intestine and rectum, gastric cancer affects stomach and endometrial cancer affects the endometrium. Cancers are further classified according to the tissue they originate from e.g. carcinomas are derived from epithelia, sarcomas from connective and muscle tissue, leukemia from blood and lymphoma from lymphatic tissue (Weinberg, 2007). Cancer mainly occurs as a result of changes in the genes that control normal cell growth and death. The transition of a normal somatic cell to a cancer cell is generally the result of many genetic changes because a single genetic change is rarely sufficient for the development of a malignant tumor. The major genetic changes include activation of proto-oncogenes and inactivation of tumor suppressor genes. These changes allow the cell to escape the normal cellular control mechanisms. The frequency of these somatic mutations leading to cancer in human beings is dictated largely by chemicals in the cellular microenvironment and to a small extent by heritable genetic predisposition. Numerous environmental factors have been found to be associated with development of cancer (Brennan et al., 2006), the major ones include physical carcinogens, such as ultraviolet and ionizing radiation, chemical carcinogens such as asbestos, components of tobacco smoke, aflatoxin (a food contaminant), arsenic (a drinking water contaminant) and biological carcinogens such as infections from certain viruses (hepatitis B and liver cancer, human papilloma virus and cervical cancer, human immunodeficiency virus and Kaposi sarcoma), bacteria (Helicobacter pylori and stomach cancer) or parasites (schistosomiasis and bladder cancer) (W.H.O. 2009). Although cancer is considered to be a genetic disease (Potter, 1999), however, only about one percent of cancers are unmistakably inherited, including certain childhood cancers (Fearon, 1997) e.g. retinoblastoma (hereditary mutation in the retinoblastoma gene) and some adult cancers like breast, ovarian (an inherited mutations in the genes BRCA1 and BRCA2) and colon cancer (an inherited mutation of the APC gene "familial adenomatous polyposis"). Hormone production during reproductive cycles and other internal factors have also been found to stimulate excessive cell growth.

Aging is another fundamental factor for the development of cancer. The incidence of cancer rises dramatically with age because of the build up of risk for specific cancers due to the less effective cellular repair mechanism (World Health Organisation, 2009). The most deadly aspect of cancer is its ability to spread or metastasize. It has been estimated that 90% of cancer deaths occur due to metastasis. Cancer is a threat concomitant with human history. Although we have stepped into the very advanced twenty first century with considerable progress in cancer treatment, it is still a very difficult disease to treat and is the second most common disease that causes mortality.

Cells in different tissues contain the same genetic material that orchestrates cell growth, proliferation, differentiation as well as cell death. The process of tumorigenesis is proposed to include six essential alterations in cell physiology, including self-sufficiency in growth signals, insensitivity to growth inhibiting signals, evasion of apoptosis, unlimited potential to replicate, continual angiogenesis, tissue invasion and metastasis (Fig 2.1) (Hanahan and Weinberg, 2000). The understanding of the molecular mechanisms of tumor growth and metastasis is one of the most important issues in cancer research. Different classes of proteins and enzymes including cell-cell adhesion molecules (CAMs) like members of the immunoglobulin, calcium-dependent cadherin families, integrins (Hanahan et al., 2000), matrix metalloproteases (MMPs) (Kleiner and Stetler-Stevenson, 1999) and plasminogen activators/plasmin system (Wang, 2001) have been found to play a vital role in tissue invasion and metastasis. Cancer mostly occurs through changes in the genome and could therefore be considered a genetic disease, however, it has been observed that majority of cancers are not heritable. The critical cancer genes are grouped according to their normal function in a cell. Proto-oncogenes play a vital role in regulation of cellular growth, proliferation and differentiation. Proto-oncogenes get activated to oncogenes through point mutation, amplification or translocation. Although, oncogenes are the dominantly inherited genes (one mutation is sufficient to cause altered cell function) these are rarely found to be the cause of inherited cancer susceptibilities (Aittomaki and Peltomaki, 2006). Tumor suppressor genes on the other hand are recessive genes requiring two hits "(both alleles to become inactivated) for cancer development. The inactivation of these genes allows cells with DNA

damage to proliferate continuously and thus promoting tumor growth. Tumor suppressor genes have been found in several hereditary forms of cancer where the first hit has been found to be inherited and the second hit occurring in somatic cells (Knudson, 1996). Tumor suppressor genes are further divided into gatekeepers, caretakers, and landscapers (Kinzler and Vogelstein, 1997; Kinzler and Vogelstein, 1998). The Gatekeeper genes are able to directly inhibit cell growth or promote cell death, e.g. a mutation in the APC gene leads to familial adenomatous polyposis coli (FAP). In contrast, the caretaker genes e.g. mismatch repair (MMR) genes cannot directly promote tumor development, however, their inactivation leads to genetic instability that in turn causes increased mutation rate in both oncogenes and tumor suppressor genes. The third class of genes i.e. landscaper genes induce changes in the landscape i.e. the microenvironment and thus promoting tumorogenesis.

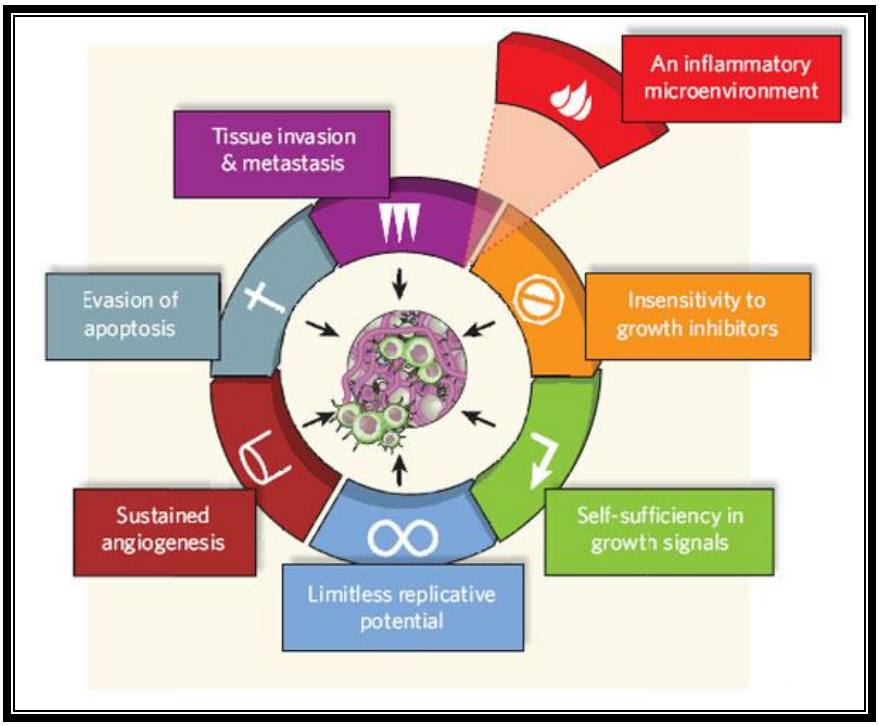


Fig.2.1. Characteristics of cancer cell

Cancer is a major burden of diseases worldwide. Each year, tens of millions of people are diagonised with cancer around the world, and more than half of the patients eventually die from it. Although many different approaches such as surgery, radiation therapy, harmone therapy, biological therapy, gene therapy, vaccine therapy and

others are employed for treatment but complete cure of cancer is still not possible. Treatment for cancer went through a slow process of development. Early in the 20th century, the only curable cancers were small and localized enough to be completely removed by surgery. Later, radiation was used after surgery to remove small tumor growths that were not surgically removed. Finally chemotherapy was added to destroy small tumor growths that had spread beyond the reach of surgeon and radiotherapist. Most of the cancer chemotherapeutic agents are mainly derived from plants. Therefore, plants are being actively explored as a source of new molecules that can curtail cancer growth and possess enormous potential to provide drugs being the reservoir of natural chemicals.

For thousands of years, natural products, especially plants have been used for the treatment of different diseases. Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient time and an impressive number of modern drugs have been developed from them. The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians (Samuelsson, 1999). "Ebers Papyrus", the best known Egyptian pharmaceutical record documented over 700 drugs and represents the history of Egyptian medicine dated from 1500 BC. The Chinese "Materia Medica," which describes more than 600 medicinal plants has been well documented with the first record dating from about 1100 BC (Cragg et al., 1997). Documentation of the Ayurvedic system recorded in "Susruta" and "Charaka" dates from about 1000 BC (Kappor, 1990). The Greeks also contributed substantially to the rational development of the herbal drugs. Dioscorides, the Greek physician (100 A.D.) described more than 600 medicinal plants in his work "De Materia Medica" (Samuelsson, 1999). The World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care (Farnsworth et al., 1985). Large number of plants used in traditional medicine has become an important part of modern world health care system (Fabricant and Farnsworth, 2008). The addition of natural remedies to the existing healthcare system has become a trend in many parts of the world. Worldwide the sales of medicinal plants, crude extracts and finished products amounted to 23 billion US dollars in 2002 (Dubey et al., 2004).

The biodiversity and chemodiversity in nature provide opportunities to discover plant products which may represent previously unknown agents with novel and potentially relevant mechanisms. Drug development is a very expensive and risky business. On an average a drug takes 15 years and cost $802 billion from discovery to reach the market. Internationally, the scientific community is concentrating on developing plant related drugs for the treatment of many major diseases such as cancer, diabetes, hepatitis etc, because of its low toxic potential and high effectiveness. Considerable efforts have been made by public organization and the private companies to expedite the process of drug discovery and development by expanding on the promising results from preliminary *in vitro* screening tests. A medicine is considered a phyto pharmaceutical preparation or herbal medicine if it is derived solely from a plant and retains the plant's chemical complexity, however, if it is an isolated compound and prepared according to specific legal and technical procedures, it is considered a drug. Natural products offer large structural diversity and the modern techniques used for the separation, structural elucidation, screening and combinatorial synthesis have led to the revitalization of plant products as source of new drugs (Steinbeck, 2004). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today (Lawrence, 1951; Cragg et al., 1997; McChesney et al., 2007).

Plants have long been used in the treatment of cancer (Hartwell, 1982). More than 3,000 different plant species have repeatedly been reported to treat cancer worldwide. Plants have played an important role as a source of effective anticancer agents and it is significant that over 60% of currently used anti-cancer agents are derived from natural sources including plants, marine organisms and micro-organisms (Cragg and Newman, 2005) either directly as pure as native compounds, as semisynthetic analogues or as models for synthetic compounds. In 1937, the United States National Cancer Institute (NCI) was established with its mission being "to provide for foster and aid in coordinating research related to cancer." In 1955, NCI set up the Cancer Chemotherapy National Service Center (CCNSC) to promote a cancer chemotherapy programme, involving the procurement, screening, preclinical development and clinical evaluation of new agents. All aspects of drug discovery and preclinical

development are now the responsibility of the Developmental Therapeutics Program (DTP) a major component of the Division of Cancer Treatment (DCT) (Perdue, 1976). During the past 35 years, over 400,000 chemicals, both synthetic and natural plant products submitted by investigators and organizations worldwide have been screened for antitumor activity and about seven per cent of these species and several hundred cytotoxic (*in vitro*) and/ or anti tumor (*in vivo*) compounds were discovered in the NCI programme. NCI has played a major role in the discovery and development of many of the available commercial and investigational anticancer agents of the plant origin (Driscoll, 1984). A number of plant products have been studied for anticancer activity on various experimental models. This has resulted in the availability of nearly 30 effective anticancer drugs (Ramakrishna et al., 1984). National Cancer Institute (NCI) has set forward exemplary strategies for the discovery and development of novel natural anticancer agents. Over the last 40 years NCI has been involved with the preclinical and clinical evaluation of the overwhelming majority of compounds under consideration for the treatment of the cancer. The screen developed by NCI currently comprises of 60 cell lines derived from nine cancer type and organized into subpanels representing leukemia, lung, colon, central nervous system, melanonma, ovarian, renal, prostrate, and breast cancer. A protein staining procedure using sulphorhodamine B (SRB) is used as the method of choice for determining cellular growth and viability in the screen. Anticancer drugs of natural origin and their semisynthetic analogues exert their effects (on the cancer cell) with distinct and definable mechanism. For example the topoisomearse inhibitors of which podophyllotoxins, topotecan and etoposide are examples; interfere with transcription, DNA synthesis and mitosis by blocking the enzymes DNA topoisomerases I and II. In contrast the *Vinca* alkaloids and taxanes e.g. vincristine and paclitaxel block the polymerisation and depolymerisation respectively of microtubuli, thereby interfering with key steps in cell division such as organisation of the mitotic spindle and thus the mitotic arrangement of the chromosomes. Camptothecin promotes DNA strand breaks thus disturbing the DNA replication, whereas anthracyclins, including doxorubicin and daunorubicin likely have multiple

sites of action that include intercalation into DNA, inhibition of topoisomerase-II, production of free radicals and binding to plasma membranes.

Experimental agents derived from natural products are offering us a great opportunity to evaluate not only totally new chemical classes of anticancer agents but also novel and potentially relevant mechanism of action. The beauty of botanical compounds is that they support the treatment of all phases of cancer and very often these compounds interact with several of these targets simultaneously and synergistically. Moreover anticancer botanicals are cheaper and less toxic than the conventional anti-cancer therapeutics. Plant derived anti-cancer drugs act through multi-targets simultaneously and/or synergistically. Many of these drugs are also chemopreventive which prevent both primary and secondary recurrence of the disease. There are different strategies which result in herbal medicine or in an isolated active compound. However, apart from this consideration, the selection of suitable plant for a pharmacological study is very important and decisive step. There are several ways in which this can be done, including consideration like traditional use, chemical content, toxicity, randomized selection or a combination of several criteria (Soejarto, 1996). The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures (ethnobotany or ethnopharmacology). Information on how the plant is used by an ethnic group is extremely important. The preparation and procedure is important. The preparation procedure may give an indication of the best extraction method. The formulation used will provide information about pharmacological activity, oral versus non-oral intake and the doses to be tested.

2.1. Plant Species used in Clinical Cancer Treatment

Almost 15 species (belonging to ten genera and nine families) have been utilized in cancer chemotherapy at a clinical level. In fact even fewer genera (*Campotheca*, *Podophyllum*, *Taxus* and *Vinca*) (Table 2.1) are considered to be the sources of mainstream and commercialized, antitumor agents. The timeline of the natural compound based anticancer drugs obtained from these species and approved by FDA for clinical use is given below (Fig.2.2).

Campotheca acuminata is a native tree of Southern China and Tibet. Although the species is protected, however, it can be cultivated also (Duke and Ayensu, 1985; Duke

and Foster, 1990). The bark, wood and young leaves of the tree have traditionally been used in China to treat different types of cancers especially cancers of the stomach, liver, and leukemia. It contains the quinoline alkaloid, camptothecin (CPT) which inhibits topoisomerase I and therefore DNA replication. Due to its high (particularly hematological) toxicity, several semisynthetic derivatives of CPT like topotecan, irinotecan and 9-nitro camptothecin have been developed and are found to have fewer side effects. CPT is therefore the second most important source of anticancer drugs (Armstrong and O'Reilly, 1998; Bookman, 1999).

Podophyllum peltatum, a perennial herb of the North American continent has been used traditionally as an emetic, vermifuge, and hepatoprotective (Mack, 1992). The roots of the herb are found to be rich in poisonous podophyllotoxins (a class of neutral crystalline lignans) which are classical spindle poisons causing inhibition of mitosis by blocking microtubular assembly. Some of the other compounds including podophylloresin (amorphous resin), diphyllin and aryltetralin (podophyllum lignan), etoposide (VP-16) and teniposide (semisynthetic derivative of 4-demethylepipodophyllotoxin) (Damayanthi and Lown, 1998) have also been obtained from the same. Etoposide, in particular, appears to be one of the most active drugs for small cell lung cancer, testicular carcinoma and malignant lymphoma (Schacter, 1996). Two more species namely, *P. hexandrum* and *P. emodi* have shown increased production of podophyllotoxin (Smollny et al., 1998; Grieve, 1994). Apart from Podophyllum species, podophyllotoxin is also accumulated in roots of *Dysosma pleianthum* (Bajiaolian) (Sarin et al., 1997).

Vinca rosea is a widely distributed tropical weed cultivated commercially for its medicinal uses in Australia, Africa, India, and southern Europe. Traditionally, the plant was used for soothing and healing of inflammatory ailments of the skin, eye irritations and infections. In modern medicine the plant extract has been used for the treatment of diabetes, high blood pressure, asthma, constipation, and menstrual problems (a renowned commercial product is Vinculin, a herbal tea derived from rose periwinkle). The aerial parts of the plant are found to contain alkaloids ajmalicine, vindoline and catharanthine. Studies have shown that vindoline on enzymatic coupling with catharanthine produces powerful cytotoxic dimeric alkaloids,

vinblastine (VBL) vincristine (VCR) and leurosidine. The vindoline and dimeric alkaloids are restricted to the leaves and stem of the plant, whereas catharanthine is found to be distributed equally throughout the aerial and underground tissues (Chu et al., 1996; St-Pierre et al., 1999). Extracts from *Madagascar periwinkle* have proved to be effective in the treatment of leukemia, skin cancer, lymph cancer, breast cancer and Hodgkin's disease (Jordan et al., 1991; Canellos, 1992).

Mistletoe is a semi parasitic, evergreen shrub growing on more than 400 different host species worldwide (Barney et al., 1998). It is commonly found on fir, apple, poplar, ash, hawthorn and lime. It was considered a sacred plant by the Druids and was also respected for its allegedly magical properties in many different countries. Although the shrub is known to be poisonous, however, if consumed in moderate to large quantities, *mistletoe* preparations are well tolerated by humans with no significant toxicities (Bussing, 2000). Leaves and young twigs of the shrub contain viscotoxin, *mistletoe* alkaloids and three lectins (lactose-specific lectin, galactose-specific lectin, N-acetylgalactosamine-specific lectin) (Franz, 1986). It has been observed that *Mistletoe* exerts direct cytotoxic action on tumor cells due to viscotoxin, *mistletoe* lectins on the other hand stimulate an immune response of the organism including the secretion of the cytokines, tumor necrosis factor (TNF), interleukin (IL) IL-1 and IL-6 by human monocytes (Ziska et al., 1978; Hauser, 1993; Werner et al., 1998).

Taxus baccata and *Taxus brevifolia* are the members of the yew family (Taxaceae). *T. baccata* and *T. brevifolia* are commonly referred as English and Pacific Yew (Western or American yew) respectively. In the traditional system of medicine, the leaves of *Taxus baccata* have been used for the treatment of cancer (Hartwell, 1982). The taxane diterpene, paclitaxel, which was initially isolated from the bark of the *Taxus brevifolia*, binds to the microtubules and inhibit their depolymerisation (molecular disassembly) into tubulin (Horwitz and Parsons, 1999), thus blocking the cell's ability to break down mitotic spindle during mitosis (cell division) and making it unable to divide into daughter cells (this is in contrast to drugs like colchicine and the Vinca alkaloids, which block mitosis by keeping the spindle from being formed in the first place). Paclitaxel is given intravenously (it irritates skin and mucous membranes on contact) and is most effective against ovarian carcinomas and advanced breast

carcinomas. The semisynthetic derivative of taxane, docetaxel (taxotere) has been found to be an effective anticancer agent (Khayat et al., 2000). Although the mechanism of action is same as that of paclitaxel, however the clinical trials have demonstrated that it is about twice as effective as paclitaxel.

Chelidonium is an urban weed which has traditionally been used against jaundice, eczema, and scrofulous diseases. It contains the alkaloid chelidonine and its semisynthetic compound Ukrain. Ukrain has been found to be able to restore macrophage cytolytic function (Sotomayor et al., 1992) and this property has proved to be useful for the treatment of Kaposi's sarcoma (Voltchek et al., 1996). On clinical level the combined cytotoxicity and immunostimulatory properties were demonstrated to be effective against astrocytoma (Steinacker et al., 1996).

The genus Paeonia is named after the physician Paeon who cured gods of wounds with the help of this plant during the Trojan War. Preparations from the roots of three Asian Paeonia species, *P. alba* L., *P. lactiflora* L., and *P. suffruticosa* L., have been used for the treatment of intestinal metaplasia and atypical hyperplasia of the gastric mucosa (Liu et al., 1992; Sakamoto et al., 1992).

Rabdosia rubescens is a Chinese medicinal plant containing diterpenoid oridonin which is a promising anticancer agent demonstrating potential for the clinical treatment of esophageal carcinoma (Wang et al., 1991).

Rubia cordifolia is a crawling Indian plant containing various anthraquinones, naphthoquinones, naphthohydroquinones (Itokawa et al., 1993; Takeya et al., 1993) and a series of bicyclic hexapeptides designated RA-XI, -XII, -XIII and -XIV (with a potent antitumor activity against P-388 leukemia) in the roots (Morita et al., 1992) and have been tested in a phase I clinical study conducted by six institutions. The administration of the hexapeptides was found to be associated with toxicity and other undesirable side effects (Yoshida et al., 1994). Another hexapeptide isolated from the plant and designated as RA-VII or O-methyl deoxybouvardin was found to be an inhibitor of protein biosynthesis *in vitro* and *in vivo* (Wakita et al., 2001).

Scientific classification of organisms used for isolation of anticancer compounds				
	Taxol	**Camptothecin**	**Podophyllotoxin**	**Vinblastine/ Vincristine**
Kingdom	Plantae	Plantae	Plantae	Plantae
Phylum	Pinophyta	Magnoliophyta	Magnoliophyta	Magnoliophyta
Class	Pinopsid	Magnoliopsida	Magnoliopsida	Magnoliopsida
Order	Pinales	Ranunculales	Cornales	Gentianales
Family	Taxaceae	Berberidaceae	Cornaceaeb	Apocynaceae
Genus	Taxus	Podophyllum	Camptotheca	Vinca

Table 2.1 Scientific classification of organisms used for isolation of anticancer compounds

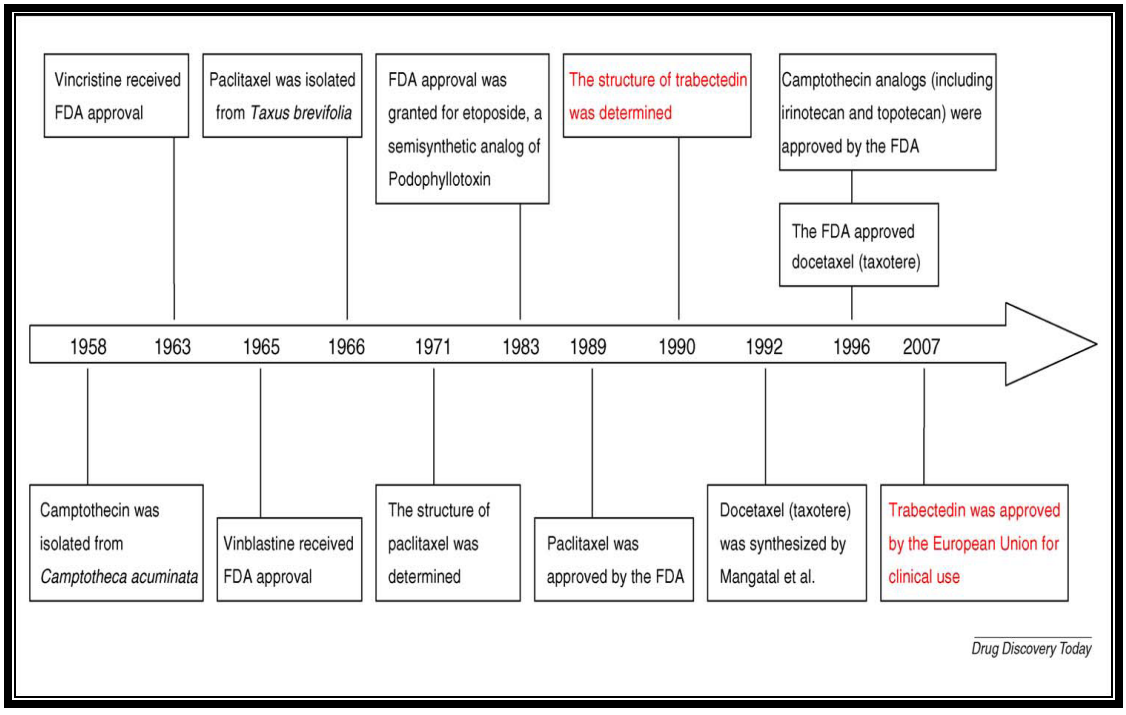


Fig.2.2.Timeline of natural-compound based anticancer drug development of the drugs approved by FDA for clinical use.

The list of plant species containing bioactive constituents that are either active against cancer cell lines or exhibit chemotherapeutic properties on diseased animals (usually mice) under experimental conditions is far more extensive than the list of the species already used in cancer chemotherapy at a clinical level. The bark of the Ethiopian tree *Brucea antidysenterica* has been found to contain bruceoside C, bruceanic acid A and its methyl ester bruceanic acid B, C and D along with the quassinoid, glucosides, bruceosides D, E, and F, bruceantinoside C, yadanziosides G and N-bruceanic acids. These compounds display very strong cytotoxicity *in vitro* against non-small-cell lung, colon, CNS, melanoma and ovarian cancer (Toyota et al., 1990; Fukamiya et al. 1992; Wang, 1991). This plant has also been found to contain various alkaloids (1, 11-dimethoxycanthin-6-one, 11-hydroxycanthin-6-one and canthin-6-one) which are active against leukemic cells (Toyota et al., 1990). A vital source of green tea, *Camellia Sinensis*, has been found to contain polyphenols (+)-gallocatechin (GC), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG) which were found to exhibit anticarcinogenic effects in rodent tumor bioassay systems as well as human cancer cell lines (MCF-7 breast carcinoma, HT-29 colon carcinoma, A-427 lung carcinoma and UACC-375 melanoma) (Katiyar et al., 1992; Valcic et al., 1996). It has also been observed that EGCG inhibits the proliferation of the NBT-II bladder tumor cell line (Chen et al., 2004) and a human cervical cancer cell line (CaSki cells) (Ahn et al., 2003). However, the administration of green tea to the patients with androgen-independent prostate carcinoma failed to manifest a considerable effect (Jatoi et al., 2003). *Acronychia* is a rainforest tree, the bark and stem of the which contains flavonols (5, 3-di hydroxy-3, 6, 7, 8, 4-pentamethoxy flavone, 5-hydroxy-3, 6, 7, 8, 3, 4-hexamethoxy flavone, digicitrin, 3-O-demethyl digicitrin, 3, 5, 3- trihydroxy-6, 7, 8, tetramethoxy flavone and 3,5 dihydroxy 6, 7, 8, 3, 4 pentamethoxy flavone) and alkaloids (1, 2, 3-trimethoxy-10 methyl acridone, 1, 3, 4 trimethoxy-10-methyl-acridone,des-N methylacronycine, normelicopine and noracronycine) which are tubulin inhibitors and active against human nasopharyngeal carcinoma *in vitro* (Funayama and Cordell, 1984). Some of the related species with similar properties are A. porteri L., A. pedunculata L., and A. Baueri L. (Zhou and Min, 1989; Svoboda et al., 1966; Lichius

et al., 1994). Another perennial herb *Saffron,* the stigmas of the flower of which are used commercially as a valuable food dye (one pound of *saffron* is derived from approximately 60,000 stigmas) have been found to contain the carotenoids crocin, crocetin, picrocrocin, and safranal, exhibiting potent activity against various human and mice cancers (Nair et al., 1995; Escribano et al., 1996). The extracts from the bark of a sausage tree, *Kigelia pinnata,* containing Dichloromethane has been found to be cytotoxic against four melanoma cell lines and a renal carcinoma cell line (Houghton et al., 1994). Similarly, the fruits of a perennial shrub *Piper longum,* contain 1% volatile oil, resin, the alkaloids piperine, piperlonguminine, a waxy alkaloid (N-is obutyldeca-trans-2- trans-4-dienamide) and a terpenoid substance. Roots contain piperine, piperiongumine or piplartine. A common use of its fruit is in the prevention of recurrent attacks of bronchial asthma as well as of chronic malaria (Gogate et al., 1983). An alcoholic extract of the fruits of this plant and its component piperine has been studied for their immunomodulatory and antitumor activity. Both the extract and piperine were found to be cytotoxic to Dalton's lymphoma ascites (DLA) cells, Ehrlich ascites carcinoma (EAC) cells and even against solid tumors in mice induced with DLA cells (Sunila and Kuttan, 2004). *Curcuma longa,* commonly known as Turmeric has been shown to possess variety of pharmacological properties including anti-inflammatory, anti-carcinogenic and anti-oxidant activities. Turmeric has also been found to activate lymphocytes and induces apoptosis in tumor cells (Araujo and Leon, 2001). *Inula racemose* is another well documented Indian medicinal plant. Preparations of its roots are used in the folk medicine against a variety of ailments including asthma, cough, lung disorders, indigestion, infectious and helminthic diseases (Cantrell et al., 1999).The antiproliferative activity of root extracts of *Inula racemose* has been reported in two human and murine neoplastic cell lines (Konishi et al., 2002). Recently, it has been observed that the root extact of *Inula racemose* induces apoptosis in human leukemia HL-60 cells by activation of intracellular caspases and cleavage of poly (ADP-ribose) polymerase (Pal et al., 2010). The cannabinoids from an annual herb *Cannabis sativa* have been found to possess numerous biological properties, including analgesic, antiemetic, anti-inflammatory, anti-convulsive and anticancer effects (Williamson et al., 2000).

Recently, it has been observed that the leaf extract of *Cannabis sativa* induces antiproliferative activity by depolarizing mitochondrial membrane potential of human leukemia HL-60 cells (Sehar et al., 2010). An aqueous extract of an evergreen tree *Polyalthia longifolia* has been found to depresses heart rate, decrease blood pressure and respiration rate in experimental animals (Achari and Lal, 1952). The crude extracts of the seeds of this plant have also showed remarkable antibacterial activities (Sayeed et al., 1995). An alcoholic extract and chloroform fraction of *Polyalthia longifolia* has recently been reported for its anticancer potential (Verma et al., 2008).

Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Current drug discovery from terrestrial plants has mainly relied on bioactivity-guided isolation methods, which have led to discoveries of important anticancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminate* (Kinghorn, 1994). Presently a number of plant derived compounds are in clinical trials for different cancers e.g. Phenoxodiol, a synthetic analog of daidzein, a well known isoflavone from soybean (Glycine max), has been developed as a therapy for cervical, ovarian, prostate, renal, and vaginal cancers, and induces apoptosis through inhibition of anti-apoptotic proteins including XIAP and FLIP (Kamsteeg et al., 2003). Phenoxodiol is currently undergoing clinical studies in the United States and Australia (Constantinou et al., 2003). Additionally, semi synthesis processes of new compounds, obtained by molecular modification of the functional groups of lead compounds have been able to generate structural analogues with greater pharmacological activity and fewer side effects. PG490 – 88 (succinyl triptolide sodium salt), a semisynthetic analog of triptolide, exerts antiproliferative and proapoptotic activities on primary human prostatic epithelial cells as well as tumor regression of colon and lung xenografts (Fidler et al., 2003). Protopanaxadiol, a derivative of a triterpene aglycone of several saponins from ginseng (Panax ginseng), exhibits its apoptotic effects on cancer cells through various signalling pathways, and is also reported to be cytotoxic against multidrug resistant tumors (Shibata et al., 1963; Jia et al., 2004). Epothilones derived from taxanes have a similar but not identical mechanism of action to the taxanes.They are microtubule de-polymerization inhibitors but remain effective against a variety of

paclitaxel-resistant cancers and appear to have lower toxicity (Goodin et al. 2004). Epothilones have been delivered in cremophor and non-cremophor vehicles and were found to cause significant neuropathy (Pronzato 2008). Epothilones have demonstrated effectiveness against ovarian, prostate, breast, colon, stomach, and kidney cancers (Goodin 2008). Larotaxel is another semi synthetic compound derived from the needles of yew trees. Larotaxel was found to be closer to docetaxel than paclitaxel. It acts by stabilizing microtubules, leading to cell death through apoptosis. In early clinical trials it has shown activity against paclitaxel resistent and non-resistant cancers, and was able to cross the blood-brain barrier, therefore making it a potential therapy for brain metastases (Morris and Fornier 2009). Larotaxel has been evaluated for its effectiveness against non-small cell lung, breast, pancreatic, and bladder cancers. A semisynthetic labdane-derived diterpenoid, Demalonyl thyrsiflorin A has been found to induce apoptosis and necrosis in human epithelial cancer cells at lower doses than the parent molecule (Garbarinoa et al., 2007).

The review of the literature above, clearly demonstrates that, small molecules derived from plant sources have a significant role in cancer therapy. As a class, they offer the advantage of having greater chemical diversity than typical synthetic chemical libraries. They also tend to have greater chemical complexity, often with complex stereochemistry. It is these features that make natural products very specific for particular targets, and it is these features that combinatorial libraries of relatively simple synthetic compounds try to mimic. However, complex natural products are normally difficult, if not impossible, to produce on a commercial scale by total chemical synthesis, except by using fermentation technologies, which have been used for many antibiotics and other promising anticancer agents such as epotholones. One way around this problem is to use understanding of the chemistry of the complex natural product to complete its synthesis from a more easily obtained naturally occurring precursor. This semi-synthesis has been the approach used to manufacture several known and vital anticancer anticancer agents including paclitaxel and many others described above. Keeping into consideration, the significance of semisynthetic derivatives of plants as a source of potent anticancer agents, we have selected two potent semisynthetic derivatives of 11-keto-β-boswellic acids from *Boswellia serrata*

for the current study and evaluated their anticancer potential in detail, so that a potent anticancer lead molecules could be generated for cancer chemotherapy.

2.2. *Boswellia serrata*

Boswellia serrata is a moderate to large branching tree (growing to a height of 12 feet) having straight, buttressed trunk with a clear bole and widespread branches. The trunk and branch bark are gray in colour having hard, sharp and conical prickles (Fig.2.3). It is commonly known as salai guggal and is mostly concentrated in tropical parts of Asia and Africa. In the traditional system of medicine *Boswellia serrata* has been used as an anti-inflammatory, anti-arthritic and anti-atherosclerotic agent. Previously, it has also been found to have expectorant effects and improves immunity (Chopra et al., 1959).

2.2.1. Taxonomical classification

Kingdom: Plantae

Division: Angiospermae

Class: Dicotyledoneae

Order: Geraniales

Family: Burseraceae

Genus: Boswellia

Species: Serrata

2.2.2. Nomenclature

Botanical Name: *Boswellia serrata.*

Latin Name: *Boswellia serrata* Roxb. Ex colebr/B. glabra Roxb.

Common Name: Indian frankincense, Salai, Indian olibanum, Luban, Gond.

English Name: Boswellia, Salai guggal, Boswellin, Indian frankincense.

Hindi Name: Kundur, luban, salai, vellakkunturukkam, gandhamula, guggu, kunduru, maheruna, rasala, salakhi, salasi, sallaki, vanakarnika, vasamaharuba .

Arabic name: Bastaj, kundur, luban.

Tibetan name: Bogadkarpo, bogdkarpa.

Urdu name: Kundur, lobana.

Part Used: Bark, oleo-gum-resin.

Fig. 2.3 *Boswellia serrata*

2.2.3. Geographical distribution: *Boswellia serrata* is widely distributed in Indian subcontinent including Rajasthan, Madhya Pradesh, Gujarat, Bihar, South East Punjab, Madras, Andaman West Bengal, Burma, Sri Lanka and some African countries like Somalia, Ethiopia, Eritrea, Kenya, Sudan Tanzania, Madagascar and a few regions of southern Arabia like Yemen and Omam.

2.2.4. Chemical constituents

The root, stem and leaf of the tree contain glycosides, alkaloids and tannins. The bark of the stem contains lupeol and β-sitostrol. The root bark has naphthalene derivatives related to gossypol (toxic principle of cotton seed) and called as 'semi-gossypol'. Flowers contain β-sitosterol, traces of essential oil, kaempherol and quercetin. Oleo-gum resins are the main chemical constituents of *B. Serrata*. Salai guggal contains 8-9

% essential oils, 20-23% gum, and about 50% resin (Bhargava et al., 1978; Pardhy and Bhattacharya., 1978)

2.2.4.1. Resin: The resin of boswellia serrata has been found to contain pentacyclic as well as tetracyclic triterpenes. Resin porition is mainly composed of pentacyclic triterpene acid of which boswellic acids (α-BA: 13.2%; β-BA: 18.2%) are the active moiety (Kokate et al., 1999) along with other pentacyclic triterpene acids such as 11-Keto-β -boswellic acid (6.1%), Acetyl-β-boswellic acid (10.5%) and Acetyl-11-keto-β-boswellic acid (3.7%) (Sterk et al., 2004).

2.2.4.2. Gum: The gum porition of *B. serrata* consists of pentose and hexose sugars with some oxidizing and digestive enzymes. The gum resin is the main active biological constituent which on hydrolysis yields arabinose, galactose, galacturonic acid and rhamnose.

2.2.4.3. Essential oil: The essential oil of *B. serrata* is predominantly composed of monoterpenoids, of which α-pinene (73.3%) is the major constituent. Other monoterpenoids identified included β-pinene (2.05%), cis-verbenol (1.97%), trans-pinocarveol (1.80%), borneol (1.78%), myrcene (1.71%), verbenone (1.71%), limonene (1.42%), thuja-2,4-diene (1.18%) and p-cymene (1.0%), while α-copaene (0.13%) is the only sesquiterpene identified in the oil (Kasali et al, 2009).

2.2.5. Historical Importance

The Indian frankincense is related to the tree that brought forth the frankincense given as a gift to baby Jesus by the wise men. For thousands of years the ancient health system of Ayurvedic medicine in India has practiced different parts of the tree for asthma, rheumatism, dysentery, skin ailments, ulcers, blood purification, bronchial conditions, and wound treatment (Broadhurst, 1998). Frankincense is also used to perfume clothes, hair and rooms. It is enjoyed at traditional festivities such as weddings or religious celebrations (Ghazanfar, 1994). Boswellia has attracted much attention in the world medical community because it possesses anti-inflammatory and anti-cancer properties that are similar to prescription medications and have been effective against most types of induced arthritis and cancer.

2.2.6. Pharmacological activity

2.2.6. 1. Anti-inflammatory and Anti-arthritic activity

Studies on paw oedema revealed the anti-inflammatory activity of a mixture of boswellic acids in rats and mice (Singh and Atal, 1984). In the chronic test of formaldehyde induced arthritis, it exhibited 45-67% antiarthritic activity in a similar dose range. The fraction was found to be effective in both adjuvant arthritis (35-59 %) as well as established arthritis (54-84%).

2.2.6.2. Anticancer Activity

An alcoholic extract of *boswellia serrata* showed growth inhibition of Ehrlich ascitic carcinoma and S-180 tumor models in mice by interfering with the biosynthesis of DNA, RNA and proteins (Tsukada et al., 1986). Boswellic acids have been found to induce concentration dependent inhibition of glioma cell proliferation and showed anti-edema effect in glioblastoma patients (Boker et al., 1997). Acetyl-11-keto-β-boswellic acid has been found to induce apoptosis in HL-60 and CCRF-CEM cells and by inhibiting topoisomerase I (Hoernlein et al, 1998). Recently, it has been found that a propionyloxy derivative of 11-keto-β-boswellic acid induces apoptosis in HL-60 cells by inhibiting both topoisomerase I &II (Chashoo et al., 2011).

2.2.6.3. Hypoglycemic Activity

Herbal formulation containing *B. serrata* oleo-gum-resin as one of the ingredients has been reported to produce significant anti-diabetic activity on non-insulin dependent diabetes mellitus in streptozocin induced diabetic rat model where reduction in blood-glucose level was comparable to that of phenformin (Awadi et al., 1991).

2.2.6.4. Antimicrobial activity

Studies with Gram positive and Gram negative bacteria including S. aureus OGSUTH 108, E.coli LASUTH 54 and Proteus mirabilis UCH28 showed antimicrobial activity of the essential oil from the bark of *B.serrata* (Kasali et al., 2002).

2.2.6.5. Immunomodulatory activity

The gum resin extract of *B. serrata* containing 60% acetyl 11-keto-β- boswellic acid (AKBA) along with other constituents such as 11-keto-β-boswellic acid (KBA), acetyl-β-boswellic acid and β-boswellic acid showed the inhibition of the passive paw anaphylaxis reaction in rats in a dose-dependant manner (Pungle et al., 2003).

2.2.6.6. Antidiarrhoeal

Boswellia serrata extract (BSE) was found to be effective in treating diarrhoea in patients with inflammatory bowel syndrome without causing constipation. It was also found effective against acetylcholine and barium chloride induced diarrhoea by inhibiting contraction of intestinal smooth muscles. The extract also inhibited gastrointestinal transit in croton and castor oil induced diarrhoea in mice. However, intestinal motility remained unaffected in control mice by BSE (Borrelli et al., 2006).

2.2.6.7. Hypolipidemic activity

The water soluble fraction of *B. serrata* extract showed decrease in total cholesterol (38-48%) and increase in HDL in the rats fed on atherogenic diet, thus showing its hypolipidemic potential (Zutsi et al., 1986).

2.2.6.8. Hepatoprotective activity

An alcoholic extract of salai guggal causes hepatoprotection in galactosamine/endotoxin induced liver damage in mice which was reflected by reduced titre of SGOT, SGPT, aminotransferase and serum enzymes (Gerlach, 1983).

2.2.6.9. Analgesic and Psychopharmacological effects

Menon et al., 1970 revealed that the gum resin of *B. serrata* possess marked analgesic activity in experimental animals. Due to the reduction in the spontaneous motor activity, it causes plosis in rats.

2.2.6.10. Anti-asthmatic activity

Gupta et al., 1998 in a double placebo control clinical study established the anti-asthmatic potential of the alcohol extract of salai guggal (300mg thrice daily dose for 6 weeks), here 70% of the patients with prolong history of asthma showed improvement in physical symptom and sign of dyspnoea, bronchi, number of attacks, increase in stimulation of mitogen activated protein kinase MAPK and mobilization of intracellular Ca^{2+} .

2.2.7. Clinical data

Boswellia serrata extract has shown beneficial effects in reducing the brain edema in patients suffering from glioma. A prospective clinical study with brain tumor patients was conducted by Heldt et al., 1996 and Böker et al., 1997. Improvement in clinical symptoms (regression in edema, with no effect on tumor size) was found in the patients at the highest daily dose (1200 mg/d), however, patients who received lower

doses also showed a lower urinary excretion of leukotrien E4 (LTE4) (as a measurement of leukotrien synthesis in the body) after seven days of intervention compared to their LTE4 excretion prior to treatment. Further, Janssen et al (Janssen et al., 2000) evaluated the use of the Boswellia extract H15 retrospectively in 8 female and 11 male children (age 0.5 to 18 years). Results were presented for 17 children who were being treated for different progressive or relapsed brain tumors. Streffer et al., 2001 published a prospective case series of 12 adult patients with progressive cerebral edema with or without over tumor progression. Most of the patients reported clinical improvement and reduction in perifocal edema.

2.2.8. Products in the market

'Salai Guggal Extract' (a product of Boswellia serrata extract) is a free flowing easy to encapsulate powder extracted from the oleogum-resin. It consists of 50-75% of boswellic acids and is used as a stimulant, anti-rheumatic, anti-diahoretic, nervous, skin diseases, urinary disorder, obesity and scrofulous affection. 'Shallaki' is a Herbal Supplement well known for its anti-inflammatory, anti-atherosclerotic and anti-arthritic activities. Other products include Rumalaya gel, Rumalaya forte, Boswellin etc.

2.2.9. Toxicity profile

The preclinical toxicology studies have shown the alcoholic extract of gum resin of boswellia serrata has no adverse effect on primates and rats (Singh et al, 1996). Singh et al., 1996 has evaluated and reported the safety and efficacy of the boswellic acids in acute, sub-acute and chronic models. The study provides a favourable framework for the cosmetic industry, as there were no adverse effects like eye irritation and an LD50 > 2g/kg.

Boswellia serrata gum extracts and their triterpenic constituents, especially boswellic acids, have drawn the attention of medicinal chemists, biochemists and pharmacologists since the first report of their anti-inflammatory activity. Their effectiveness in the treatment of rheumatoid arthritis and chronic inflammation without many side effects (such as the ulceration and heart failures generally associated with NSAIDs) combined with almost negligible toxicity and distinctive mode of action *via* inhibition of the 5-lipoxygenase (5-LO) pathway makes it a

preferred therapeutic agent in its category. Triterpenoids are structurally diverse organic compounds characterized by a basic backbone which can be modified in multiple ways allowing the formation of more than 20,000 naturally occurring triterpenoid varieties. Triterpenoids have been found to possess antitumor and anti-inflammatory properties, which can further be improved by synthesizing their synthetic and semi-synthetic derivatives. Triterpenoids are highly multifunctional and the antitumor activity of these compounds is measured by their ability to block nuclear factor-κB activation, induce apoptosis, inhibit signal transducer, and activate transcription and angiogenesis (Petronelli et al, 2009). Pentacyclic triterpenes (PT_S) are widely distributed among higher plants and plethora of PTs with diverse bioactive properties have been identified and characterized. The most important source for bioactive PTs constitutes Boswellia species (*B. spec*) including *B. sacra, B. carterri, B. frereana* and *B. serrata*, the later being the most attractive source. Preparations of the gum resin of *Boswellia species* (olibanum or 'salai-guggal') have been applied for many centuries in folk medicine in Asia and Africa for the treatment of diverse diseases. Analysis of the ingredients of gum resin revealed that the pentacyclic triterpenic acids also called boswellic acid such as β-boswellic acid, 11-keto-β-boswellic acid, acetyl-β-boswellic acid and acetyl-11-keto-β-boswellic acid are the major chemical constituents responsible for its anti-inflammatory, anti-proliferative and anticancer activities (Safayhi et al., 1992). Although the review of literature describes the pharmacological profile of its activities, still there are certain gaps in understanding the anticancer activities of its novel constituents, synthetic and semisynthetic derivatives. Since *Boswellia serrata* has a tremendous potential and a high safety profile, the scientific fraternity needs to pay a special attention towards it to emerge as a milestone in cancer treatment. The present dissertation is an effort in elucidating the anticancer potential, molecular mechanism of action and other preclinical studies of the semisynthetic derivatives of boswellic acids in order to generate novel and more potent anticancer therapeutic lead molecules.

2.3. Molecular Targets for Anticancer therapy

A vast number of multi-pronged strategies are designed to eradicate cancer, these include target based chemotherapy utilizing modern bioinformatic tools for drug

designing, immunotherapy using designer cancer vaccines based on tumor associated cell surface antigen, anti-angiogenesis therapy and development of tumor specific vehicles for drugs. A significant progress had been made in the development of targeted therapy drugs that act specifically on detectable molecular abnormalities in certain tumors, and which minimize damage to normal cells. Mostly it is influenced by the type of cancer, stage or extent of the disease. In addition the presence of specific molecular markers can also be useful in the treatments. Recent advances in deciphering the human genome have launched a tremendous potential for cancer treatment. New drugs are now being designed to target cancer cell's specific molecular features, including genetic mutations, gene expressions, changes in protein structure and signalling pathways **(**Shoshan and Linder, 2008**)**. Molecularly targeted interventions can be classified into five general strategies that will guide future research to discover and develop new anticancer agents that specifically target the cancer cells without affecting the normal cells. Targeting these events should have potent and specific therapeutic consequences. One of these events in cell deregulation is obligate compensatory suppression of apoptosis (programmed cell death, PCD), which provides support for neoplastic progression. In this proposal, an attempt has been made to induce apoptosis in cancer cells. Whatever be the mechanism involved, if a test compound induces apoptosis, it becomes a potential candidate for anticancer lead optimization.

2.3.1. Apoptosis and Cancer

Apoptosis (programmed cell death) is an important regulator of cell growth and proliferation. It is an essential cellular homeostasis mechanism that ensures correct development and function of a multicellular organism particularly during embryogenesis and metamorphosis (Gluecksmann, 1951; Lockshin, 2001). The term programmed cell death was introduced in 1964, proposing that cell death during development is not of accidential nature but follows a sequence of controlled steps leading to defined self-destruction (Fig.2.4) (Lockshin, 1964). Apoptosis is beneficial as a natural anticancer mechanism. It has been found that, once the DNA of a cell gets damaged it undergoes apoptosis to preserve the healthy state of an organism, further, cells becoming irrepairably damaged due to a disease also undergo apoptosis. Studies

have revealed that most of the known cancers carry mutations in one of the key regulators of the apoptotic pathways and the resistance towards apoptosis is a key factor for their survival. It has also been observed that defects in the apoptosis signalling further contribute to the drug resistance of tumor cells. Targeting apoptosis has therefore become a major goal for oncologic treatments and it has turned out to be an exciting challenge to translate the growing knowledge of apoptotic pathways into clinical applications (Schulze-Bergkamen and Krammer, 2004). Since apoptosis is a normal physiological process, it therefore affects the life span of both normal and cancer cells. It is believed that a successful anticancer drug kills or incapacitates cancer cells without causing excessive damage to normal cells. Literature reveals a large variety of plant products which are known to induce apoptosis in neoplastic cells but not in normal ones (Chiao et al., 1995; Kaufman and Earnshaw, 2000). A large number of known anticancer agents including ionizing radiation, alkylating agents such as cisplatin and 1,3- bis (2-chloroethyl)-1-nitrosourea (BCNU), topoisomerase inhibitor: etoposide, cytokine: tumor necrosis factor (TNF) (Shih, 1995), taxol (Gibb et al., 1997), and N-substituted benzamides such as metoclopramide and 3-chloroprocainamide (Pero., 1997) show their anticancer potential *via* apoptosis. The induction of apoptosis has therefore been a new target for innovative mechanism based drug discovery (Fisher, 1994; Workman, 1996). It is thus considered important to isolate, characterize or modify anticancer agents from plants and to evaluate their apoptotic potential.

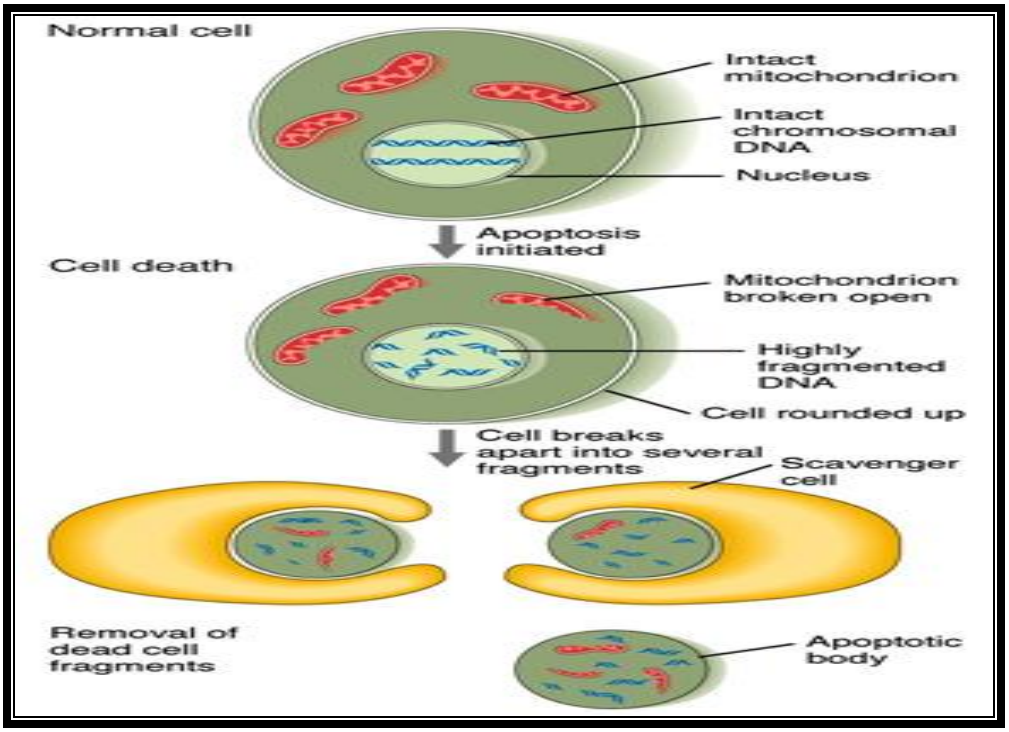


Fig.2.4. Programmed Cell Death

2.3.2. Molecular mechanism of apoptosis

Apoptosis is a normal physiological process involving many genes and requires the active consumption of energy in the form of ATP to safely dispose of the cells once they have fulfilled their intended biological role. Apoptotic cell death can be induced by a variety of stimuli, including ligation of cell surface receptors, serum deprivation, growth factor deprivation, heat shock, hypoxia, exposure to ultraviolet radiation, DNA damage, viral infection and exposure to cytotoxic or chemotherapeutic agents. The process of apoptosis is characterized by a number of stereotypical morphological features which include cell shrinkage, nuclear and cytoplasmic condensation, release of cytochrome c from mitochondria, caspase activation, plasma membrane blebbing, phosphatidylserine externalization on the plasma membrane, DNA fragmentation and the formation of apoptotic bodies (Fig.2.3) (Wilson, 1998; Allen et al., 2007 and Thompson, 1995). Nuclear condensation during apoptosis is usually accompanied by the activation of nucleases that first degrade chromosomal DNA into large 50 to 300 kb subunits and then into smaller units of ~180 base pairs (Wyllie, 1980). Since the plasma membrane integrity is maintained throughout the process of apoptosis, therefore, this form of cell death is normally not associated with an inflammatory

response. This is in contrast to passive forms of cell death like necrosis which is always associated with inflammation, often resulting from an overwhelming cellular insult that causes cell and organelle swelling, breakdown of the plasma membrane, release of lysosomal enzymes and spillage of the cell contents into the extracellular milieu (Fig.2.3) (Trump and Berezesky, 1995). Even though apoptosis and necrosis can occur simultaneously, studies have suggested that the intracellular energy levels are important determinants of the mode of cell death (Nicotera, 1998; Leist, 1997). The signalling for apoptosis occurs *via* both intracellular (Intrinsic) and extracellular (Extrinsic) mediators that are in turn initiated from triggering events from either within or outside the cell (Nicotera et al., 1999). Although, the intrinsic and the extrinsic pathways are known to occur independently, however, a cross-talk between the two pathways has also been observed. The death ligands/receptors and their respective intracellular signalling pathways have been found to act *via* specific death signals and all these signalling pathways converge on a common machinery of cell destruction that is activated by a family of cystein proteases known as caspases (Strasser et al., 2000), which play a key role in the progression towards the final morphological changes taking place in this process (Gallaher et al., 2001). Although cancer can arise due to the dysfunctioning of any of the two pathways, however, due to its sensitivity cancer arises more oftently *via* intrinsic than the extrinsic pathway (Johnstone et al., 2002).

The extrinsic pathway (death receptor pathway) gets activated by clustering of various death receptors on the cell surface. The binding of the ligands to the respective receptors e.g. Fas ligand (FasL) to Fas receptor and Tumor Necrosis Factor (TNF) to TNF receptor triggers the formation of a death inducing signalling complex (DISC) that recruits and activates pro-caspase 8 to active caspase-8 (Budihardjo et al., 1999; Hengartner, 2000), which further activates caspase-3 and thus initiating the final step of apoptosis (Fig.2.5). An intrinsic pathway on the other hand is a mitochondria dependant pathway as here most of the responses of this pathway get derived from this organelle (Hengartner, 2000; Okada et al., 2004). In this pathway the oligomerization of the bax results in the permeabilization of the outer membrane of mitochondria (Kluck et al., 1999) leading to the release of pro-apoptotic materials like

cytochrome c and apoptosis inducing factors (AIF) from the mitochondria into the cytosol. These pro-apoptotic factors are considered to be the central players of this pathway (Budihardjo et al., 1999; Hengartner, 2000; Okada et al., 2004). After its release from mitochondrion, cytochrome c gets combined with the apoptotic protease activating factor (Apaf-1) and caspase-9 resulting in the formation of apoptosome which further triggers the activation of caspase-3 (Hengartner, 2000) and thus resulting in apoptosis (Fig 2.3). The intrinsic pathway of apoptosis is mainly controlled by the proteins of Bcl-2 family by regulating the exit of cytochrome c from mitochondria (Hengartner, 2000) but once the cytochome c gets released the process of apoptosis goes on with no return (Goldstein et al., 2000).

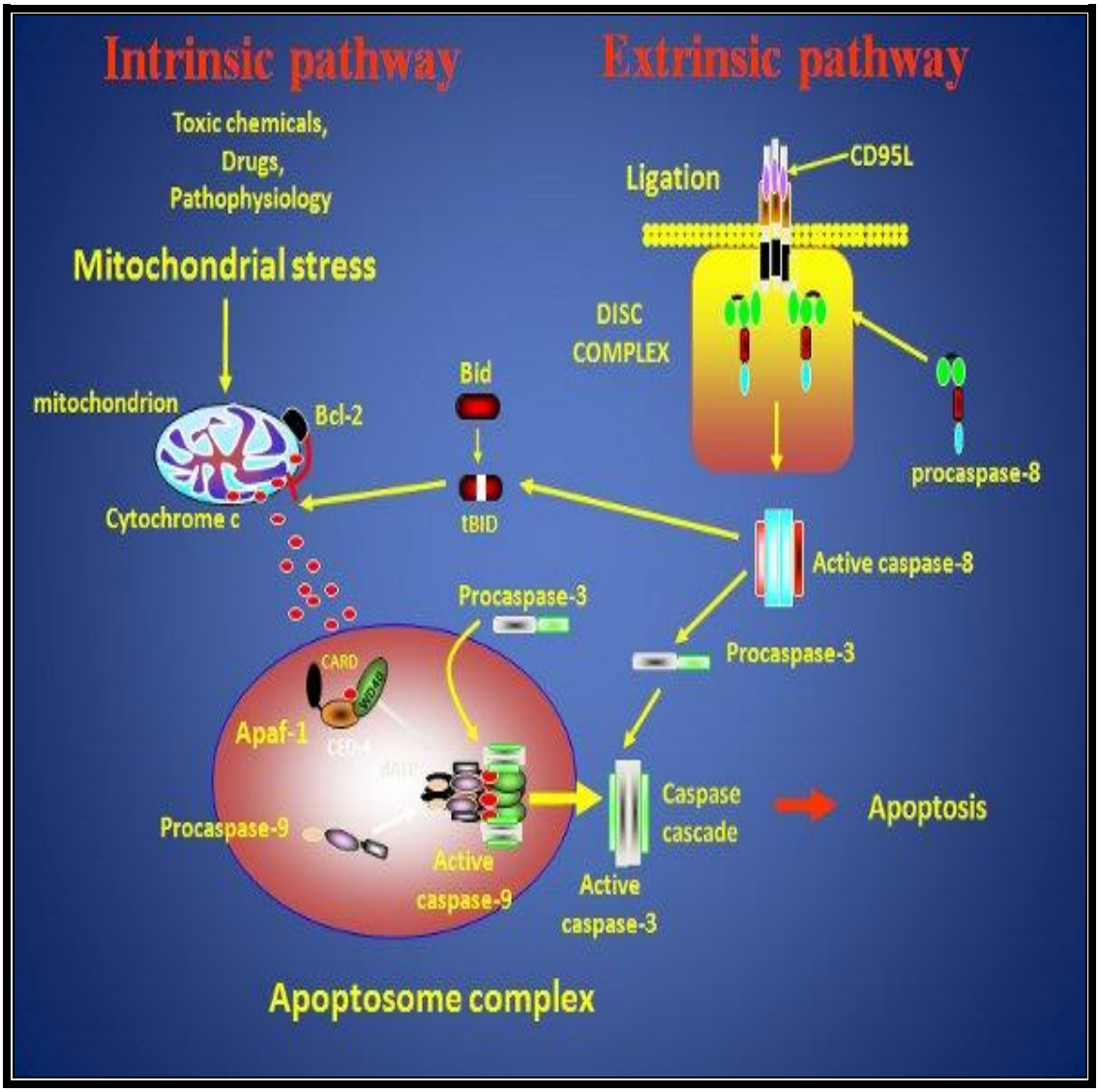


Fig 2.5: Extrinsic and Intrinsic apoptotic pathways.

2.3.3. Different Apoptotgenic factors

2.3.3.1. Caspases

Caspases are known to be the initiators of apoptosis. Caspases are a set of cysteine proteases possessing an active cysteine site and cleave their substrates at aspartic acid residues. Caspases have been found to mediate over 100 substrates in a cell which are very important and have a well defined function. During the process of apoptosis caspase-3 removes the inhibitory subunit of ICAD allowing CAD (Caspase activated DNase) to cut the genomic DNA into 180 base pair fragments which forms the basis of the DNA laddering test for apoptosis. Further nuclear shrinking, budding and active blebbing are due to cleavage of nuclear lamins (Buendia et al., 1999; Rao et al., 1996) and PAK2 (Rudel et al., 1997) by caspases respectively. Although caspases are the main initiators of apoptosis, their activation is in turn tightly regulated by other apoptotic regulators such as proteins from IAP and Bcl-2 family (Hengartner, 2000). Based on the pro apoptotic function of caspases, these are further divided into two groups i.e. initiator and effector caspases. The initiator (apical) caspases including caspase -2, -8, -9, -10 and -11 activate the effector caspases including caspase -3,-6 & -7 (Fig.2.5, 2.6). The effector (downstream) caspases further degrade multiple substrates including the structural and regulatory proteins in cell nucleus, cytoplasm and cytoskeleton which leads to the deregulation of vital cell processes and ultimately to cell death. It has been observed that caspase-1 promotes the activation of effector caspases-3 -7 and -6. Caspase-9 on the other hand undergoes activation of caspases -2, -3, -6, -7, -8 and -10 in a cytochrome c dependant manner. Caspase-3 has been found to activate caspases-2,-6,-8,-10 and also cytochrome c dependent activation of caspase-9 (Loss et al., 1995). Further, caspase-8 activates caspases-1, -2, -3, -6, -7, -9 and -11 (Slee et al., 1999). In the final stage of caspase cascade, caspase-6 catalyzes the activation of caspase-8 &-10 and caspase-2, -7, -8 and -10 cleave the target protein substrates directly (Loss et al., 1995). Caspases and their regulators are therefore potentially attractive targets for the development of new cancer therapies.

2.3.3.2. Cytochrome c

Cytochrome c is a component of the electron transport chain and is involved in the production of ATP. Both *in vitro* and *in vivo* studies have demonstrated the release of

cytochrome c from mitochondria during apoptosis (Yang et al., 1997; Kluck et al., 1997). After getting released, cytochrome c interacts with Apaf-1 and procaspase-9 in the cytosol thus forming an apoptosome complex (Fig 2.6) (Li et al., 1997), which further undergoes cleavage and activation of procaspase-9 and other procaspases responsible for the executive stages of apoptotic cell death.

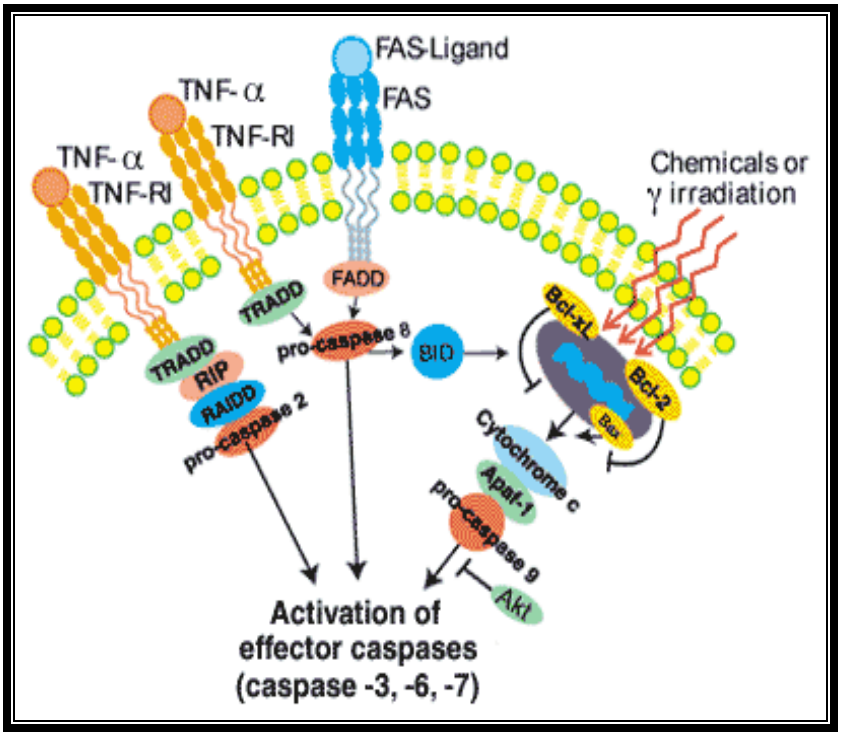


Fig.2.6. Role of caspases and Cytochrome c in apoptosis

2.3.3.3. Poly (ADP-ribose) polymerase

Poly (ADP-ribose) polymerase (PARP) is an essential DNA repair enzyme playing its role in the repair of single-stranded breaks in DNA *via* the base excision repair pathway. PARP has emerged as an important target in cancer therapy (Fig.2.7). It has been observed that the cleavage of PARP during apoptosis facilitates cellular disassembly and in particular of the nucleus, ensuring the completion and irreversibility of this process. The cleavage of PARP has been observed in almost all forms of apoptosis (Duriez and Shah 1997). During apoptosis the fragmentation of nuclear DNA produces numerous single-strand nicks in the linker regions of chromatin since PARP interacts preferentially with single-stranded DNA breaks (Le et al. 1994), it recruit the base excision repair (BER) proteins to repair the damaged DNA. Studies by Smulson et al. (1998) have shown that the cleavage of the PARP by

caspase-3 results in the isolation of its DNA-binding domain and therefore it binds irreversibly to the internucleosomal DNA in apoptotic cells. Further it has been proposed that the cleavage of PARP by caspases can promote apoptosis *via* two ways, either, the absence of PARP disables the key aspects of the cellular genomic surveillance mechanism and prevents the unnecessary DNA repair that would delay chromatin degradation (Casciola et al. 1996) or the cleavage of the PARP improves the access of the endonuclease to the chromatin. The localization of PARP to the nuclear envelope has also suggested that its cleavage during apoptosis participates in nuclear disassembly and facilitates downstream events which are otherwise delayed (Dantzer et al. 1998).

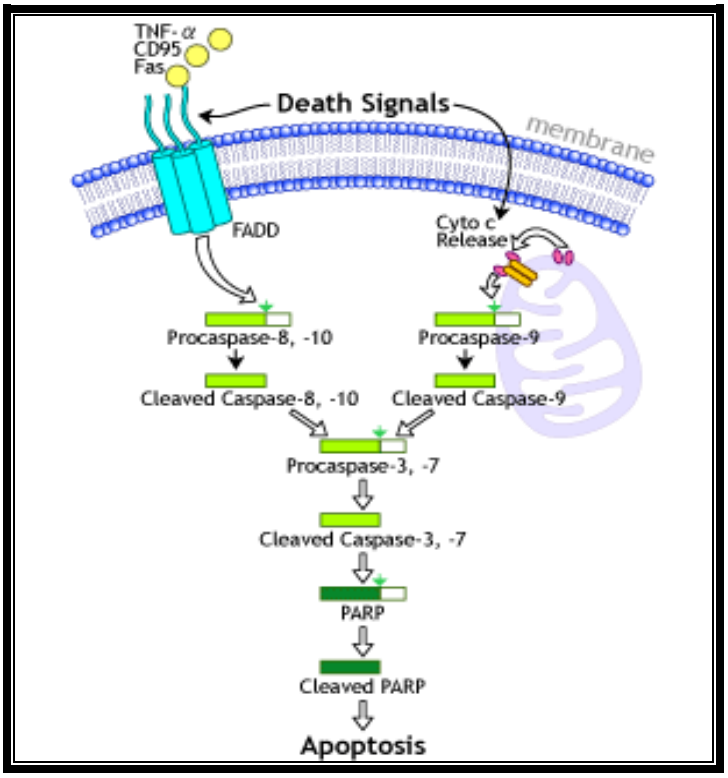


Fig.2.7. Role of PARP in apoptosis

2.3.3.4. Bcl-2 family

Bcl-2 family is a group of evolutionary conserved proteins that regulate apoptotic processes converging on the mitochondria as well as endoplasmic reticulcum (ER) **(**Hengartner and Horvitz, 1994**).** Bcl-2 the first recognised member of the family was found in B-cell lymphoma hence the name Bcl. The proteins are divided into three subgroups determined by their structure and function. The antiapoptotic fractions

including Bcl-2, Bcl-$x_{L,}$ Bcl-w, A1, Mcl-1 and Boo. The proapoptotic Bax/ Bak- like group comprising Bad, Bik, Bid, Hrk, Bim, Bmf, Noxa and Puma. Most anti-apoptotic proteins like Bcl-2 and Bcl-x_L show strong sequence conservation in all four domains, whereas pro-apoptotic proteins, including Bad, Bik and Bid frequently lack the BH4 domain. The later are divided in the bax subfamily, members of which contain BH1, BH2 and BH3 and the 'BH3-domain-only' proteins that show sequence homology only within the BH3 domain (Bid, Bad) (Reed, 1998). Inviable cells and anti-apoptotic proteins are localized in membranes such as the outer mitochondrial membrane, the endoplasmic reticulum or the nuclear membrane while pro-apoptotic proteins are found in the cytosol (Wolter et al., 1997). The formation of Bcl-2/Bax heterodimers and the interaction of Bad with Bcl-2-Bcl-x_L within the outer mitochondrial membrane, respectively suggests a neutralising competition of the proteins (Fig 2.8) (Ottilie et al., 1997). Furthermore Bad, Bcl-2 and Bcl-x_L can be inactivated by phosphorylation (Kharbanda et al., 2000). In response to apoptotic signal, Bax, for example dimerises and translocates to mitochondria where it becomes an integral pore forming membrane protein (Gross et al., 1998). Aditionally, following CD95 treatment, Bid is cleaved at its amino terminus by caspase-8. Truncated Bid (tBid) also translocates to the mitochondria, inserts into the membrane and interacts with bax to cause mitochondrial permeability transition (Desagher et al., 1999). In contrast, Bcl-2 antagonises the pore forming activity of the Bax, thus preventing the efflux of apoptosis-activating factors (Martinou and Green, 2001).

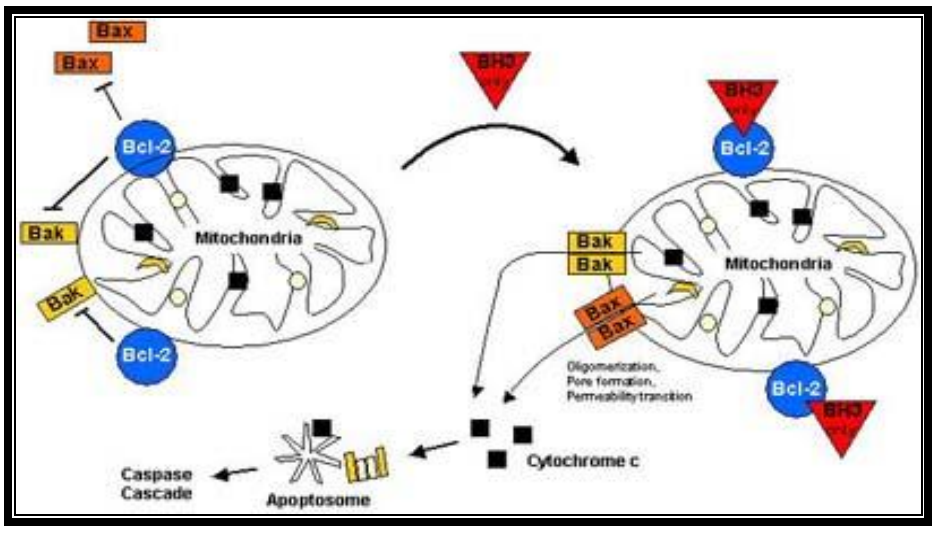


Fig.2.8. Role of Bcl-2 family in apoptosis

2.3.4. Topoisomerases

Topoisomerases are the enzymes that modulate superhelicoidal density of DNA and act by introducing single (type I) or double (type II) strand DNA breaks. These enzymes are involved in DNA repair, replication, transcription and chromosome segregation during mitosis. In prokaryotes these topoisomerases maintain DNA in a supercoiled state by altering the linking number without changing its primary structure. In higher organisms the wrapping of DNA around histones requires the action of DNA topoisomerases to resolve the topological constraints imposed during wrapping and thus maintaining the supercoiled structure.

2.3.4.1. Topoisomerases as targets for anticancer drugs

Topoisomerases are the targets of an increasing number of anticancer drugs that block the reaction that reseals the breaks in the DNA mediated by these enzymes. Drugs targetting topoisomerase can either be classified as topo posions or topo catalytic inhibitors. The former acts by stabilizing the enzyme DNA cleavable complexes leading to DNA break and the latter acts by stabilizing the enzyme where both DNA strands remain intact and no DNA breaks occur. The binding of drug is often found to be reversible, however, once the replication fork runs into the blocked topoisomerase, a piece of gapped DNA strand which is not bound by the topoisomerase gets released, creating a permanent breakage in the DNA and thus leading to the cell death. It has been found that topoisomerase I inhibitors induce single-strand breaks into DNA (Fig 2.9) and show their inhibitory activity *via* different mechanisms. Certain drugs like camptothecin (CPT) inhibit the dissociation of topoisomerase and DNA (Hsiang et al., 1989) resulting in a replication-mediated DNA damage which is repaired more efficiently in normal cells than in cancer cells (deficient for DNA repair). Topoisomerase I inhibitors have also been observed to cause gene inactivation through chromatid aberrations.

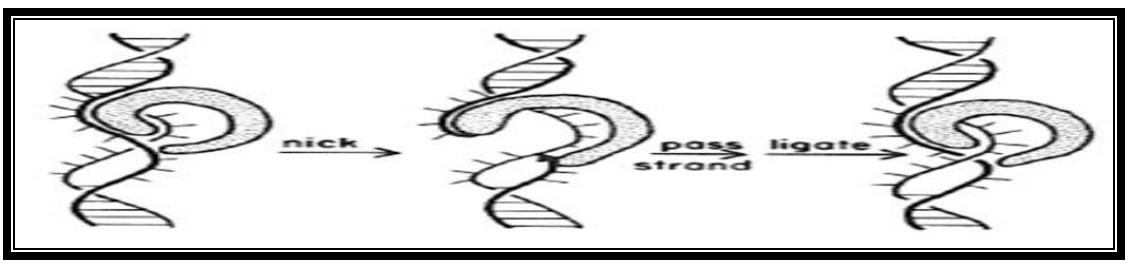


Fig.2.9. Single strand breaks introduced by Topoisomerase-I

The inhibitors of topoisomerase II like Etoposide (ETO) are amongst the most widely used anti-cancer agents. Being the potent inducers of double strand breaks in DNA (Fig 2.10) (Gorczyca et al., 1993) these drugs arrest the cell cycle at the G2 stage by disrupting the interaction between topoisomerase II and regulators of the cell cycle such as Cdc2. These inhibitors result in a wide range of chromosomal aberrations and show their activity by either stabilising topoisomerase II-DNA complexes that can easily be cleaved or by interfering with the catalytic activity of the enzyme. The inhibitory action of CPT and ETO has widely been studied on various cell lines (Fulda et al., 1997; Shao et al., 1997). Dual inhibitors that target both topoisomerase I & II have also been observed and are found to be more potent as anticancer agents. These inhibitors work either by recognising structural motifs present on both enzymes, by linking separate topoisomerase inhibitors together into a hybrid drug, or by using inhibitors that bind to and intercalate DNA.

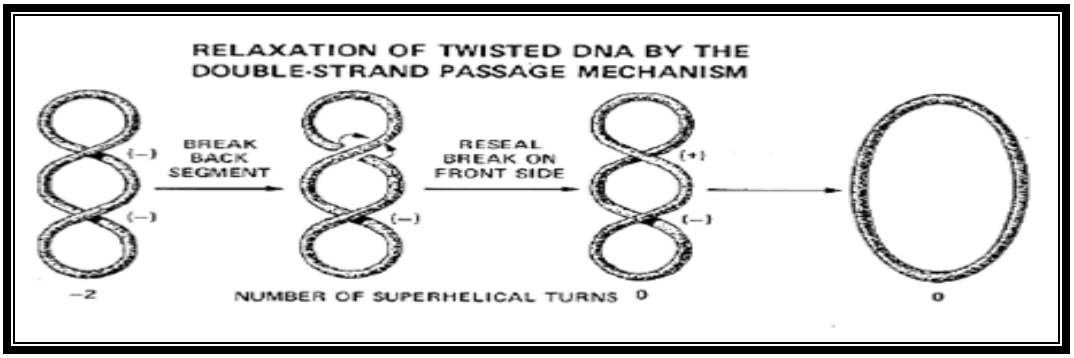


Fig.2.10. Double strand breaks introduced by Topoisomerase-II

2.4. Tumor Angiogenesis

Angiogenesis (formation of new blood vessels) is one of the central mechanisms that allow sustained growth of tumors by providing continuous supply of oxygen and nutrients. In the earlier stages of tumorogenesis no vasculature has been observed, however, for proper growth and metastasis a tumor creates its own blood vessel supply by intussusceptive angiogenesis and postnatal vasculogenesis. Several reports have shown that tumor angiogenesis occurs due to the upregulation of several pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and its receptors VEGF-R1 and VEGF-R2, angiopoietins (Ang-1, Ang-2), fibroblast growth factor-2 (FGF2) and its receptors, matrix metalloproteinases (MMPs), plasmin

activator receptors and inhibitors and collagenase prolyl hydroxylases (Otrock et al, 2007) (Fig 2.11)

2.4.1. Tumor Angiogenesis as target for anticancer drugs

Vascular targeting has become a novel strategy to treat malignant tumors. Antiangiogenic drugs have been found to exert their beneficial effects either by disabling the agents that activate and promote cell growth or by blocking the growing blood vessel cells directly. Literature reveals that more than 300 different substances show angiogenesis inhibitory properties and these range from molecules produced naturally such as green tea extract to new chemicals synthesized in the laboratory. Some of the known medicines approved by U.S. Food and Drug Administration (FDA) which include celecoxib (Celebrex), bortezomib (Velcade) and interferon have also been found to possess antiangiogenic properties. A large number of new inhibitors are currently being tested in clinical trials for anti-angiogiogenesis activities. Bevacizumab (also known as Avastin, anti-VEGF, RhuMabVEGF) is the only FDA-approved antiangiogenic drug for colon-rectal cancer (Frankish and Helen et al., 2002). It is an antibody that neutralizes the VEGF protein by sticking to it and thus preventing it from triggering the growth of blood vessel.

2.4.2. Pro-angiogenic factors

2.4.2.1. Angiopoietins

Angiopoietins are the protein growth factors that promote angiogenesis. There are generally four types of angiopoietins and these include Ang1, Ang2, Ang3 and Ang4. Ang1 and Ang2 are mostly required for the formation of mature blood vessels (Thurston, 2003). Both of these angiopoietins bind to the extracellular domain of Tie2 at the same position and with similar affinities (Maisonpierre, 1997; Fiedler, 2003). It has been observed that the binding of Ang-1 to Tie2 mediates rapid receptor autophosphorylation that promotes endothelial cell migration and survival. In contrast to this the binding of Ang-2 to the Tie2 does not elicit any rapid autophosphorylation, suggesting that Ang-2 functions as an antagonist ligand of Tie2. Ang-1-Tie2 signaling is an important factor for vascular maintenance (Wong et al., 1997). Ang-1-mediated Tie2 phosphorylation signals primarily through the protein kinase B (PKB)-Akt pathway that transduces survival signals (Kim et al., 2000; Papapetropoulos et al.,

2000). Genetic manipulation experiments in mice and cell-culture experiments suggest that Ang-2 antagonizes Ang-1 mediated Tie2 functions (Maisonpierre, 1997; Sato et al, 1995; Scharpfenecker, 2005), however, the functioning of Ang-2 has been found to be context dependent. Ang-2 facilitates angiogenesis if it functions in concert with VEGF and leads to vessel regression in its absence (Hanahan D, 1997). Certain evidence show that Ang-2 functions as a Tie2 agonist in a concentration dependent manner. It has also been observed that long term sustained stimulation of endothelial cells with Ang-2 results in Akt signaling promoting endothelial cell survival, sprouting and migration (Korff et al., 2001; Kim et al., 2000; Mochizuki et al., 2002). Since Ang-1 functions constitutively, the functioning of Ang-2 is dynamically regulated and thus Ang-2 manipulatory therapies are preferred and many Ang-2 neutralizing reagents have been developed as potential anti-angiogenic tumor drugs (Oliner, 2004).

2.4.2.2. Matrixmetalloprotinases

Matrix metalloproteinases (MMPs) is a family of nine or more highly homologous Zn (++) endopeptidases that collectively cleave most of the constituents of the extracellular matrix. The family is further divided into collagenases (MMP-1, -8, & -13), gelatinases (MMP-2 & 9), stromelysins (MMP-3 &10), matrilysins (MMP-7 & 26) and the membrane type MMPs (MMP-14 to 17 & 24). MMPs are important in many normal biological processes including embryonic development, angiogenesis and wound healing as well as in pathological processes such as inflammation, cancer and tissue destruction. Besides cleaving extracellular matrix, MMPs are involved in the breakdown and remodelling of many tissues and organs. MMPs play an important role in angiogenesis for tumor progression and metastasis. Endothelial and tumor cells show increased expression of MMPs which is correlated with the tumor grade (Overall and Lopez-otin, 2002; Slack- Davis and Parsons, 2004; Takaha et al., 2004). The breakdown of tissue matrix by MMPs to facilitate the movement of newly formed endothelial cells for vessel formation is a critical step in angiogenesis (Overall and Lopez-otin, 2002). MMP-2 and MMP-9 are known to associate to tumor invasion and progression. (Mehta et al., 2003; Takaha et al., 2004). It has been observed that MMP-2 participates in extracellular matrix degradation (ECM) of a wide range of

substrates including type I, IV, V, VII and X collagens, laminin, elastin, fibronectin and proteoglycans (Nagase et al., 1999; Woessner 1999; Sternlicht et al., 2001). Studies conducted by Gerhards et al. (2001) have showed a clear correlation between high levels of urinary MMP-2 with tumor grade and stage. Targeting MMPs had therefore turned out to be a promising anticancer therapy *via* angioprevention.

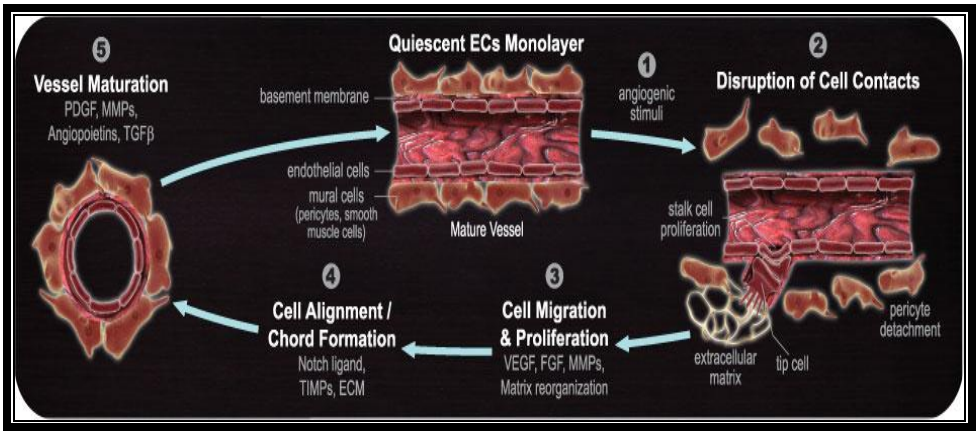


Fig.2.11. Pro-angiogenic factors and tumor angiogenesis

2.5. Drug delivery system

The challenge of modern drug therapy is the optimization of the pharmacological action of the drugs coupled with the reduction of their toxic effects *in vivo*. The prime objectives in the design of drug delivery systems (DDS) are the controlled delivery of the drug to its site of action at a therapeutically optimal rate and dosage to avoid toxicity and improve the drug effectiveness and therapeutic index. It has been observed that many of the problems that hinder the clinical applications of particulate DDS get solved by several DDS formulations. It has shown advantages in *vivo* delivery of new drugs and ligand targeted therapeutics. DDS has improved many of the pharmacological properties of conventional ("free") drugs including particulate carriers which are primarily composed of lipids and/or polymers and their associated therapeutics. It alters the pharmacokinetics (PK) and biodistribution (BD) of the associated drugs or functions as drug reservoir or both. Some of the problems exhibited by free drugs that can be ameliorated by the use of DDS are given below (Table.2.2).

Problem	Implication	Effect of DDS
Poor solubility	A convenient pharmaceutical format is difficult to achieve, as hydrophobic drugs may precipitate in aqueous media. Toxicities are associated with the use of excipients such as Cremphor (the solubilizer for paclitaxel in Taxol).	DDS such as lipid micelles or liposomes provide both hydrophilic and hydrophobic environments, enhancing drug solubility.
Tissue damage on extravasation	Inadvertent extravasation of cytotoxic drugs leads to tissue damage e.g. tissue necrosis with free doxorubicin.	Regulated drug release from the DDS can reduce or eliminate tissue damage on accidental extravasation.
Rapid breakdown of the drug *in vivo*	Loss of activity of the drug follows administration e.g. loss of activity of camptothecins at physiological pH.	DDS protects the drug from premature degradation and functions as a sustained release system. Lower doses of drug are required.
Unfavorable pharmacokinetics	Drug is cleared too rapidly, by the kidney, for example, requiring high doses or continuous infusion.	DDS can substantially alter the PK of the drug and reduce clearance. Rapid renal clearance of small molecules is avoided.
Poor biodistribution	Drugs that have widespread distribution in the body can affect normal tissues, resulting in dose-limiting side effects such as the cardiac toxicity of doxorubicin.	The particulate nature of DDS lowers the volume of distribution and helps to reduce side effects in sensitive, nontarget tissues.
Lack of selectivity	Distribution of the drug to normal	DDS can increase drug

for target tissues	tissues leads to side effects that restrict the amount of drug that can be administered. Low concentrations of drugs in target tissues will result in suboptimal therapeutic effects.	concentrations in diseased tissues such as tumors by the enhanced permeability and retention (EPR) effect. Ligand-mediated targeting of the DDS can further improve drug specificity.

Table 2.2. Non-ideal properties of drugs and their therapeutic implications.

2.5.1. Rationale of Nanoparticle based drug delivery system

The major goals in designing nanoparticles as a delivery system are to control particle size, surface properties and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen. Liposomes have been used as potential carriers with unique advantages including protecting drugs from degradation, targeting to site of action and reduction in toxicity or side effects. The applications of liposomes were found to be limited due to the inherent problems such as low encapsulation efficiency, rapid leakage of water soluble drug in the presence of blood components and poor storage stability. Polymeric nanoparticles have been found to offer specific advantages over liposomes. They help to increase the stability of drugs/proteins and possess useful controlled release properties (Vila et al., 2002; Mu et al., 2003). Nanoparticles offer wide advantages in drug delivery system. The particle size and surface characteristics of nanoparticles can easily be manipulated to achieve both passive and active drug targeting after parenteral administration. They control and sustain the release of the drug during transportation and at the site of localization, altering its organ distribution and subsequent clearance so as to achieve increase in drug therapeutic efficacy and lower side effects. It has been found that controlled release and particle degradation of such nanoparticles could be readily modulated by the choice of matrix constituents. Further, drug loading is relatively higher and the drugs can be incorporated into the systems without any chemical reaction, which is an important factor for preserving the drug activity. These particles can further be modified to achieve site-specific targeting *via* attachment of targeting ligands to their surface or use of magnetic

guidance. Nanoparticle based drug delivery system can be administered through various routes including oral, nasal, parenteral, intra-ocular and so on.

2.5.2. Nanoparticle based drug delivery system for cancer

The conventional chemotherapy has encountered several problems like normal tissue toxicity, poor solubility, stability and high incidence of drug resistant tumor cells. Cytototoxic agents which are administered conventionally are found to bind extensively and indiscriminately to body tissues and serum proteins in a highly predictable manner, with the result only a small fraction of the drug reaches the tumor site (Ratain and Mick, 1996). Further, cancer cells have a defence mechanism characterised as "cellular" drug resistance or multidrug resistance (MDR) phenotype which involves active efflux of a broad range of cytotoxic drugs out of the cytoplasm by membrane bound transporters In addition cancer cells tend to be more resistant to chemotherapy due to various drug permeation barriers which makes it difficult to achieve high intratumoral drug concentration in solid tumors. This type of drug resistance or sometimes referred as "non-cellular" drug resistance may further lead to compromised clinical outcomes even though an anticancer drug may has strong *in vitro* efficacy. The most important goal of drug delivery is to minimize the exposure of normal tissues to these drugs while maintaining their therapeutic concentration in tumors. The problems related to cancer chemotherapy can partially be overcome by direct intratumoral delivery of controlled release biodegradable nanoparticles (NPs). NPs are colloidal carrier systems, which have been shown to improve the efficacy of the encapsulated drug by overcoming drug resistance as well as by providing sustained drug effect (Brigger et al., 2002). Biodegradable poly (hydroxy acids) such as the copolymers of PLA (poly lactic acid) and PLGA (d, l-lactide-co-glycolide) are being extensively used in biomedical applications because of their biocompatibility, ability to encapsulate various drug molecules and sustained release properties. One particularly interesting application of nanoparticule is the drug brain delivery accompanied with the local sustained release of the new large therapeutic molecules available to treat the CNS. Due to their poor stability in biological fluids, rapid enzymatic degradation, unfavourable pharmacokinetic properties and lack of diffusion toward the CNS, they may be advantageously formulated in brain targeted protective

nanocontainers (Pardridge, 2001). These drugs in comparison to conventional drugs possess a high intrinsic pharmacological activity.The small dose requested for therapeutic efficiency easily fits the loading capacity of nanoparticles and do not require the administration of large amount of potentially toxic nanoparticle excipient. Further, it has been found that transferrin receptors are over expressed in most cancer cells by two to tenfold more than in normal cells. The transferrin-conjugated nanoparticles have been demonstrated to have enhanced cellular uptake and retention than unconjugated nanoparticles (Sahoo et al., 2005).

2.5.3. Rationale of PLGA nanoparticles for anticancer drug delivery

Particulate drug carrier systems encapsulating drugs have emerged as promising approach in anticancer treatment by improving the therapeutic index of drugs by preferential localization at target sites (Bisht and Maitra, 2009). Nanoparticles formulated from the biocompatible and biodegradable polymer poly (d, l-lactide-co-glycolide) (PLGA) have shown the potential for various drug delivery applications (Sahoo and Labhasetwar, 2003). These nanoparticles can be designed to slip between intercellular spaces, enter cells or transport directly through biological barriers to access disease sites either by modifying the surface characteristics or by attaching any suitable ligand on their surface (Das et al., 2009). Biodegradable and biocompatible PLGA is perhaps the most widely investigated biomaterial for making NPs for controlled release and sustainable drug delivery (Blanco et al., 2005; Kilic et al., 2005; Olivier, 2005). It has been found that PLGA has a solid safety profile and sustained drug release (Lupi et al., 2004; Gavini et al., 2004; Panyam et al., 2002). PLGA like other natural polyesters undergoes hydrolysis upon implantation into the body, forming biocompatible and metabolizable moieties such as lactic acid and glycolic acid that are eventually removed from the body by the citric acid cycle (Panyam et al., 2003). PLGA NPs are generally made by emulsion solvent evaporation or by solvent displacement techniques (Jain, 2000). Drugs encapsulated inside the NPs can be released at a sustained rate through diffusion and by the degradation of the NPs. Many lines of evidence suggest that the degradation rate of PLGA can be controlled by changing block copolymer composition and molecular

weight (Lin et al., 2000). Accordingly, the release rate of encapsulated drugs can be altered from lasting for days to months.

2.5.3.1. Characterization of PLGA Nanoparticles

2.5.3.1.1. Particle size

Particle size and size distribution are the most important characteristics of nanoparticle systems. These determine the *in vivo* distribution, biological fate, toxicity, targeting ability, drug loading, drug release and stability of nanoparticles. Studies have demonstrated that nanoparticles of sub-micron size have a number of advantages over microparticles as a drug delivery system (Panyam and Labhasetwar, 2003). Due to their small size and relative mobility, nanoparticles have relatively higher intracellular uptake and are available to a wider range of biological targets. Particle size affects the drug release rate of the particles. Since smaller particles have larger surface area, most of the drug associated remains at or near the particle surface, which leads to fast drug release, however, due to the large cores, larger particles allows encapsulation of more drug and therefore slow diffusion outside (Redhead and Davis, 2001). Particle size also affects the rate of degradation of the polymer which is found to increase with increasing particle size (Dunne and Corrigan, 2000). Photon-correlation spectroscopy or dynamic light scattering is the fastest and the most routine method of determining particle size. It requires the viscosity of the medium to be known and determines the diameter of the particles by Brownian motion and light scattering properties (Swarbrick and Boylan, 2002). Results obtained by this technique are usually verified by scanning or transmission electron microscopy (SEM or TEM).

2.5.3.1.2. Zeta Potential

The zeta potential of a nanoparticle is commonly used to characterise its surface charge (Couvreur et al, 2002). It reflects the electrical potential of the particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the nanocapsule or adsorbed onto the surface.

2.5.3.1.3. Morphology

To ascertain the overall shape and morphology of PLGA nanoparticles, atomic force microscopy (AFM) and/or electron microscopy techniques such as scanning electron microscopy (SEM) and Transmission electron microcopy (TEM) are used.

2.5.3.2. Surface properties of nanoparticles

After intravenous administration nanoparticles are easily recognized by the immune system of the body and are then cleared by phagocytes from the circulation, the process referred to as opsonisation (Muller and Wallis, 1993). For successful drug targeting , there should be minimum opsonisation and prolonged circulation of these particles *in vivo* which can either be achieved by surface coating of nanoparticles with hydrophilic polymers/surfactants or formulation of these particles with biodegradable copolymers with hydrophilic segments such as polyethylene glycol (PEG), polyethylene oxide, polyoxamer, poloxamine and polysorbate 80 (Tween 80). Studies have shown that PEG conformation at the nanoparticle surface is of utmost importance for preventing opsonisation due to the opsonin repelling function of the PEG layer.

2.5.3.3. Stability and sterilisation

In general lyophilised polymeric nanoparticles exhibit a high stability, they do not require special storage conditions and may be stored in the refrigerator at 2 - 4°C (Huncharek et al., 1998). Radiation sterilisation is the method of choice for the sterilisation of nanoparticles. Indeed, routine sterilization techniques are often inapplicable due to physicochemical properties of the nanoparticles. The sterile filtration is not feasible because the sizes of the nanoparticles are close to or above the pore size of the sterile filters, heat sterilisation is not possible because of the heat sensitivity of most nanoparticle materials (Kreuter, 2006). It has been found that irradiation not only protects the physicochemical parameters (mean particle size, polydispersity, molecular weights and aggregation stability) of the nanoparticles (both empty and drug loaded) but also prevents the radiolysis of the drug (Maksimenko et al., 2008).

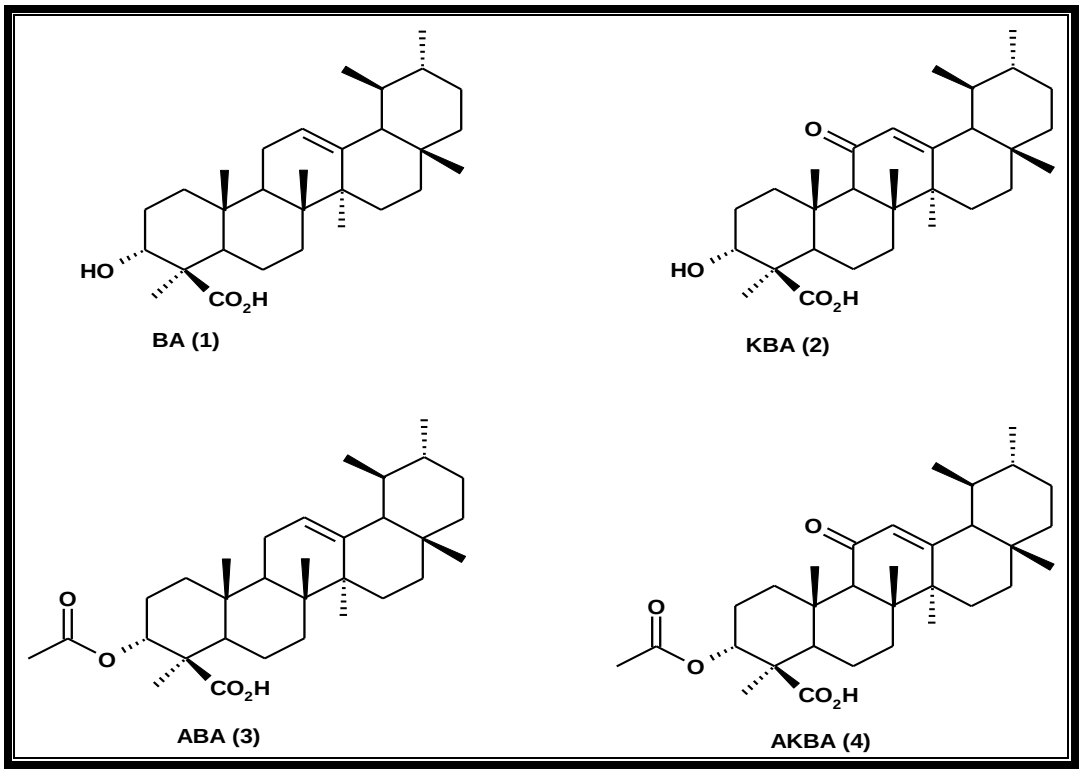
2.5.3.4. Nanoparticles for drug delivery into the brain

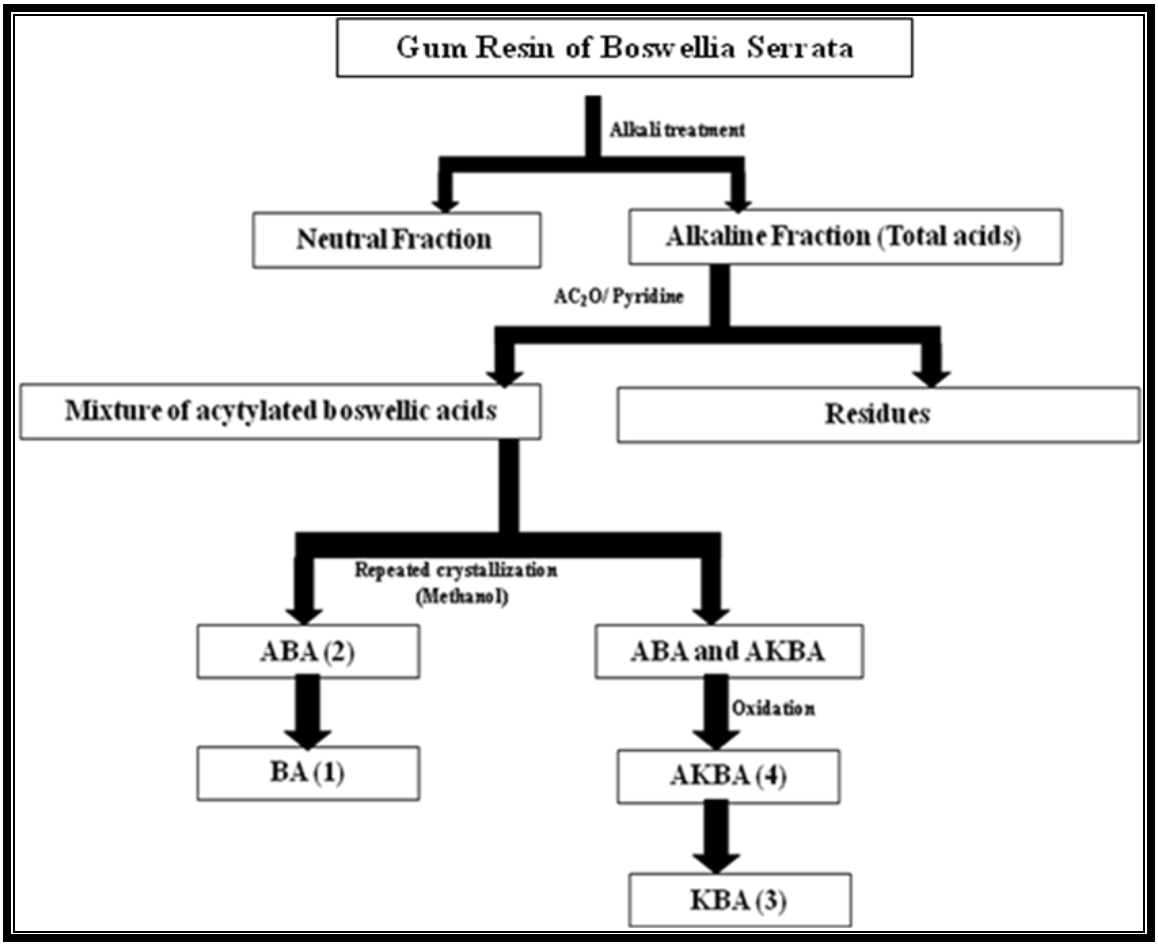
The blood-brain barrier (BBB) is the most important factor limiting the development of new drugs for the central nervous system. The BBB is characterized by relatively impermeable endothelial cells with tight junctions, enzymatic activity and active efflux transport systems. It effectively prevents the passage of water soluble molecules from the blood circulation into the CNS and can also reduce the brain concentration of lipid soluble molecules by the function of enzymes or efflux pumps (Chen et al., 2004). Consequently, the BBB only permits selective transport of molecules that are essential for brain function. Strategies for nanoparticle targeting to the brain rely on the presence and interaction of nanoparticles with specific receptor-mediated transport systems in the BBB. Polysorbate 80/LDL, transferrin receptor binding antibody (such as OX26), lactoferrin, cell penetrating peptides and melanotransferrins are found to be capable for delivering a self non transportable drug into the brain *via* the chimeric construct that can undergo receptor-mediated transcytosis (Kreuter, 2004; Ji et al., 2006; Gabathuler et al., 2005). Poly (lactic-co-glycolic acid) (PLGA) transferrin-conjugated nanoparticles have also been found to transport cytostatics across the blood-brain barrier (BBB) (Jorg and Svetlana, 2008).

3.1. Preparation of Compounds

3.1.1. Isolation of Boswellic acids

Boswellic acids (BAs) were isolated by the method analogous to that developed by Winterstein and Stein (1932). The methods using alkali-acid treatments of the concentrated alcoholic extract provide comparatively higher purity of BAs. The chromatographic methods were used for the separation of the individual acids and other constituents. The methanolic extract of gum resin of *Boswellia serrata* was given alkali (KOH) treatment to prepare the sodium salt of the acids, which is soluble in water. The extract was then given hexane washing to isolate neutral fraction (mixture of terpenoids lacking acid functionality). The acid's salt was further treated with dil. HCl to liberate free acids which are filtered off. The precipitates filtered were acetylated using acetic anhydride and pyridine to yield a mixture of acetylated BAs. The acetylated BAs i.e. ABA and AKBA were subjected to column chromatography on silica gel to yield pure ABA and AKBA. The acetylated BAs were hydrolyzed back into corresponding acids BA and KBA respectively. The flow diagram (Scheme-1) describes the isolation of total acid fraction in >90% purity.



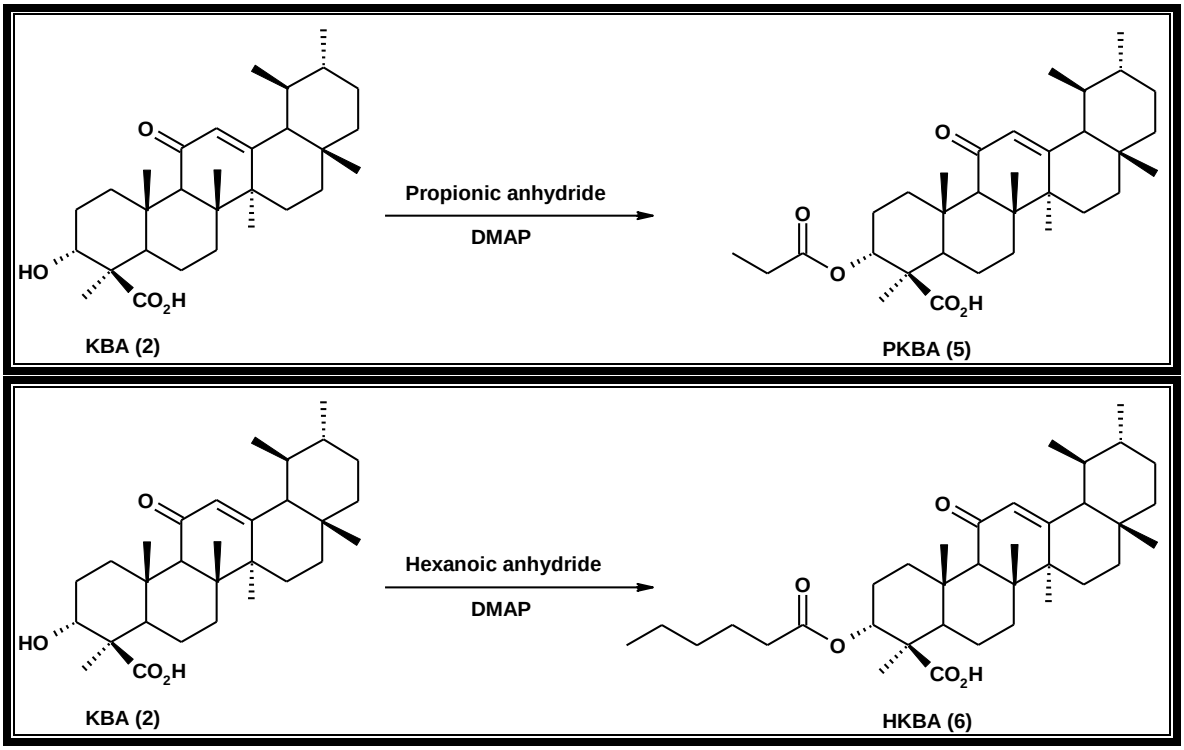


Scheme 1: Flow chart for isolation of various terpenoids

3.1.2. Synthesis of 3-α-propionyloxy-11-keto-β-boswellic acid (PKBA) and 3-α-hexanoyloxy-11-keto-β-boswellic acid (HKBA).

The synthesis of 3-α-propionyloxy-11-keto-β-boswellic acid (PKBA) and 3-α-hexanoyloxy-11-keto-β-boswellic acid (HKBA) was accomplished from 11-keto-β-boswellic acid (KBA, a triterpenic constituent of boswellic acids). 11-keto-β-boswellic acid (KBA) was isolated from a mixture of boswellic acids by method reported in literature (Shah et al., 2009). In order to prepare PKBA and HKBA, KBA (1 g, 2 mmol) was treated with propionic anhydride and hexanoic anhydride (1.2 eq) in presence of dimethyl amino pyridine (DMAP) as a catalyst in dry DCM (10 ml) to produce PKBA and HKBA (Scheme-2), respectively in quantitative yields. After completion, the reactions were treated with 10% sodium bicarbonate ($NaHCO_3$) solution, extraction with dichloromethane (DCM) (3x100 ml), usual workup and

chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave compound PKBA & HKBA respectively in 95% yield as white powder.



Scheme-2: Synthesis of PKBA and HKBA

3.1.3. Spectral analysis

3.1.3.1. 3-O-Propionyloxy-11-keto-β-boswellic acid (PKBA)/SS-167

White solid, $[\alpha]_D^{25}$ +66.6 (*c* 1.0 $CHCl_3$), mp 264-266 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.83, 0.93, 1.0, 1.12, 1.15, 1.24 (24H, 23, 25, 26, 27, 28, 29 & 30 -CH_3 & -CH_2CH_3), 1.40-2.39 (m, nH), 2.33 (t, *J* = 7.3 Hz, 2H, -CH_2CO), 5.31 (bs, 1H, H-3), 5.56 (bs, 1H, H-12). ^{13}C NMR (50 MHz, $CDCl_3$): δ 9.4, 13.2, 17.5, 18.4, 18.9, 20.5, 21.2, 23.5, 23.9, 27.3, 27.5, 28.1, 28.9, 30.9, 32.9, 34.0, 36.9, 37.4, 39.3, 39.4, 40.9, 43.8, 45.1, 46.6, 50.6, 59.1, 60.4, 72.8, 130.5, 165.2, 173.9, 181.9, 199.2; ESI-MS (*m/z*): 549 $[M+Na]^+$. Anal. Calc. for $C_{33}H_{50}O_5$: C, 75.25; H, 9.57. Found: C, 75.11; H, 9.72.

3.1.3.2. 3-O-Hexanoyloxy-11-keto-β-boswellic acid (HKBA)/SS-243

Colourless semisolid, $[\alpha]_D^{25}$ +37 (*c* 1.0 $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.83, 0.89, 1.12, 1.15, 1.20, 1.34 (24H, 23, 25, 26, 27, 28, 29 & 30 -CH_3 & -CH_2CH_3), 1.34-

1.89 (m, nH), 2.35 (t, *J* = 7.3 Hz, 2H, -CH_2CO), 5.31 (bs, 1H, H-3), 5.56 (bs, 1H, H-12). ^{13}C NMR (50 MHz, $CDCl_3$): δ 12.1, 12.9, 16.4, 17.4, 19.4, 20.1, 21.3, 22.5, 22.9, 23.6, 23.9, 27.3, 27.8, 28.9, 30.2, 33.6, 34.0, 36.4, 37.3, 37.8, 38.2, 39.2, 39.3, 40.9, 42.7, 44.1, 49.5, 56.5, 58.0, 59.4, 71.8, 129.4, 164.0, 171.8, 177.7, 197.4; ESI-MS (*m/z*): 567 $[M+1]^+$. Anal. Calc. for $C_{36}H_{56}O_5$: C, 76.01; H, 9.92. Found: C, 76.13; H, 10.03.

3.1.4. High Performance Liquid Chromatography (HPLC)

3.1.4.1. Preparation of samples for HPLC analysis

A solution of 1 mg/ml PKBA was prepared in acetonitrile (HPLC grade) and filtered through 0.22 µm millipore filter.

3.1.4.2. Analysis of HPLC Profile

10 µl of each sample was analyzed using Waters HPLC at 30°C. The conditions used were as under:

Solvent	HPLC grade (Rankem, India)
HPLC Column	RP-18 (250 mm x 4.0, I.D. 5µm)
Mobile Phase	Acetonitrile: 0.5% acetic acid (95:5), Flow rate 1.0 ml / min.
UV Detection	271 nm

The HPLC chromatograms of PKBA and HKBA are shown in Fig 3.1& 3.2.

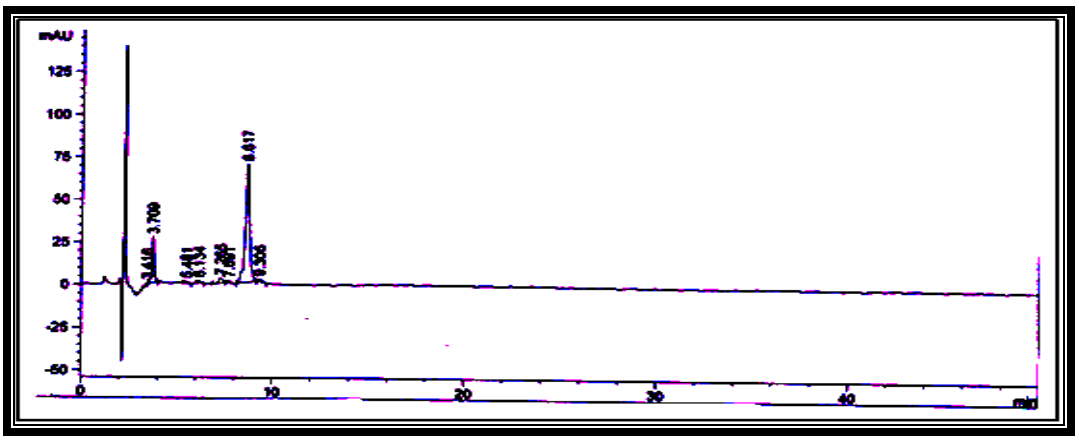


Fig.3.1. HPLC chromatogram of PKBA

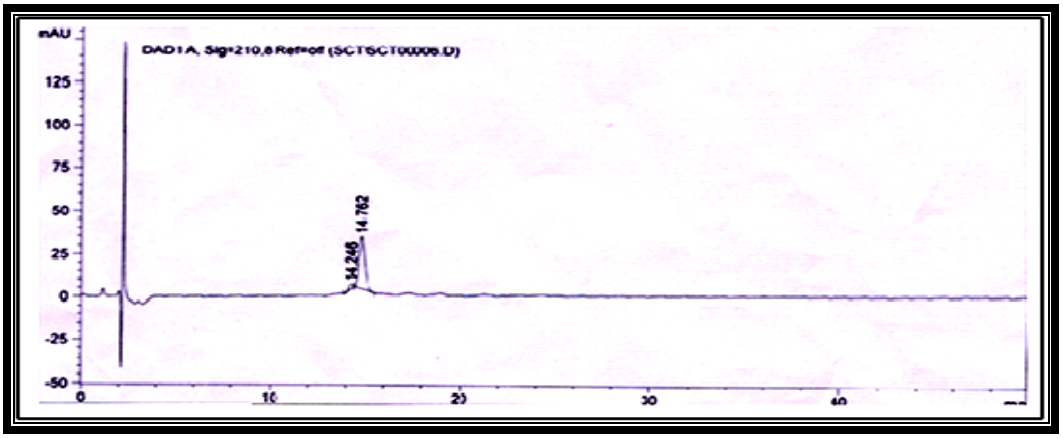


Fig.3.2. HPLC chromatogram of HKBA

3.2. Determination of *in vitro* cytotoxicity using human cancer cell lines

3.2.1. Chemicals

Roswell Park Memorial Institute medium (RPMI-1640), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), phosphate buffer saline (PBS), minimum essential medium (MEM), glacial acetic acid, sulphorhodamine Blue (SRB), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), mitomycin C, doxorubicin, 5-Fluorouracil, paclitaxel, trypsin and gentamycin were obtained from Sigma Aldrich, USA. Glacial acetic acid, isopropyl alcohol and trichloroacetic acid were procured from Sisco Research Laboratories, Mumbai. Ethylenediamine tetraacetic acid, disodium salt (EDTA) and tris buffer were from HiMedia Laboratories, Mumbai.

3.2.2. Apparatus

96-well flat bottom tissue culture plates (Iwaki, Cat No. 3860-096), cryovials (Nunc, Cat No. V 7384); 15 or 50 ml radiation sterilized centrifuge tubes (Tarsons Products Pvt. LTD, Cat No. 546020/546040); microcentrifuge tubes (Tarsons Products Pvt. LTD); micropipettes (Tarsons Products Pvt. LTD); sterile disposable syringes (10ml, 5ml, 2ml) (Dispovan, Hindustan Syringes and Medical Devices LTD); syringe driven filter unit (0.22μ) (Millipore, Cat no. SLGP033RS, Carrigtwohill, Ireland); micropipette-tips (Tarsons Products Pvt. LTD); pipette dispenser (Accu-jet$^{@}$ pro, Brand, Germany); Tissue culture flasks (T-25 – 75 & T-150 cm^2) (Nunc, Cat no. 156499, Denmark) and cryo 1^{o}C freezing container or cryobox (Mr. Frosty, Sigma Chem. Co, USA) were used for the study.

3.2.3. Instruments

Carbon dioxide incubator (Heraeus, Germany), centrifuge (Beckman,USA), deep freezer (Model 702, Thermofisher), filtration assembly, weighing balance (Sartorius, Germany) hemocytometer (Sigma Chem. Co., USA), mechanical shaker (IKA, Germany), microscope (Nikon, Japan), sunrise ELISA reader (Tecan, Switzerland), vertical laminar flow - clean air work station (Klenzaids), liquid nitrogen container (Thermo Electron Corporation, USA) were used in the current study.

3.2.4. Preparation of reagents required for cell culturing and cytotoxicity assays

3.2.4.1. Preparation of Media

3.2.4.1.1. Growth media

Growth medium: RPMI-1640 medium with 2 mM L-glutamine or MEM was prepared by dissolving the contents of the respective supplied vials in double distilled water as per the supplier's instructions followed by the addition of streptomycin (100 mg/liter) and sodium bicarbonate (1.2 gm/liter). The pH of medium was adjusted to 7.2 and sterilized by filtering through 0.2 μm filters in laminar flow hood under sterile conditions. The media was stored in refrigerator (2-8^{o}C).

Complete growth medium: For making complete growth medium, growth medium was supplemented with 10 % FCS and Penicillin (100 IU/ml, before use).

3.2.4.1.2. Freezing medium

For cryopreservation, freezing medium was prepared by addition of 20 % FCS and 10 % DMSO (cell culture grade) in growth medium in case of adherent cells. For suspension cells the freezing medium was prepared by addition of 95 % FCS & 5 % DMSO.

3.2.4.2. Phosphate buffer saline (PBS)

PBS was prepared by dissolving the contents of a vial of PBS in double distilled water, diluted up to 1 liter and filtered with 0.2 micron filter under sterile condition.

3.2.4.3. Trypsin-EDTA

50 mg Trypsin (0.05%) and 20 mg EDTA, disodium salt (0.02%) were dissolved in PBS, diluted upto 100 ml and filtered with 0.2 micron filter under sterile condition.

3.2.4.4. Trichloro acetic acid (TCA)

50% (w/v) TCA solution was prepared in double distilled water.

3.2.4.5. Acetic acid

Glacial acetic acid was diluted to 1% with double distilled water.

3.2.4.6. SRB Dye

SRB Dye was prepared by dissolving 4 g SRB (0.4%) in 1% acetic acid and diluted up to 1000 ml.

3.2.4.7. Tris-buffer

Tris-buffer (10 mM) was prepared by dissolving 1.21 g of Tris-base in 950 ml of distilled water, pH was adjusted to 10.5 and diluted upto 1 L.

3.2.5. Culturing of human cancer cell lines

3.2.5.1. Handling of cell line on arrival

After receiving the cells in cryovials from NCI, USA, these were transferred to liquid nitrogen container immediately and were used as per requirement. The cells received in Tissue Culture Flask from NCCS, Pune were observed under microscope under aseptic conditions. If the flask was found to contain healthy cells without contamination, the supernatant of the flask was transferred to centrifuge tube and after centrifugation the cells with required quantity of medium were placed back into the same flask. After 24 hour, cells were observed under microscope. At sub-confluent stage, the cells were subcultured as described later.

3.2.5.2. Revival of cell lines

Cryovials containing the cells were removed from the liquid nitrogen container and thawed quickly by shaking in water bath at 37^{o}C. Cryovials were wiped with 70% alcohol to avoid contamination and transferred to laminar flow. Contents of the vial were transferred into a sterile centrifuge tube containing 10 ml complete growth medium and centrifuged. Supernatant was discarded and the cells were suspended in fresh complete medium. Cells were mixed properly to ensure uniform distribution in the medium. Cells were transferred to the Tissue Culture Flask (T-25) containing 7 ml of complete growth medium aseptically and incubated in CO_2 incubator at 37^{o}C, 5 % CO_2 atmosphere and 90 % RH.

3.2.5.3. Method of cell culture of adherent cell lines

Most of the cell lines used in the current study is included in the panel recommended by NCI, USA (Monks *et al.,* 1991). The cell lines were grown in RPMI-1640 (or)

MEM growth medium depending on cell type as mentioned in the Table 3.1. Human cancer cell lines were grown in tissue culture flasks with complete growth medium at 37ºC in an atmosphere of 5 % CO_2 and 90 % RH in a carbon dioxide incubator. Cells were checked daily for their proper growth. The medium of the cells was changed when the color turned yellow from orange. To change the medium, the medium in the flask was aspirated with pipette-man and discarded. The fresh medium (5-7 ml in case of TCF-25) was placed in the culture flask under sterile conditions. The flasks were properly marked and incubated in CO_2 incubator. Depending on the mass doubling time of cells, sub-culturing of cells was done, when they were at sub-confluent stage. It is a stage of rapid growth of cells, here some of the space remains vacant between the growing cells. The cells are in log phase of their growth and can be used for experimental purposes as well as sub-culturing or cryopreservation.

3.2.5.4. Subculture of the cell lines

It involves detachment of the cells from the growth surface (substratum) of the culture flask and re-inoculation of the cells into fresh medium in new culture flask i.e. TCF-25, TCF-75 or TCF-150 (depending on the number of cells). The medium of the flask at sub-confluent growth was changed one day in advance. The entire medium from the flask was taken out and discarded. Cells were washed with PBS. The minimum quantity of Trypsin-EDTA (Pre warmed at 37°C) was added just enough to make a thin layer and incubated for approximately 5 minutes at 37°C. Complete growth medium was added to the flask after incubation to make a cell suspension. An aliquot of this suspension was taken out, cells were counted and checked for viability with trypan blue. Cell stock with more than 98% cell viability was accepted for determination of *in vitro* cytotoxicity. The cell density was adjusted to $1x10^6$ cells/ml by the addition of more complete growth medium and inoculated into fresh TCF-75 or TCF-150 and incubated in CO_2 incubator to continue the culture.

Tissue	Cell lines	Medium	No. of cells/well
Breast	MCF-7	MEM	15,000
Colon	SW-620	RPMI-1640	10,000
	Colo-205	RPMI-1640	15,000
	HCT-15	RPMI-1640	10,000
	HT-29	RPMI-1640	5,000
Ovary	OVCAR-5	RPMI-1640	20,000
Cervix	HeLa	MEM	10,000
	SiHa	RPMI-1640	10,000
CNS	SK-N-SH	MEM	15,000
	IMR-32	MEM	10,000
	SF-295	RPMI-1640	15,000
Lung	HOP-62	RPMI-1640	15,000
	A-549	RPMI-1640	10,000
Liver	Hep-2	MEM	10,000
Prostrate	DU-145	MEM	10,000
	PC-3	RPMI-1640	10,000

Table 3.1. List of human cancer cell lines, related tissues, medium and required cell density

3.2.5.5. Method of cell culture for suspension cell lines

Human suspension cancer cell lines HL-60 & Molt-4 were seeded and grown in tissue culture flasks in complete growth medium (RPMI-1640) at 37ºC in an atmosphere of 5% CO_2 and 90 % RH in a carbon dioxide incubator. Cells were observed daily for proper growth and maintenance. The medium of the flasks was replaced when the color turned yellow with the fresh complete growth medium (pre-warmed at 37^{o}C). For changing the medium, the exhausted medium was taken out from the flask and poured into 50 ml centrifuge tube aseptically and centrifuged at 1200 rpm for 10 minutes, the supernatant was discarded the pellet was resuspended in fresh RPMI-

1640 medium and again seeded into a new TCF-75 flask for continuation. The cell cultures were maintained for further storage and experiments. The flasks were marked properly and incubated at 37^{o}C & 5% CO_2 atmosphere in an incubator.

3.2.5.6. Cryopreservation of cell lines

In order to minimize the genetic drift, senescence or transformation in infinite cell lines and guard against accidental loss by contamination or otherwise, the common practice is to freeze (cryopreserve) aliquots of cells in liquid nitrogen vapours. For cryopreservation, cells are brought primarily to -80°C at a cooling rate of 1°C/min. The adherent cells at sub confluent stage in which medium was changed one day in advance were trypsinised. The cell suspension was made and centrifuged at low speed (500 g). Cells with more than 98% viability as determined by trypan blue exclusion technique were selected for cryopreservation. The pellet was suspended in freezing medium for cryopreservation and the cell density was adjusted to $1x10^8$ cells/ml. The aliquots of 1.0 ml were transferred into cryovials. The temperature of the vials was brought down to -80 ºC by using a specially designed box (Sigma Chem. Co, USA Cat no. C-1562), which is commercially available. The whole box was put into -80ºC refrigerator for 24 hours. Later, the vials were transferred into liquid nitrogen container.

In case of suspension cell lines, cells of the flask were harvested and cell suspension was centrifuged at low speed, 800 rpm for 10 minutes at temperature of 4^{o}C. The pellet so obtained was suspended in freezing medium containing 90% FCS and 10% DMSO and the cell density was adjusted to $1x10^8$ cells/ml. The cells were preserved following the same procedure as has been mentioned above for adherent cell lines.

3.2.6. Preparation of test material

(a) Stock solution: Dimethyl sulphoxide (DMSO) is often used as a solvent to dissolve the test materials as it is safe to the cells below 1% w/v, however, proves to be cytotoxic at high concentrations. Both PKBA and HKBA were dissolved in DMSO. A stock solution of 20 mg/ml of both the semisynthetic compounds was prepared.

(b) Working solution: The stock solutions (20 mg/ml) were serially diluted with complete growth medium containing 50 µg/ml of gentamycin to obtain working test

solutions of 100, 70, 50, 30, 20 & 10 µg/ml. The working test solutions were not filtered/ sterilized but microbial contamination was controlled by addition of gentamycin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

3.2.7. Preparation of Positive control

(a) Stock solution: A stock solution of $2x10^{-2}$M concentration of the positive control was prepared. The solvents used for Paclitaxel and Doxorubicin were DMSO, 5-Fluorouracil and Mitomycin C were dissolved in distilled water and stored at -20^{o}C.

(b) Working solution: The stock solutions were serially diluted to obtain working test solutions with complete growth medium containing 50 µg/ml of gentamycin to obtain working test solutions of $2x10^{-6}$ M or $2x10^{-5}$ M as per the requirement.

3.2.8. *In vitro* Cytotoxicity Assays

3.2.8.1. Cell lines

The following cell lines were used for evaluating the *in vitro* cytotoxicity of test compounds.

- **Breast** MCF-7
- **Neuroblastoma** IMR-32, SF-295
- **Colon** Colo-205, HCT-15
- **Lung** A549
- **Liver** Hep-2
- **Prostate** PC-3
- **Ovary** OVCAR-5
- **Leukemia** HL-60, MOLT-4, THP-1

Besides the above mentioned cancer cell lines, the test samples were also evaluated for their cytotoxicity effect against Normal Monkey Kidney **CV-1**cell line.

3.2.8.2. Cell cytotoxicity evaluation by SRB assay

SRB assay was a conventional method to assess the cytotoxicity of cells growing in microtiter plates. It is a sensitive, simple, reproducible, rapid, gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement**.** The assay is based on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B (SRB) by basic amino acids in the cells

in acidic medium. The greater the number of cells, the greater amount of dye is taken up and, after fixing, the released dye in alkaline medium will give a more intense color and greater absorbance (Skehan et al., 1990).

Cell lines at sub-confluent stage which were healthy with no signs of contamination were used for performing the *in vitro* cytotoxicity assay. Cells were harvested from the TCF after trypsinization and a single cell suspension was prepared. An aliquot of this suspension was taken and the cells were counted by using haemocytometer to measure the cell density of the original suspension. Depending upon the cell line, cell suspension was further diluted with appropriate growth medium to obtain the required cell density (Table 3.1).

SRB assay was performed in a 96-well cell culture plates. A 100 µl of cell suspension of desired density was added into each well of the plates and incubated (5% CO_2, 37^{o}C, 90 % RH) for 24 h. After 24 h of incubation, 100 µl of test samples were added in the wells containing cells and further incubated for 48 h. Suitable controls, blanks and positive controls were also included in each experiment. After incubation period, the cells were fixed with 50 µl of ice-cold TCA (50%) for 1 h at 4ºC followed by washing with distilled water and plates were allowed to dry in the air. After complete drying, 100 µl of SRB dye was added to each well of the plates and allowed to stain at room temperature for 30 min. The excess of the dye was removed by washing the plates five times with 1% acetic acid and allowed to dry in air. The adsorbed dye was solubilised by the addition of 100 µl of 10 mM Tris Buffer (pH 10.5) to each well and plates were shaked for 5 min on a shaker platform. The plates were read in a 96-well ELISA plate reader (Molecular Devices, Sunnydale, USA) at 540 nm. The flow chart below gives the outline of the assay (Fig 3.3).

Each sample was run in triplicate and the mean values of three similar experiments were used to obtain the result. The concentration of DMSO added to the cultures was maintained to < 1%.

3.2.8.2.1. Calculations

(a) Calculation of % Growth Inhibition of test sample

The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in the presence of test

material was calculated considering the growth in the absence of any test material as 100 % and in turn percent growth inhibition in the presence of test material was calculated.

% Cell growth = $\frac{\Delta \text{ OD in presence of test sample}}{\Delta \text{ OD in absence of test sample}} \times 100$

% Growth Inhibition by the Test sample = 100 - % Cell growth of test sample

(b) Calculation of IC_{50} value for a Test Sample:

For IC_{50} value determination the slope & intercept of Log (concentration of sample) Vs Log (response at that concentration) for three to five different concentrations of a sample is calculated.

$$X = \frac{Log_{50} - \text{Log Intercept}}{\text{Log Slope}}$$

$$\downarrow$$

IC_{50} value = Log (1/X)

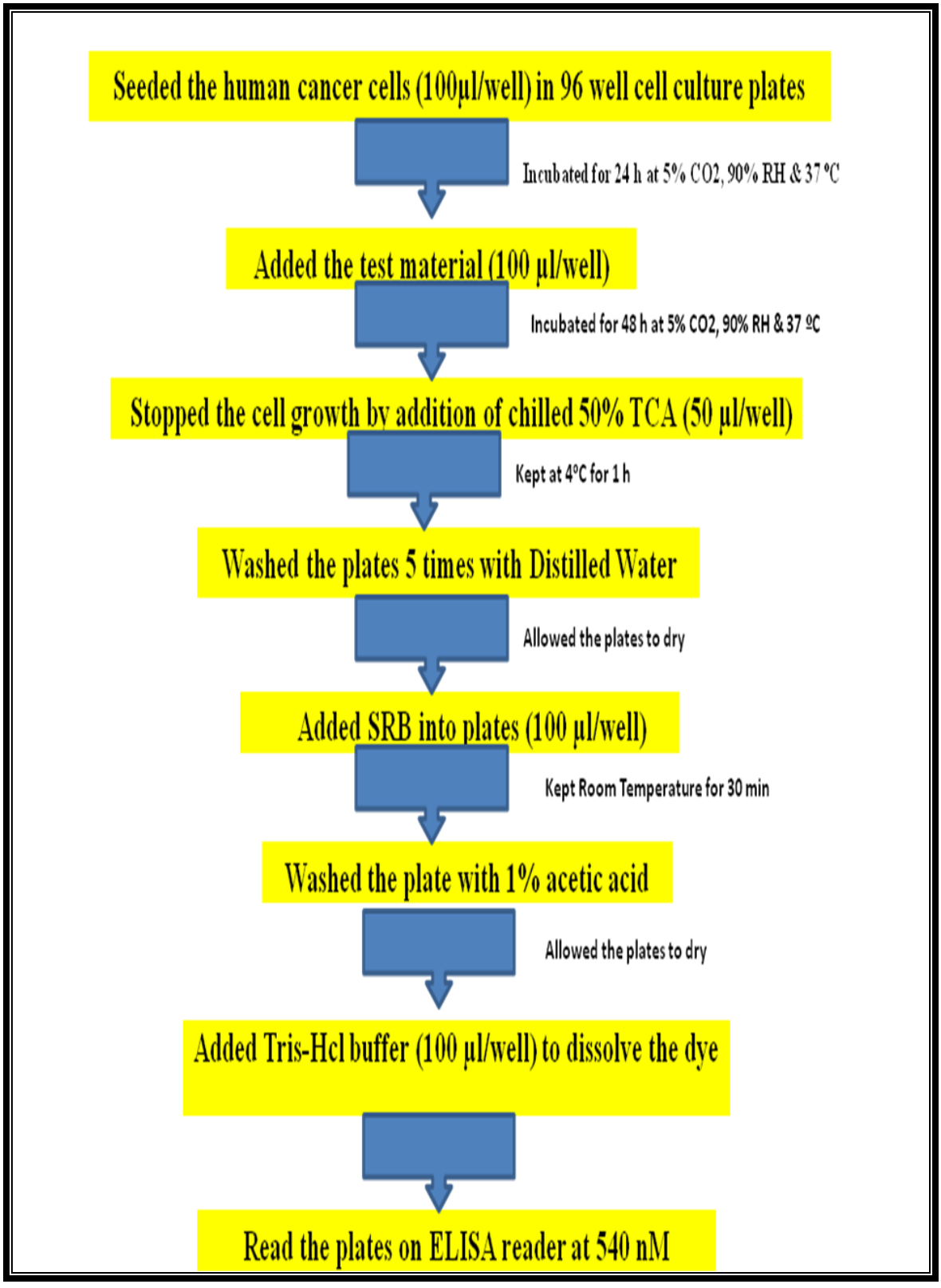


Fig. 3.3. Flowchart of *in vitro* cytotoxicity assay using SRB dye

3.2.8.3. Cell viability evaluation by MTT test

This method uses 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The assay works on the principle that when MTT is added to cells on a microtitre plate, the yellow coloured MTT is metabolically reduced by the mitochondria in viable cells to purple formazan. A solubilisation solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of the coloured solution can be quantified by measuring absorbance (usually between 500 and 600 nm) by a spectrophotometer. The maximum absorption is dependent on the solvent employed. Higher colour intensity indicates more cell viability & less cytotoxic effect of the drug sample which is tested.

MTT assay was performed in 96-well tissue culture plates. A 100 µl of cell suspension (of suspension cell lines, HL-60 & MOLT-4) of desired density was added into each well of the plates and incubated initially for 1 h (24 h in case of adherent cell lines). After incubation, 100 µl of test samples were added in the wells containing cells and further incubated for 48 h. Before 4 h of complete incubation (i.e. after 44 h) a 20 µl of MTT dye (2.5 mg/ml) was added to each well (50 µg/well) and incubated to further 4 h. Once the incubation period was complete the plates were centrifuged at 1500 rpm for 15 min, the supernatant was discarded and 150 µl of DMSO was added to each well. The plates were shaken on a plate shaker for 10 min and the optical density was determined using a microplate reader at 570 nm. IC_{50} values were calculated by plotting OD against concentration.

3.3. *In vivo* anticancer studies

The *in vivo* experiments were based upon the percent growth inhibition of ascitic and solid tumors (implanted in the mice) by the test sample in comparison to normal saline treated tumor bearing animals.

3.3.1. Tumor Models used in the current study

For evaluating the *in vivo* anticancer potential of test samples in the present study, the following murine tumor models were used (Fig. 3.4).

- Ehrlich Ascitic Carcinoma (Ascitic)
- Sarcoma-180 (Solid)
- Ehrlich Tumor (Solid)

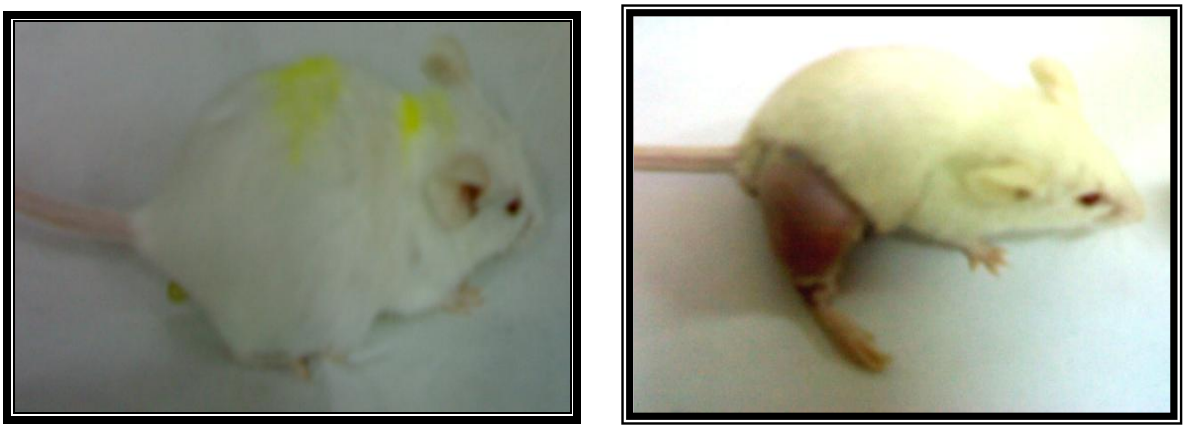


Fig. 3.4. Ascitic tumor bearing mice (a) and solid tumor bearing mice (b)

3.3.2. Animal Maintenance

Inbred BALB/c and non-inbred Swiss albino mice from in house colonies, maintained at anticancer vivarium of Indian Institute of Integrative Medicine, Jammu were used in the present study. The animals were housed in standard polycarbonate cages providing internationally recommended space for each animal. Animal room temperature and humidity were maintained at 23± 2 ^{o}C and 50 %, respectively. Animals were provided commercially available pelleted feed supplied by M/s Ashirwad Industries, Chandigrah (India) and autoclaved water was available *ad libitum*. Animals were cared as per the guide for the care and use of laboratory animals (1996), ILAR Washington DC. They were housed in controlled conditions of temperature (23 ± 2^{o}C), humidity (50-60 %) and 12:12 hr of light: Dark cycle. The

study and the number of animals used were approved by the Institutional Animal Ethics Committee, Indian Institute of Integrative Medicine, Jammu. The study was conducted as per the protocols of National Cancer Institute (NCI), USA. The experiments were carried on healthy mice and free from any disease. Mice were selected in the age range of 18-23 g and around 2 month's age. In order to ensure the uniformity of results, mice of same sex and single strain were used for the experiment.

3.3.3. Propagation of Ehrlich Ascitic Carcinoma (EAC)

Transplantation refers to the induction of cancer in animals by injecting cancer cells in their bodies that are taken from animals bearing 8-10 days old tumor for further propagation. The animals were injected $1x10^7$ cells intraperitoneally. Ehrlich ascites carcinoma (EAC) and Sarcoma-180 Ascites cells were maintained in the peritoneal cavity of animals of respective strains by transplanting them after every 8-10 days in fresh healthy animal. Approximately 2 ml of normal saline was taken in a syringe and about 1 ml was injected into the intraperitoneal cavity of animal from where the fluid was to be collected. The ascitic fluid was aspirated slowly and carefully from the animal. The required volume of fluid in the test tube was collected and the cells were mixed repeatedly and properly throughout the fluid by inverting the test tube several times. The number of cells in the fluid/ml was counted using hemocytometer/Neubauer's chamber. The number of cells in the ascitic fluid was adjusted approximately to $5x10^7$ cells/ml and 0.2 ml of fluid containing $1x10^7$ cells were injected intraperitoneally into naive animals in which transplantation has to be carried out.

3.3.4. Preparation of test material

Two different doses of PKBA (100 & 150 mg/kg i.p.) and HKBA (50 & 60 mg/kg i.p.) were selected for the evaluation of *in vivo* anticancer activity. The total duration of treatment of all experiments was 9 days. The test material was prepared on day 1 for four days and on day 5 for next five days according to the average body weight of the treatment groups. The calculation of the amount of test material was carried out by the following formulae.

W= [Average b.wt. of test group (g) x No. of animals x No. of days x dose of test material (mg)] / 1000

Where 'W' is the weight of test material in mg.

The calculated amount of test material was dissolved in gum acacia. The solution of test material was titurated in a pestle-mortar and final volume was made with normal saline.

3.3.5. Positive control and Normal control

Positive control is essential to use for comparison of the activity of the test material. 5-Fluorouracil was used as a positive control in case of both ascitic and solid tumors at a dose of 20 and 22 mg/kg respectively. The dose of positive control is adjusted as per the tumor as well as the mortality inhibition so that an appreciable effect is obtained without death of animal in the experiment. The normal saline (0.85 % w/v) was administered to tumor bearing animals being referred to as normal control.

3.3.6. Experimentation

Test materials were evaluated for their *in vivo* anticancer activity against ascites and solid murine tumor models as per the protocol described by Geran et al., (1972). The protocol involves the following steps:

3.3.6.1. Induction of Ascites Tumor

On day 0 the induction of tumor in the mice is done. For tumor induction the peritoneal fluid was collected from an animal bearing 8-10 days old Ehrlich ascitic carcinoma and the number of tumor cells per ml of ascitic fluid was determined by counting the cells with the help of hemocytometer. The ascitic fluid was diluted with normal saline in such a way that 0.2 ml of fluid contained $1x10^7$ EAC cells. All animals selected for conducting the experiment were injected intraperitoneally with 0.2 ml of ascitic fluid containing $1x10^7$ EAC cells.

3.3.6.1.1. Treatment

Day 1: Mice were randomized and divided in different treatment and control groups. Normal control group contained 10 animals and all other groups (treatment and positive control) contained 7 animals each. Based on the average body weight of each group, test materials were prepared for four days in gum acacia in such a way that each dose was contained in 0.2 ml volume. Test drugs were administered from days 1-4.

Day 5: Animals in each group were again weighed and based on the average body weight for each group, suspensions of test drugs were prepared in gum acacia for the next 5 days. The treatment continued upto 9^{th} day.

Day 9: The body weight of each individual mice of each group was recorded.

Day 12: The body weight of each individual mice of each group was recorded. All mice were then sacrificed by cervical dislocation. The Ascitic fluid from each animal was collected in a clean, dry, pre-weighed graduated centrifuge tube with the help of a glass funnel.

3.3.6.1.2. Evaluation and Calculation of % tumor growth inhibition

Ascitic fluid volume (ml) and ascitic fluid weight (g) of each tube was recorded. The total number of tumor cells in ascitic fluid was counted with the help of hemocytometer. The percent tumor growth inhibition with respect to the total number of tumor cells in ascitic fluid was calculated as follows.

Percent tumor growth inhibition =

$$\frac{\text{Av. no. of cells in control group} - \text{Av. no. of cells in treated group}}{\text{Av. no. of cells in control group}} \times 100$$

3.3.6.2. Outline of protocol of Ascites model

Propagation:

Animals	Swiss albino or Balb/c mice
Weight	20 $\pm$3 g
Age	2-3 months
Sex	Same sex for all experimental and control groups.
No. of animals per test group	7
No. of animals per control group	10
Schedule of drug administration	Daily from day 1 – 9

Day 0	Inject 10^7 cells (i.p) from 8-12 days old tumor bearing mice
Day 1	Divide into test and control groups and weigh on day 1, prepare test drug for four days
Day 1-4	Treatment with test material.

Day 5 Weigh individual mouse of each group and prepare test material for next 5 days

Days 5-9 Treatment continued up to 9^{th} day.
Prepare drug for the next five days.

Day 12 Sacrifice mice and collect ascitic fluid

Evaluation On the basis of tumor volume, weight and cell count.

Parameter of effect for acceptable control on day 12

S. No.	Parameters	**Acceptable Control (12^{th} day)**
1.	Av. Number of tumor cells	40×10^7
2	Av. Volume of tumor	3 ml
3.	Av. Weight of tumor	2 g
4.	Excessive 'No takes' in control	≤ 3

Percent inhibition of tumor:

$$\frac{\text{Av. no. of cells in Controls} - \text{Av. no. Of cells in treated groups}}{\text{Av. no. Of cells in Controls}} \times 100$$

3.3.6.3. Induction of Solid tumor

Peritoneal fluid (Ehrlich Ascites Carcinoma or Sarcoma-180 Ascites) was collected aseptically either from Swiss albino or Balb/c mice bearing 8-10 days old ascitic tumor $1x10^7$cells contained in 0.2 ml of ascitic fluid were injected intramuscularly into the right thigh of each mouse on day 0.

3.3.6.3.1. Treatment

Day 1: The mice were randomized and divided in different treatment and control groups. Normal control group contained 10 animals and all other groups (treatment and positive control) contained 7 animals each. Based on the average body weight of each group, test drugs were prepared for four days in 1 % gum acacia in such a way that each dose was contained in 0.2 ml volume. Test drugs were administered from day 1-4.

Day 5: Animals in each group were again weighed and based on the average body weight for each group, suspensions of test drugs were prepared in 1 % gum acacia for the next 5 days. The treatment continued upto 9^{th} day.

Tumor bearing thigh of each mice was shaved with hair remover cream. The body weight of each group was recorded. Longest (length) and shortest (width) diameters of the tumor were measured with the help of vernier caliper to determine the weight of the tumor.

Day 9 &13: Body weight and tumor dimensions were again recorded

3.3.6.3.2. Evaluation and Calculation

Shortest and largest diameters of the tumor were measured with the help of vernier caliper and the weight of tumor was calculated as follows.

$$\text{Tumor Weight (mg)} = \frac{\text{length (mm)} \times [\text{width (mm)}]^2}{2}$$

Percent inhibition of tumor =

$$\frac{\text{Av. Tumor wt. of Controls} - \text{Av. Tumor wt. of treated animal}}{\text{Av. Tumor wt. of controls}} \times 100$$

3.3.6.4. Outline of protocol of Solid Tumor model

Animals	Swiss albino or Balb/c mice
Weight	20 $\pm$3 g
Age	2-3 months
Sex	Same sex for all experimental and control groups.
No. of animals per test group	7
No. of animals per control group	10
Schedule of drug administration	Daily from day 1 – 9

Day 0	Inject 10^7 cells (i.p) from 8-12 days old tumor bearing mice
Day 1	Divide into test and control groups and weigh on day 1, prepare test drug for four days
Day 1-4	Treatment with test material.
Day 5	Weigh individual mouse of each group and prepare test material of next 5 days
Days 5-9	Treatment continued up to 9^{th} day. Measure tumor dimensions (longest & shortest diameter) of individual mouse of each group on 5^{th} and 9^{th} day

Day 13 Weigh individual mouse and measure tumor dimension of each group

Evaluation On the basis of tumor weight.

Parameter of effect for acceptable control on day 13

S. No.	Parameters	**Acceptable Control (13th day)**
1.	Av. Weight of tumor	2 g (1.5-3.0)
2.	Excessive 'No takes' (≤39 mg) in control	≤ 3

$$\text{Tumor weight (mg)} = \frac{\text{Length (mm) x [width (mm)]}^2}{2}$$

Percent inhibition of tumor =

$$\frac{\text{Av. Tumor wt. of Controls} - \text{Av. Tumor wt. of treated animals}}{\text{Av. Tumor wt. of controls}} \times 100$$

3.4. Apoptotic assays

3.4.1. Cell line used for apoptotic assays

Human leukemia suspension cell line HL-60 was used for studying apoptosis.

3.4.2. Preparation of cell suspension

Healthy cells at the log phase, having no signs of contamination were used for apoptotic studies. Medium of the cells was changed one day in advance. Cells were centrifuged at 1200 rpm and the pellet was re-suspended in fresh medium. An aliquot of cells was taken out for counting and viability determined with trypan blue. Cell stock with more than 98% cell viability was accepted for the mechanistic studies. The cell density was adjusted as per the requirement from $1x10^6$-$1x10^7$ cells/ml by the addition of complete growth medium and was seeded in 6 well tissue culture plate and incubated in CO_2 incubator followed by treatment with desired test compound for desired incubation period.

3.4.3. Determination of nuclear morphology

DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes and can be used for living as well as fixed cells. DAPI staining helps in differentiating normal cells over apoptotic cells. It has been found that, in contrast to normal cells, the nuclei of apoptotic cells contain highly condensed chromatin material which when stained with DAPI, a DNA binding dye represents the entire nucleus as a single or a group of featureless, bright spherical beads and these morphological changes can be visualized by fluorescence microscopy.

HL-60 cells ($2x10^5$/2 ml/well) were seeded in 6-well plates and treated with test compounds for 18 & 24 h. Cells were collected, washed with PBS and smears were prepared. For fluorescent 4'-6-Diamidino-2-phenylindole (DAPI) staining, air dried slides were fixed in methanol at -20^{o}C for 20 min, air dried and stained with 1 µg/ml DAPI (Sigma) for 20 min in the dark, mounted in glycerol PBS (9:1) and observed under inverted fluorescence microscope (Olympus 1×70) (Shougang et al., 2000).

3.4.4. Determination of morphological changes

Light and electron microscopy have been decisive tools to identify the specific type of cell death definitely and so far has been the gold standard for the most precise

detection of apoptosis based on the original morphological criteria described by (Wyllie et al., 1980). However, some recent studies have advocated the use of SEM for the accurate assessment of mechanism of cell death in human cancer cells (Rello et al., 2005).

HL-60 cells were seeded in a 6 well plate and then treated with the test compounds at various concentrations along with the control. Cells were harvested, washed twice with PBS and the pellet was collected. Cells were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 1 h at 4ºC on cover slips and post-fixed with osmium tetraoxide (OsO_4) for 1h in the same buffer, then dehydrated with acetone, dried in a Critical Point Dryer using CO_2 and coated with gold using a Sputter Coater (Rello et al., 2005). The specimens were examined with a JEOL-100CXII Electron Microscope (EM) with ASID at 40 KV. The photography was done using a 120 Roll film.

3.4.5. Determination of DNA Fragmentation by DNA ladder assay

The presence of oligonucleosome fragmentation of DNA can demonstrate apoptotic cell after treatment with test compound.

DNA fragmentation was determined by electrophoresis of extracted genomic DNA from leukemia HL-60 cells. Briefly, exponentially growing cells ($2x10^6$cells/ml) in a 6 well plate were treated with test compound at different concentration for 24 h. Cells were harvested, washed with PBS, pellets were dissolved in lysis buffer [10 mM EDTA, 50 mM Tris pH 8.0, 0.5% w/v SDS and proteinase K (0.5 mg/ml)] and incubated at 50 ºC for 1 hr. Lysate was further incubated with RNase A (0.5 mg/ml) at 50ºC for 1 hr. Additionally, phenol: chloroform: isoamylalcohol (25:24:1) were added into each tube to remove proteins and other wastes from DNA solution by centrifugation at 14,000 rpm for 15 min. DNA fragments could be dissolved in upper supernatant which were collected and stored at 4^oC before gel electrophoresis.

Solution of DNA fragments was mixed with loading dye into suitable ratio and dropped into each well of 1.5 % agarose gel (pre stained with EtBr) and electrophoresis was carried out at 100 V for 2-3 hrs.

3.4.6. Determination of anti-topoisomerase activity

Eukaryotic topoisomerases I and II are the targets of an increasing number of anti-cancer drugs that act to inhibit these enzymes by blocking the reaction that reseals the breaks in the DNA. Often the binding of the drug is reversible, but if a replication fork runs into the blocked topoisomerase, then a piece of the gapped DNA strand not bound by the topoisomerase could be released, creating a permanent breakage in the DNA that leads to cell death.

Supercoiled PryG DNA was incubated with test compounds and four units of human topoisomerase I & II separately (TOPO GEN), in relaxation buffer containing 10 mM Tris-HCl, pH 7.9, 10 mM EDTA, 1.5 mM NaCl, 0.1mg/ml bovine serum albumin, 1mM Spermidine and 50% glycerol. Camptothecin (100 µM) and Etoposide (100 µM) were used as a positive controls for topoisomerase I & II respectively. Each reaction volume was made upto 20 µl with H_2O, incubated at 37°C for 30 min and stopped by the addition of SDS to a final concentration of 1% and was treated further with proteinase K (0.5 mg/ml) and incubated at 37^oC for 15 min. Products were resolved by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) and stained with 0.5 µg/ml ethidium bromide (EtBr) for 15 min and destained with distilled water for 15 min at room temperature (Mark et al., 1985).

3.4.7. Determination of DNA damage by Comet assay

Comet assay measures, double as well as single strand breaks, alkali labile sites, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-Drug cross linkage and DNA repair. The principle of the assay rests on strand breakage of the supercoiled double helical DNA which leads to the reduction in the size of large molecules where in strands of the DNA can be stretched out by electrophoresis. Similarly, under highly alkaline conditions there is denaturation, unwinding of the double strand and the expression of alkali labile sites as single strand breaks. Comets form when the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode. DNA migration is a function of both the size and the number of broken ends of the DNA. Due to the damage, the Tail increases in length and reaches its maximum which is dependent on the electrophoretic conditions and not on the size of the fragments (Laiq et al., 2007). In order to quantify the DNA damage

induced by test compounds, comet assay was performed by the method given in the literature (Singh et al., 1988).

Exponentially growing HL-60 cells ($2x10^6$cells/ml/well) were treated with different concentrations of test compounds and incubated for 24 h. The cells were harvested and washed twice with PBS and its 50 µl aliquot was embedded in 450 µl of warm (45^oC) low melting point agarose (0.75%). The resulting mixture was spreaded over precoated microscopic slides (0.1% agarose routine). The gel was covered with glass cover slip and left to set at 4^oC for 5-10 min. Gel embedded cells were lysed in lysing buffer (2.5M NaCl, 100 mM disodium EDTA,10 mM Trizma base, 8g/l NaOH, pH10) for 20 min at 4^oC to allow DNA unwinding. Electrophoresis was performed at 400 mA and 50 V for 20 min. The slides were stained with 20 µg/ml ethidium bromide and examined under an Olympus fluorescence microscope (IX 41) equipped with an excitation filter (BP 510nm) and a barrier filter (590nm). Slides were analyzed using computerized imaging analysis system (KOMET 5.5). To evaluate the amount of DNA damage, computer generated tail moment values were used. Approximately hundred cells were used to access the DNA damage by (a) olive tail moment (b) tail length (c) tail coefficient variance.

3.4.8. Determination of Phosphatidylserine by Annexin V-FITC assay

The appearance of phosphatidylserine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell is an important parameter, used to detect and measure apoptosis. The presence of PS on the cell surface creates one of the specific signals for recognition and removal of apoptotic cells by macrophages. These PS changes can be detected with the anticoagulant, Annexin V, which has a high affinity for binding to PS. Annexins are a structurally conserved family of proteins characterized by reversible Ca^{2+} dependent membrane binding to negatively charged phospholipids like PS and shows minimal binding to phosphatidylcholine and sphingomyeline. By conjugating FITC to Annexin V it is possible to identify and quantitate apoptotic cells on a single-cell basis by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) allows (bivariate analysis) the discrimination of

intact cells (FITC '-' and PI '-'), early apoptotic (FITC '+' and PI '-') and late apoptotic or necrotic cells (FITC '+' and also PI '+').

Exponentially growing HL-60 cells ($2x10^6$/ml/well) were treated with different concentrations of test compound for 24 h, washed with PBS and stained with annexin V-FITC antibody and PI as per the instructions given by the manufacturer (BD Apoalert 630109). The cells were scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels. The fractions of cell population in different quadrants were analyzed using quadrant statistics. Cells in the lower right quadrant represented apoptosis while cells in the upper right quadrant represented necrosis or post apoptotic necrosis (Shashi Bhushan et al., 2007).

3.4.9. Determination of DNA content on Cell cycle analysis

Measurement of DNA content in the apoptotic cells can be identified through cell cycle phase specificity. Most commonly used dye for DNA content/cell cycle analysis is Propidium iodide (PI). PI intercalates into the major groove of double stranded DNA & produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600nm. Since PI can also intercalate to double stranded RNA, it is necessary to treat cells with RNase for optimal DNA resolution. PI binds to DNA in cells at all stages of the cell cycle and the intensity with which a cell nucleus emits fluorescent light is directly proportional to its DNA content. Apoptotic cells unlike necrotic ones, have reduced DNA stainability following staining with a variety of fluorochromes. Therefore, the presence of cells with DNA stainability lower than that of G_1 cells (hypodiploid or sub-G_1 peaks) has been considered a marker of cell death by apoptosis. The reduced stainability of apoptotic cells is the direct consequence of partial loss of DNA due to the activation of endogenous nucleases and diffusion of low molecular weight DNA outside the cells.

HL-60 cells ($2x10^6$/ml) were treated with test compound for 24 h at different concentrations and washed twice with ice-cold PBS, harvested, fixed in cold 70% alcohol in PBS and stored at -20°C for 30 min. After fixation, the cells were incubated with RNase A (0.1 mg/ml) at 37°C for 30 min, stained with propidium iodide (50 µg/ml) for 30 min on ice in dark (Waxman and Schwartz, 2003) and then measured for nuclear DNA content using BD-LSR Flowcytometer (Becton Dickinson, USA)

equipped with electronic doublet discrimination capability using blue (488 nm) excitation from argon laser. The fluorescence intensity of sub-G_1 cell fraction represented the apoptotic cell population.

3.4.10. Determination of Mitochondrial Membrane Potential

Mitochondria are supposed to act as central coordinators of cell death that get affected in the early apoptotic process. Rhodamine-123, a fluorescence probe which selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria, whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential (Lemasters et al., 1993).

Exponentially growing HL-60 cells ($1x10^6$/ml/well) were treated with test compound for 24 h. Rhodamine-123 (200 nM) was added 1h before the termination of the experiment. Cells were collected, washed in PBS and incubated with propidium iodide (5 µg/ml) for 15 min (Desagher et al., 1999). The decrease in fluorescence intensity due to the loss of mitochondrial membrane potential was analyzed in FL-1 channel.

3.4.11. Determination of intracellular caspase activity

Caspases represent the family of cysteinyl endopeptidases, which cleave their substrate at specific aspartic acid residues. The caspase colorimetric assays are based on the principle of hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspases, resulting in the release of the p-nitroaniline (pNA) moiety which has a high absorbance at 405 nm. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions. p-nitroaniline (pNA) is released from the substrate upon cleavage by a caspase. Free pNA produces a yellow color that is monitored on a spectrophotometer.

The activation of intracellular caspases was measured by caspase colorimetric kits from R&D system USA. HL-60 cells (2×10^6/ ml/well) were incubated with test compounds for different time intervals at 37 °C in a 5 % CO_2 atmosphere. Cells were collected, washed in PBS and lysed in lysis buffer (25 µl of cold lysis buffer per 1 x 10^6 cells). Activities of caspases-1, -2, - 3, -6, -8, -9 & -10 in cell lysate were

determined as per the instructions of manufacturer. Camptothecin (5 µM) was used as reference compound.

3.4.12. Immunoblotting

3.4.12.1. Protein Estimation

Protein estimation was done by Quantipro BCA assay kit from sigma. The principle of the Bicinchoninic acid (BCA) assay relies on the formation of Cu^{2+}- protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{+}. The amount of reduction is proportional to protein present. BCA forms a purple-blue complex with Cu^{1+} in alkaline environment.

The assay was performed in a 96 well microtittre plate. A required amount of Quantipro working reagent was prepared. Protein standards in the range of 0.5 to 30 µg/ml were prepared. 1 ml of the Quantipro working reagent was added to 1 ml of each protein standard, blank and unknown (lysate of test compound treated HL-60 cells) and mixed thoroughly by vortexing. Each well received a respective volume of 300 µl of the standard, blank and unknown protein. The plate was incubated at 37 ^{o}C for 2 h. The plate was allowed to cool down at room temperature and within 10 min the absorbance was read at 562 nm. The protein concentration was determined by the absorbance of the unknown samples to the standard curve prepared using the protein standards.

3.4.12.2. Preparation of cytosolic and mitochondrial lysates of HL-60 cells

Different pro and anti-apoptotic factors have been found to play a vital role in the process of programmed cell death. The extent of expression of these factors is important in regulation of apoptosis and is studied *via* immunoblotting assays. Certain factors from the intermembrane space of mitochondria are released into the cytosol where they form part of activation complexes for caspases (e. g. cytochrome c, Smac) or they translocate into the nucleus where they participate in DNA fragmentation. To detect the occurrence of certain proteins in the cytoplasm the separation of cytosolic and mitochondrial fraction is necessary.

HL-60 cells were collected after treatment and washed twice with PBS. Cytosolic fractions were obtained by selective plasma membrane permeabilization with digitonin (Wang et al., 2002). Briefly, 3 x10^{6} cells were lysed for 2 min in 200 µl of

lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA and 350 µg/ml digitonin).The digitonin treated cells were centrifuged at 12,000g for 1 min. The supernatant from each sample was mixed with an equal volume of 2X gel-loading sample buffer for Western blot analysis. For mitochondrial proteins expression, the above remaining pellets were dissolved in ice-cold 200 µl of lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 137 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate and Protease inhibitor cocktail: 1µl/10^6 cells, and incubated on ice for 30 min. After centrifugation at 12,000x g for 10 min at 4°C, the cell lysates were transferred to fresh tubes and stored at -80°C for immunoblotting of proteins. The protein contents were determined by BCA protein assay kit from Sigma and aliquots normalized to equal quantities before loading.

3.4.12.3. Preparation of total cell lysate

After treatment with test compound HL-60 cells ($3x10^6$) were harvested and resuspended in 0.2 ml of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 30 mM Na_2HPO_4, 50 mM NaF, 0.5 mM $NaVO_4$, 2 mM phenylmethylsulfonyl fluoride, and 10% protease cocktail inhibitor). Cells were incubated on ice for 30 min, vortexed and centrifuged at 12000xg for 15 min. Supernatants were collected and stored at - 80^{o}C (Han et al., 2004**)**. The protein contents were determined by BCA protein assay kit from Sigma and aliquots normalized to equal quantities before loading.

3.4.12.4. Preparation of **nuclear extracts**

HL-60 cells ($1x10^7$ /10 ml/100 mm plate) were incubated with test molecules for the desired time periods. At the end of treatment cells are collected and washed twice with PBS (100xg, 5 min, 4^{o}C) and suspended in 400 µl ice cold hypotonic buffer for 10 min on ice. Suspension was vortexed and centrifuged at 15000xg for 30 sec at 4 °C. The supernatant was discarded and the nuclear pellet gently resuspended in 100 µl of ice cold saline buffer on ice for 20 min. Cells suspension was vortexed and centrifuged at 15000xg for 5 min at 4°C. The supernatant was stored at -70^{0}C as nuclear lysate and the protein contents were assayed (Shina et al., 2001). The protein

contents were determined by BCA protein assay kit from Sigma and aliquots normalized to equal quantities before loading.

3.4.12.5. Protein separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was performed for protein separation. This technique allows the electrophoretic separation of denaturated proteins according to their size. Sodium dodecyl sulfate (SDS), a highly negative charged detergent, solubilises proteins and leads to a constant net charge per mass unit. Hence, SDS-polypeptide complexes migrate toward the anode through the polyacrylamide gel according to their molecular weight. In addition, the differences in molecular shape are compensated by the loss of the tertiary and secondary structures because of the disruption of the hydrogen bonds and unfolding of the molecules. By addition of a reducing agent like dithiothreitol (DTT) disulfide bonds are cleaved and proteins are totally unfolded. The molecular weight of the investigated proteins is estimated by applying molecular weight standards (Chemichrome STM western control, Sigma). Discontinuous gel electrophoresis is commonly used. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) the following chemicals, reagents and apparatus are required:

3.4.12.6. Preparation of Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, the following reagents were prepared

(a) Acrylamide Stock Solution (30% w/v) : 100 ml of acrylamide solution was prepared by mixing 0.8 g of bisacrylamide and 29.2 g of acrylamide in double distilled water.

(b) Sample Buffer: Sample buffer was prepared by mixing the following components

- SDS (5.0 ml)
- 0.05M Tris-HCl, pH 6.8 (2.5 ml)
- Double distilled water (5.0 ml)
- Glycerol (2.5 ml)
- 2- Mercaptoethanol (0.25 ml)

- 5 % Bromophenol blue (0.2 ml)

(c) Separating gel buffer: Separating gel buffer was prepared by mixing 18. 3 g of 1M 2-Amino-2-(hydroxymethyl) propane-1, 3-diol (2-Amino-2-(hydroxymethyl) propane-1, 3-diol (Tris base)) and 24 ml of 1N HCl in double distilled water. The pH of the buffer was adjusted to 8.8.The final volume of the buffer was made upto 50 ml.

(d) Stacking gel buffer: The stacking gel buffer was prepared by dissolving 1M 2-Amino-2-(hydroxymethyl) propane-1, 3-diol (2-Amino-2-(hydroxymethyl) propane-1, 3-diol (Tris base) (6.05 g) in double distilled water and the pH was adjusted to 6.8 using dil. HCl. The final volume of the buffer was made to 50 ml.

(e) Running Buffer, pH 8.3: For preparing running buffer, the following reagents were used.

- 0.02M 2-Amino-2-(hydroxymethyl) propane-1, 3-diol (2-Amino-2-(hydroxymethyl) propane-1, 3-diol (Tris base) (1.21 g)
- 0.25M Glycine (9.4 g)
- 0.1% SDS (0.5g)

The pH of the buffer was adjusted to 8.3 and the final volume was made upto 500 ml using double distilled water.

(f) 10% (w/v) SDS: 10 % SDS was prepared in double distilled water

(g) Ammonium persulfate: 10 % Ammonium persulfate was freshly prepared in double distilled water

(h) N, N, N', N' tetramethylethylenediamine (TEMED). TEMED was directly added to gel mixtures.

(i) 10 % Separating gel : 5 ml of separating gel was prepared by using following components

- Double distilled water (1.9 ml)
- Acrylamide Stock solution (1.7 ml)
- Separating gel buffer (1.3 ml)
- 10% (w/v) SDS (50 µl)
- 10% (w/v) APS (50 µl)
- TEMED (5 µL)

(j) 4 % Stacking gel: 2ml of 4 % stacking gel mixture was prepared by mixing the following reagents

- Double distilled water (1.4 ml)
- Acrylamide Stock solution (0.33 ml)
- Separating gel buffer (0.25 ml)
- 10% (w/v) SDS (20 µl)
- 10% (w/v) APS (20 µl)
- TEMED (2 µL)

(k) Staining Solution: 500 ml of staining solution was prepared using following reagents

- Coomassie brilliant blue (0.55g)
- Methanol (250 ml)
- Glacial acetic acid (50 ml)

The final volume was made upto 500 ml using double distilled water.

(l) Destaining Solution: Destaining solution was prepared by dissolving 70 ml of methanol and 7 ml of glacial acetic acid in double distilled water and the final volume was made upto 1 litre.

(m) Transfer buffer: 2 litres of transfer buffer was prepared by mixing the following reagents

- 192 mM Glycine (28.82 g)
- 25 mM 2-Amino-2-(hydroxymethyl) propane-1, 3-diol (2-Amino-2-(hydroxymethyl) propane-1, 3-diol (Tris base)) (6.06 g)
- 15 % Methanol (300 ml)
- 0.2 % SDS (2 g)

The pH of the buffer was adjusted to 8.3 and the final volume was made upto 2 L using double distilled water.

3.4.12.7. Procedure of SDS PAGE

The plates of desired thickness (1.0 or 1.5 cm) were fixed into the plate fixing assembly (Bio Rad, Mini-PROTEN-3 Electrophoresis Module, cat #165-3002). The separating gel solution (10 %) was added in between the plates and the upper layer of

gel solution was overloaded with the water saturated n-butanol and kept for 45 min and meanwhile the stacking gel (5%) solution was prepared. A stacking gel that allows protein concentration in the gel was overlaid on to a separation gel which allows protein separation. Percentage of acrylamide/ bisacrylamide gels was chosen according to the expected molecular weight of the protein of interest. The upper layer of n-butanol was washed out and rinsed properly with water. The stacking gel solution was then added, immediately the gel comb (1or 1.5 cm) was fixed and kept for 30 min so that the gel gets polymerized. After 30 min the comb was taken out gently and the plates were fixed into SDS-PAGE electrophoresis assembly followed by addition of gel running buffer. Electrophoresis was carried out in a vertical apparatus Mini Protean II (Bio Rad, CA, USA). Two gel runs were performed in parallel.

3.4.12.8. Western Blot Analysis

For immunoblotting, the protein lysates along with the standard protein marker were subjected to discontinuous SDS-PAGE analysis. Proteins aliquots (50 µg) were resolved on SDS-PAGE run at 60 V (Power PacTM HC High current power supply, Bio Rad) for 3 h. The resolved proteins were electro transferred to polyvinylidene difluoride (PVDF) membranes (Bio-RAD) in to Western blotting transfer frames in the following order: Sponge-blotting paper - gel - PVDF membrane - western blotting paper - sponge. The transfer was allowed overnight at 4°C at 30 V in transfer buffer. Non-specific bindings of the membrane were blocked by incubation with 5 % non-fat milk in Tris-buffered saline ((10 mM Tris-HCl, 150 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were probed with respective primary anti-mouse antibodies for 2 h (1:1000 dilutions) and washed three times with TBST. The blots were then incubated with horseradish peroxidase conjugated respective secondary antibodies for 1 h (1:1000 dilution), washed again three times with TBST. PVDF membrane was incubated into ECL Pus western blot detection reagent (ECL kit, Amersham Biosciences) for 5 min on a transparency sheet, in dark. PVDF membrane was placed in to the Hyper Cassette and superimposed with high performance chemiluminescence film in the dark room for 2 min and the protein signal on to the high performance chemiluminescence's X-ray film was developed by using developer and the signal was fixed by processing chemical fixer. The film was

washed out with tap water gently and air dried. The density of the bands was arbitrarily quantified using Quantity One software of Bio-RAD gel documentation system.

3.5. Determination of anti- angiogenic activity: Ang-2 & MMP-2 inhibition

Both Ang2 and Ang1 act as ligands of the endothelial cell (EC)-specific tyrosine kinase receptor, Tie2. Through binding to Tie-2, Ang-1 promotes interactions between ECs and peri-ECs to stabilize the established vasculature. Ang 2 modulates Ang1-mediated vessel stabilization by competitively inhibiting the binding of Ang-1 to Tie-2. Ang-2 promotes tumor cell invasion and stimulates the activation of MMP-2. Effect of test compound on pro-angiogenic factors Ang-2 and MMP-2 was measured using ELISA based kits from R&D system. HL-60 cells (2×10^6 cells/ml/well) in 6-well plates were incubated with test compound at different concentrations in 6-well plate for 3, 6, 12 and 24 h, using camptothecin (5 µM) as a reference. The effect on angiogenesis was determined as per the manufacturer instructions (Lopa Leech et al., 2002). The assay was performed in 96 well polystyrene miroplates (12 strips of 8 wells) coated with mouse monoclonal antibody against Ang-2 and MMP-2 respectively. Initially, 50 µl of assay diluent was added to each well and then 200 µl of standard, control and sample (pellet of test compound treated HL-60 cells) were added to the respective wells and incubated for 1 h at room temperature. The supernatant was aspirated out and each well was washed thrice with wash buffer after which 200 µl of conjugate was added to each well and incubated at room temperature for 1 h, the supernatant was again aspirated out and the wells were washed thrice with wash buffer. Later a 200 µl of substrate solution was added to each well and incubated for 20 min in dark. Finally a 50 µl of stop solution was added to each well and the plates were read at 450 nm within 30 min.

3.6. Pharmacokinetic (ADME: Adsorption Distribution Metabolism and Excretion) studies

Pharmacokinetics (PK) deals with the important aspects of selecting a drug, dosing regimen, and monitoring the dosing for appropriate toxic effects for individual. It explores what the body does to the drug, and essentially describes the fate of a drug after administration to a living being and involves several features like the extent and

the rate of absorption, distribution, metabolism and excretion referred to as ADME. These four criteria influence the drug levels and kinetics of drug exposure to the tissue and hence influence the performance and pharmacological action of a drug.

Male Swiss mice 9-14 weeks of age weighing in the range of 18 to 28 g were used in the present study. Animals were kept under regulated environmental conditions. (Temp. 26 ± 2 ^{o}C, relative humidity 50 ± 5 %; 12 h light dark cycle) and maintained on pelleted rodent diet (Ashirwad Industries Ltd. Chandigarh, India). Water was provided *ad libitum.* The institutional ethics committee approved the animal handling protocols. The animals were fasted for 16 h before use unless otherwise indicated.

3.6.1. Dosing Solution

For oral drug administration accurately weighed compound was transferred into mortar and triturated with 2 % Gum acacia powder with slow and consistent addition of double distilled water and were stirred continuously. A dose of 100 mg/kg b.wt. was used in the present study.

3.6.2. Administration Routes

Test samples were administered *via* oral route with the help of mice cannula and syringe corresponding to the body weight of respective animal.

3.6.3. Test Groups

Swiss albino mice (5 in each group)

- Group 1: Sacrifice time-5 min
- Group 2: Sacrifice time-15 min
- Group 3: Sacrifice time-30 min
- Group 4: Sacrifice time-1hr.
- Group 5: Sacrifice time-2 hr.
- Group 6: Sacrifice time-4 hr
- Group 7: Sacrifice time-6 hr
- Group 8: Sacrifice time-8 hr

3.6.4. Sample Collection and handling

Blood was obtained by excision of jugular vein under anesthesia with diethyl ether. EDTA was used as an anticoagulant. The plasma was obtained from the samples after centrifugation of blood samples at 5000 rpm for ten minutes. The brain of the mice

was isolated by bilateral incision of the skull followed by washing thrice with normal saline. Brain was homogenized in motar driven glass with teflon pestle and subjected to extraction.

3.6.5. Drug recovery from plasma and brain

A fixed amount of plasma and brain volume (200µl) was transferred in test tubes and to this was added 3 ml of IPA (4 ml in case of brain). The samples were vortexed on a vortex mixer for two minutes and subjected to centrifugation at 3000 rpm for 10 min and the organic layer was collected. The samples were kept in solvent evaporator to get rid of the organic solvent.

3.6.6. Sample analysis

After evaporation, the residues left in the tubes were reconstituted with mobile phase (which was prepared by combining Acetonitrile: Buffer (0.5% acetic acid in water) 95: 5 and vortexed for one minute. The samples were filtered through syringe filters 0.45 µm. Samples were analyzed by HPLC by injecting *via* automatic syringe injector onto RP-18, 5 micron column employing with Photo Diode Array detector.

Bioavailability was calculated by comparing the plasma and brain levels of drug in various groups.

3.7. Preparation of Nanoparticles

3.7.1. Synthesis of PEG-PLGA Copolymer Conjugate

Copolymer of PEG-PLGA was synthesized using solution polymerization process under nitrogen, using stannous octoate as catalyst (Avgoustakis, 2004). Stannous octoate has been approved by the US FDA for surgical and pharmacological application. This compound is the most widely used initiator as it provides high reaction ratio, and high molar mass even under relatively mild conditions (Beletsi et al., 1999).

Briefly, lactide and glycolide in a molar ratio of 4:1 and the specified amount of Bis-PEG were put in thick-walled glass tubes. The total weight of the feed was about 3 g. Stannous octoate was dissolved in hexane and added at a concentration of 0.03% by the weight of the feed. Then, the tubes were heated at 190°C for the 2 hours. The resulting copolymer was purified by dissolving in chloroform and then precipitating

in an excess methanol. The purified copolymer was dried under vaccum and characterized by IR and gel permeation chromatography.

3.7.2. Preparation of PEG-PLGA copolymer based Nanoparticles

Test compound loaded PEG-PLGA NPs were formulated by oil-in-water single emulsion-solvent evaporation technique. In brief, a solution of 100 mg PEG-PLGA co- polymer and 10 mg drug test samples) (10 % w/w dry weight of polymer) in 3 ml of chloroform was emulsified in 12 ml of 2 % w/v aqueous solution of PVA to form an oil-in-water emulsion. The emulsification was prepared using a micro-tip probe sonicator set at 55 W of energy output (VC 505, Vibracell Sonics) for 2 min over an ice bath. The emulsion was stirred overnight at room temperature to allow evaporation of organic solvent and formation of NPs. NPs were recovered by ultracentrifugation at 40,000 rpm for 20 min at 4 °C (Sorvall Ultra speed Centrifuge), washed twice with water to remove excess PVA and unencapsulated drug. The nanoparticulate suspension was then lyophilized for 2 days (-47 ºC and < 10 µm mercury pressure, LYPHLOCK 12, Labconco) to obtain the powdered NPs for further use.

3.7.3. Transferrin Coupling with PEGylated Nanoparticles

Conjugation technique depends on the two interrelated chemical reactions: the reactive functional group present on the various cross-linking or derivatizing reagents and the functional group present on the surface of the target macromolecules to be modified. Protein molecules are the most common targets for modification and modified proteins can be selected to retain its ability to bind its natural target organ. Transferrin coupled PEGylated nanoparticles were prepared by coupling of transferrin to PEGylated nanoparticles using periodate method. Briefly 1ml of Tf solution (80mg/ml) was mixed with 50µl sodium periodate solution (20mg/ml). The mixture was kept in ice for 2 hours. The obtained oxidized Tf was then incubated with 1 ml of PEG-nanoparticles. After 1.5 hours at room temperature, 50µl of glycine solution was then added and the Tf-PEG-nanoparticles were purified to remove excess free Tf by centrifugation at 39,000 x g for 20 minutes (Li et al., 2003).

3.7.4. Physicochemical characterization of drug loaded NPs

3.7.4.1. Particle size analysis and zeta potential measurement

Particle size and size distribution (polydispersity index) of the formulation was determined, based on dynamic light scattering (DLS), using a Zetasizer (Nano ZS, ZEN3600, Malvern Instrument) with a wavelength of 532 nm at 25 ^{o}C with an angle detection of 90^{o}. In brief, 1 mg/ml of nanoparticulate suspension was prepared in MilliQ water, sonicated using a sonicator set at 55 W of energy output for 2 min over an ice bath for 30 sec. A 100 µl of above NPs suspension was diluted to 1 ml in water and then subjected to particle size measurement. Zeta potential of nanoformulation was determined by the same instrument, following the above protocol. All measurements were performed in triplicates.

3.7.4.2. Transmission electron microscopy (TEM)

Nanoparticles were also evaluated for size by transmission electron microscope (Philips/FEI Inc. Barcliff). For this purpose, a sample of NPs (0.5 mg/ml) was suspended in water and sonicated for 30 sec. One drop of this suspension was placed over a carbon coated copper TEM grid (150 mesh, Ted PELLA Inc.) and negatively stained with 1 % uranyl acetate for 10 min, allowed to dry and the images were visualized at 120 kV under microscope.

3.7.4.3. Scanning electron microscopy (SEM)

The surface morphology and shape of the formulated NPs were visualized by scanning electron microscopy (SEM). The powdered NPs were sputtered with gold to make them conductive and placed on a copper stub. The images were taken using SEM (JSM-T220A, JEOL) operating at an acceleration voltage of 10-20 kV.

3.7.5. Evaluation of encapsulation efficiency of the drug loaded nanoparticles

Encapsulation efficiency was determined by the reverse phase isocratic mode of high performance liquid chromatography (RPHPLC) method. Agilent 1100 HPLC (Agilent technologies, Waldbronn Analytical Division) which consists of Zorbax Eclipse XDB-C18, 150 x 4.6 mm, i.d with internal standard of dimethylphthalate. To estimate entrapped drug in NPs, 5 mg of freeze dried drug loaded NPs was dissolved in 5 ml of acetonitrile and sonicated for 2 minutes. The sample was then centrifuged at 13,800 rpm for 10 min at 25 °C (SIGMA 3K30, Germany) to extract the drug present in the solution. The collected supernatant was analyzed for drug content by RPHPLC using a mobile phase of acetonitrile and water (with 0.5 % acetic acid) in the ratio 95:5

(v/v), flow rate of 1 ml/min, at 30 ℃ with thermostat (Model No -G1316A). The drug peak was measured at wavelength of 240 nm (with DAD, Model-G 1315A) and quantitatively determined by comparing with a standard plot. The entrapment efficiency of sample test compounds was calculated according to the following equation.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Mass of drug in nanoparticle}}{\text{Mass of drug used in formulation}} \times 100$$

3.7.6. *In vitro* release kinetics of drug from nanoparticles

In vitro release of drug from NPs was carried out by dispersing 10 mg of drug loaded NPs in 3 ml of PBS (0.01 M, pH 7.4) containing 0.1 % v/v of Tween-80. The nanoparticulate suspension was equally divided in three tubes 1 ml each (as experiment was performed in triplicates) and kept in a shaker at 37 ℃ at 150 rpm (Wadegati Labequip). At particular time intervals samples were taken out from shaker and centrifuged at 13,800 rpm, 25℃ (SIGMA 3K30) for 10 min. The collected supernatants were lyophilized for 48 hrs. The precipitated NPs were resuspended in 1 ml of fresh PBS-Tween 80 solution and replaced in shaker for next readings. The lyophilized product was dissolved in 1 ml of acetonitrile and the sample was centrifuged at 13,800 rpm for 10 min at 25 ℃ to collect the drug in supernatant. 20 µl of this supernatant was injected in the HPLC to determine the amount of drug released with respect to different time intervals.

3.8. Statistical analysis

Each experiment was repeated at least in triplicate. Results were expressed as Mean ±SD, unless otherwise indicated. Comparisons were made between control and treated groups unless otherwise indicated using ANOVA and p-values < 0.01 were considered significant.

4.1. Antiproliferative assays of PKBA

4.1.1. Determination of cellular cytotoxicity/viability by SRB/MTT assay

SRB assay was performed to determine the *in vitro* cytotoxicity against adherent cell lines (Table 4.1). SRB assay is based on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B (SRB) by basic amino acids in the plasma membrane of the cells. The antiproliferative potential against suspension cell lines was determined by MTT assay (Table 4.2, 4.3). MTT assay is a reduction of tetrazolium bromide to formazon product by the mitochondrial enzyme succinate - dehydrogenase by living but not by the dead cells.

Inhibitory effect of PKBA was evaluated against twelve human cancer cell lines including IMR-32, SF-295 (neuroblastoma), PC-3 (Prostate), Colo-205, HCT-15 (both Colon), MCF-7 (Breast), OVCAR-5 (Ovarian), Hep-2 (Liver), A549 (Lung), THP-1, HL-60 & MOLT-4 (Leukemia) at different concentrations (1, 5, 10, 30, 50, 70 & 100 µg/ml). Although, PKBA was found to induce cytotoxicity in all the twelve cancer cell lines, but the degree of cytotoxicity varied from cell line to cell line, which may be due to molecular characteristic of the cells. It showed > 50 % growth inhibition at 10 µg/ml against both (IMR-32 and SF-295) the neuroblastoma cell lines, only Colo-205 from colon and all the three (THP-1, HL-60 & Molt-4) leukemia cell lines. It showed that in general PKBA was more active against neuroblastoma and leukemia cell lines than other tissues. PKBA showed increase in cytotoxicity with in the same cell line when the concentration was increased. It showed 79-99 % growth inhibitin at 100 µg/ml against all the cell lines. Thus, the cytotoxic effect of PKBA was concentration dependent (Table 4.1, 4.2 and 4.3). The efficacy of PKBA was compared with known anticancer drugs including adriamycin, paclitaxel, mitomicin-c and 5-fluorouracil.

4.1.2. Determination of IC_{50} values

The *in vitro* cytotoxicity results showed PKBA to be active against all the twelve human cancer cell lines being evaluated. In order to have a better insight into the comparative data the IC_{50} values (drug concentration at which 50 % of growth inhibition takes place) of PKBA against all the twelve cell lines was calculated. The IC_{50} values were found to be in the range of 5.95 to 18 µg/ml with IMR-32 showing

the lowest value (5.95 µg/ml) and HCT-15 showing the highest value (18 µg/ml) (Table 4.4). The lowest IC_{50} values of 5.95 and 7.11 µg/ml were observed for IMR-32 and SF-295 cell lines respectively of neuroblastoma origin, indicating that PKBA is most active against neuroblastoma cell lines. It was followed by 8.7, 9.5 and 10.3 µg/ml for the three leukemia cell lines namely HL-60, Molt-4 (Fig. 4.2) and THP-1 respectively. This indicated that PKBA has most pronounced activity against neuroblastoma followed by leukemia cell lines. It showed higher IC_{50} for the cancer cell lines from other tissues such as prostate, breast, ovarian, liver, lung & colon.

Tissue		Neuroblastoma		Prostate	Colon		Breast	Ovary	Liver	Lung	Leukemia
Cell Line		IMR-32	SF-295	PC-3	Colo-205	HCT-15	MCF-7	OVCAR-5	Hep-2	A549	THP-1
Code	Conc. µg/ml	% Growth Inhibition									
PKBA	1	29	25	11	11	14	16	13	30	20	17
	5	41	40	25	30	18	33	29	29	26	28
	10	68	66	47	60	30	47	43	38	37	61
	30	73	75	76	71	79	71	62	50	65	91
	50	78	82	81	86	88	79	74	62	71	92
	70	81	87	89	90	95	85	80	73	78	92
	100	96	94	89	98	95	90	89	87	89	99
Adriamycin	1µM	65	67	-	-	-	68	-	---	-	-
Paclitaxel	1 µM	-	-	-	-	-	-	58	-	-	-
Mitomycin c	1 µM	-	-	50	-	-	-	-	58	-	51
5-Flurouracil	20 µM	-	-	-	50	62	-	-		52	70

Table 4.1 *In vitro* cytotoxicity (percent growth inhibition) of PKBA. Cells were grown in 96 well plates and treated with various concentrations 1,5 10, 30,50, 70 and 100 µg/ml of PKBA for 48 h. The SRB assay was performed as described in material & method section.

Cell Line	HL-60 (Leukemia)			
Conc. µg/ml	**Average O.D**	**STDEV**	**%Viability**	**% Inhibition**
0	0.941	0.03528	100	0
10	0.428	0.066578	45.5	54.5
30	0.249	0.038323	26.5	73.5
50	0.21675	0.009708	23.03	76.97
70	0.20925	0.045419	22.23	77.77
100	0.194	0.014071	20.61	79.39

Table 4.2 *In vitro* proliferation and percent growth inhibition of PKBA in HL-60 cells. Cells were treated with PKBA for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Cell Line	MOLT-4 (Leukemia)			
Conc. µg/ml	**Average O.D**	**STDEV**	**%Viability**	**% Inhibition**
0	0.976	0.006782	100	0
10	0.34075	0.041604	31	69
30	0.25775	0.028745	25.7	74.3
50	0.24225	0.027825	24.7	75.3
70	0.16425	0.024019	16.5	83.5
100	0.05	0.029609	5.2	94.8

Table 4.3 *In vitro* proliferation and percent growth inhibition of PKBA in MOLT-4 cells. Cells were treated with PKBA for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Tissue	**Cell line**	**IC_{50} (µg/ml) of PKBA**
Neuroblastoma	**IMR-32**	5.95
	SF-295	7.11
Prostate	**PC-3**	15.2
Breast	**MCF-7**	15
Ovarian	**OVCAR-5**	15.9
Liver	**Hep-2**	17.4
Lung	**A549**	17.4
Colon	**Colo-205**	14.5
	HCT-15	18
Leukemia	**THP-1**	10.3
	HL-60	8.7
	Molt-4	9.5
Normal Monkey Kidney	**CV-1**	> 100 µg/ml

Table 4.4 IC_{50} values of PKBA against twelve human cancer cell lines

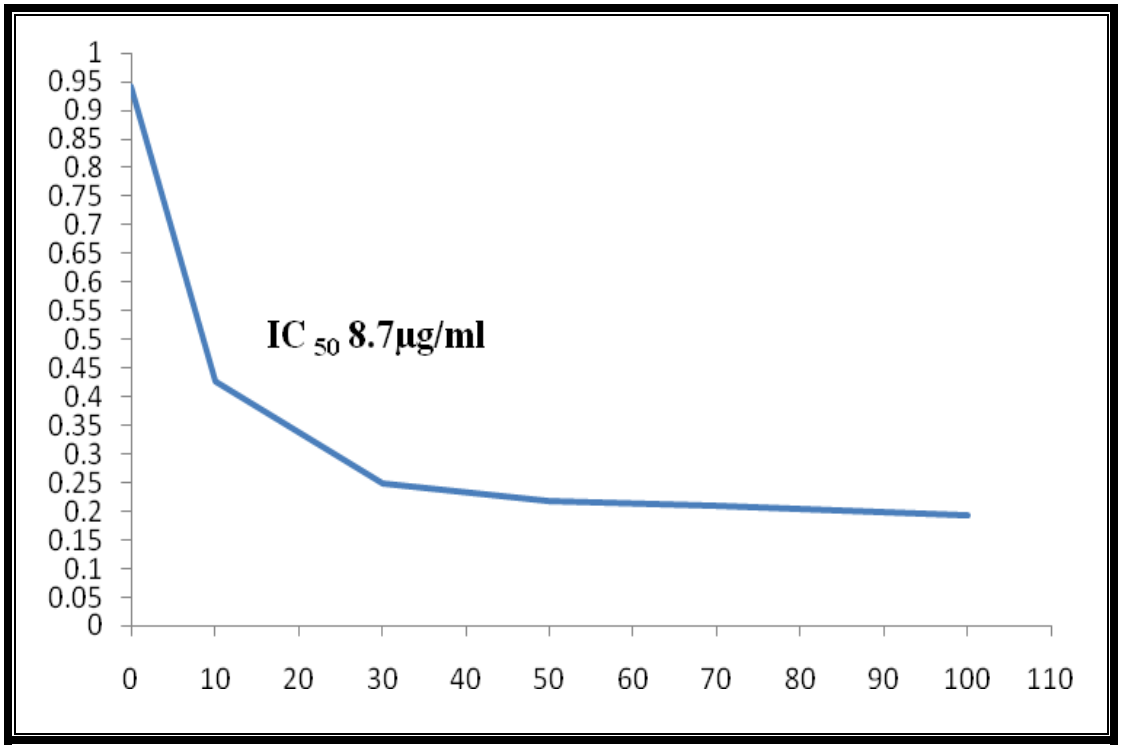


Fig.4.1 IC_{50} value of PKBA in HL-60 cell line by MTT assay

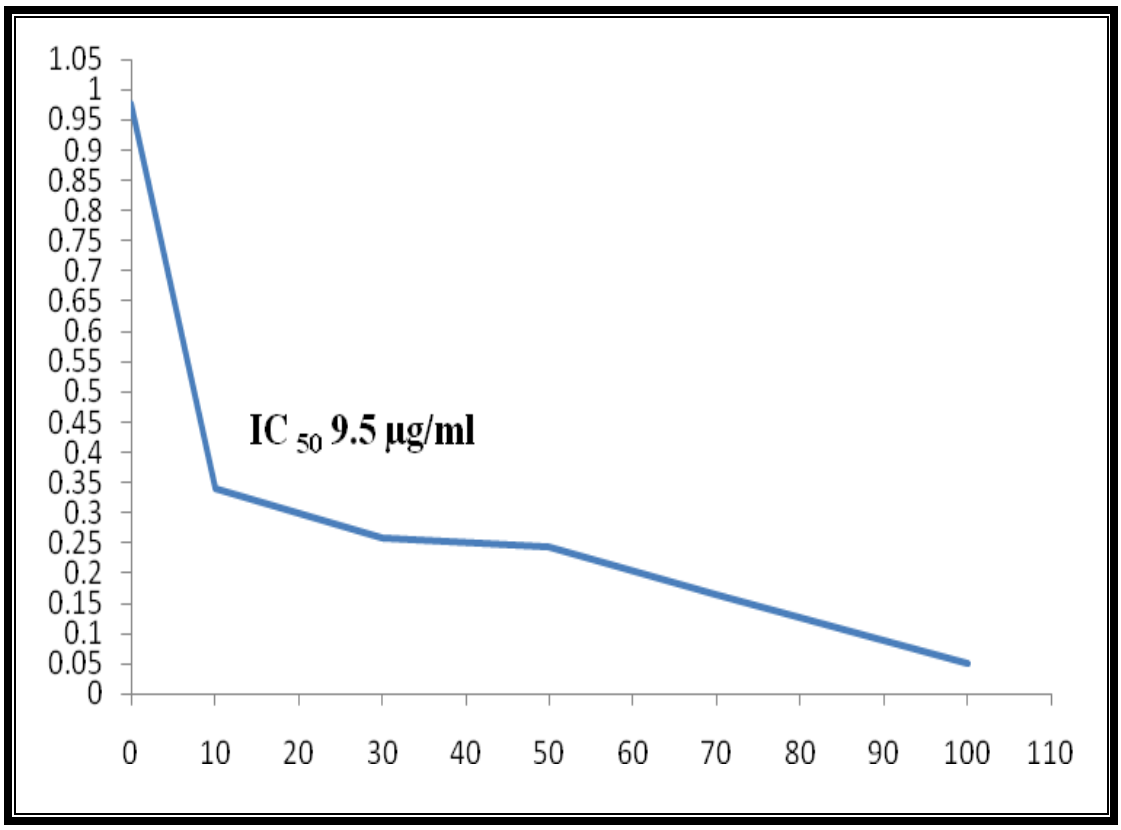


Fig.4.2 IC_{50} value of PKBA in MOLT-4 cell line by MTT assay

4.2. Determination of *In vivo* anticancer activity

4.2.1. *In vivo* anti cancer activity against Ehrlich Ascites Carcinoma

PKBA showed 100 % ± 0.56 tumor growth inhibitions against Ehrlich ascites carcinoma (EAC) when evaluated at 100 mg/kg i.p. dose. The growth inhibition at 100 mg/kg was significant (p = <0.05) as compared to control animals treated with normal saline. 5-FU taken as a positive control showed a tumor growth inhibition of 92.45 ± 1.5 at 20 mg/kg i.p. (Table 4.5).

4.2.2. *In vivo* anti cancer activity against Ehrlich tumor

The *in vivo* anticancer activity of PKBA against Ehrlich tumor (solid) is depicted in Table 4.6 PKBA showed dose dependent increase in tumor growth inhibition, being 33.78 ± 0.47 % and 66.74 % at 100 and 150 mg/kg i.p. respectively. The result at 150 mg/kg was significant (p= <0.05). 5- FU showed 45.13±1.5 % tumor growth inhibition at 22 mg/kg i.p.

4.2.3. *In vivo* anticancer activity of PKBA against Sarcoma-180

In vivo anticancer activity of PKBA against Sarcoma-180 (solid) revealed a dose dependant tumor inhibition as given in Table 4.7. At 100 and 150 mg/kg i.p. dose levels, PKBA showed growth inhibition of 34.25±0.46 & 68.02±0.44 % respectively. 5-FU (positive control) at 22 mg/kg i.p. dose level inhibited tumor growth by 47.3 ± 0.44 %.

Sample	Dose (mg/ kg i.p.)	Animals/ Mortality	Tumor Volume (ml)	Cell count (107)	% Tumor growth inhibition
Control	NS	7/0	3.11 ±0.35	100.26 ±3.26	-
PKBA	100	7/0	1.32 ± 0.16	1.33± 5.60**	100 % ±0.56**
5 FU	20	7/0	0.45 ± 0.15	7.98 ± 1.1	92.45 ± 1.5**

Table 4.5 *In vivo* anticancer activity of PKBA against Ehrlich Ascities Carcinoma. * represents significant (p= < 0.05), ** highly significant (p= < 0.01). Data are Mean ± S.D.

Sample	Dose (mg/ kg i.p.)	Animals/ Mortality	Body Weight (g)	Tumor Weight (mg)	% Tumor growth inhibition
Control	NS	7/0	22.65 ± 0.35	1799.28 ± 82.36	-
PKBA	100	7/0	21.25 ± 0.40	1205.28 ± 51.10	33.78 ± 0.47
	150	7/0	22.18 ± 0.61	598.44 ± 49.22**	66.74± 0.51**
5 FU	22	7/0	19.11 ± 0.50	989.64 ± 45.3 *	45.13± 1.5*

Table 4.6 *in vivo* anticancer activity of PKBA against Ehrlich Tumor (solid) * represents significant (p= < 0.05), ** highly significant (p= < 0.01). Data are Mean ± S.D

Sample	Dose (mg/ kg i.p.)	Animals/ Mortality	Body Weight (g)	Tumor Weight (mg)	% Tumor growth inhibition
Control	NS	7/0	22.33 ± 0.35	1972.16 ± 76.24	-
PKBA	100	7/0	21.45 ± 0.40	1296.59 ± 52.91	34.25± 0.46
	150	7/0	18.38 ± 0.61	630.69± 50.01**	68.02 ± 0.44%**
5 FU	22	7/0	18.65 ± 0.50	1039.33 ± 52.11*	47.3 + ± 0.4%.*

Table 4.7 *in vivo* anticancer activity of PKBA against Sarcoma 180 (solid) * represents significant (p= < 0.05), ** highly significant (p= < 0.01) Data are Mean ± S.D

4.3. Apoptotic assays

4.3.1. Determination of Morphological Changes by Fluorescence Microscopy

In contrast to normal cells, the nuclei of apoptotic cells contained highly condensed chromatin material which when stained with DAPI, a DNA binding dye represents the entire nucleus as a single or a group of featureless, bright spherical beads. These morphological changes can be visualized by fluorescence microscopy. The fluorescence microscopy studies of PKBA revealed nuclei of HL-60 cells to be round in shape (Fig. 4.3a), while those treated with PKBA for 18 h at 5 and 10 µg/ml showed chromatin condensation and formation of apoptotic bodies (Fig 4.3 c & d) which are characteristic features of apoptosis.

4.3.2. Determination of Morphological Changes by Scanning Electron Microscopy

Scanning Electron Microscopic studies of PKBA showed the characteristic differences between treated and control HL-60 cells. The cells were incubated with test compound (10 µg/ml) for 24 h. The control HL-60 cells were spherical in shape with a few surface projections and microvilli (Fig. 4.4 a, b & c) whereas the treated cells showed loss of surface projections, smoothening of cell surface and a few prominent surface blebs, the characteristic of apoptosis (Fig. 4.4 d, e & f). SEM studies therefore confirmed that PKBA induced apoptosis in HL-60 cells and the treatment did not reveal any sign of necrotic cell death such as formation of holes on cell surface due to membrane breakdown.

4.3.3. Determination of DNA fragmentation by DNA ladder assay

Apoptosis or programmed cell death is a regulated physiological process whereby a cell can be removed from a population marked by DNA fragmentation. During apoptosis endonucleases cleave the intra nucleosomal chromatin in multiples of 180 bp leading to DNA fragmentation resulting in a DNA ladder formation. HL-60 cells when incubated with PKBA for 24 h exhibited a typical DNA ladder formation at 10, 15 and 20 µg/ml (Fig. 4.5a). Camptothecin (5 µM), as reference exhibited a discrete ladder pattern after 6 h of treatment however, no ladder formation was observed in untreated cells. DNA isolated from untreated and PKBA treated normal monkey kidney CV-1 cells did not show any DNA ladder formation (Fig. 4.5b).

4.3.4. Determination of topoisomerase I & II inhibitory activity

Topoisomerases are the essential enzymes that control and modify the topological state of DNA. Topoisomerse I and II act by sequential breakage and reunion of single and double strands of DNA respectively. The topoisomerase targeting drugs can either be classified as topo poisons or topo catalytic inhibitors. The former acts by stabilizing DNA cleavable enzyme complexes leading to DNA break and latter acts by stabilizing enzyme where both DNA strands remain intact and no DNA breaks occur, resulting in apoptosis. PKBA at 20 µg/ml inhibited the enzymatic activity of both Topoisomerase I & II (Fig.4.6) in a way similar to that of known topo-I & II inhibitors, camptothecin (100 µM) and etoposide (100 µM) respectively.

4.3.5. Determination of phosphatidylserine by Annexin V-FITC assay

Loss of plasma membrane integrity is an early event in apoptosis, independent of the cell type, resulting in the exposure of phosphatidylserine (PS) residues at the outer plasma membrane. Annexin V in the presence of calcium interacts strongly and specifically with PS and can be used to detect apoptosis by targeting for the loss of plasma membrane asymmetry. HL-60 cells after treatment with different concentrations of PKBA were stained with annexin V-FITC and PI to access the apoptotic and necrotic cell population (Fig 4.7). The basal apoptotic population in the untreated culture was 3.5 %. Test compound showed a dose dependant increase in apoptotic population which was found to be 70.06 and 96.74 % respectively at 10 and 20 µg/ml after 24 h of incubation. Apoptosis thus appeared to be the primary mode of cell death induced by PKBA.

4.3.6. Determination of DNA damage by the comet assay

Comet assay measures, double as well as single strand breaks, alkali labile sites, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-Drug cross linkage and DNA repair. The principle of the assay rests on strand breakage of the supercoiled double helical DNA which leads to the reduction in the size of large molecules where in strands of the DNA can be stretched out by electrophoresis. Similarly, under highly alkaline conditions there is denaturation, unwinding of the double strand and the expression of alkali labile sites as single strand breaks. Comets form when the broken ends of the negatively charged DNA molecule become free to migrate in the electric

field towards the anode. DNA migration is a function of both the size and the number of broken ends of the DNA. Due to the damage, the Tail increases in length and reaches its maximum which is dependent on the electrophoretic conditions and not on the size of the fragments. PKBA treated HL-60 cells revealed the formation of comets (Fig. 4.8 c) with Head DNA of 70.15 and 43.21 % at concentration of 10 and 20 µg/ml respectively. Tail DNA that revealed the actual DNA damage was found to be 29.85 and 56.79 % at the above mentioned concentrations. The Olive Tail movement was found to be 2.83 and 4.46 % while the Tail length was found to be 10.06 and 27.07 % respectively (Table.4.8). Camptothecin (5 µM) taken as a positive control also induced comet formation (Fig. 4.8 b) in HL-60 cells with a head DNA of 66.66 % and Tail DNA of 33.34 % (Table.4.8), however, no comet formation was observed in untreated cells (Fig. 4.8 a).

4.3.7. Determination of DNA content on cell cycle analysis

Effect of PKBA on cell cycle of HL-60 cells was analyzed by propidium iodide uptake *via* flow cytometry. Propidium iodide is an intercalating dye with a specific affinity for DNA. Its quantititation, in permeabilized cells measures DNA content and is used to estimate the cell cycle distribution in the populations of cells. Flowcytometry often is called upon to distinguish diploid from anueploid cells. Ploidy refers to DNA content and normal cells are usually diploid. Normal cells pass through the cell cycle; they proceed from G_0/G_1 phase with a diploid (2N) amount of DNA, through the DNA synthesis (S) phase where the DNA can range from 2N to 4N. Cells then enter the G_2-phase with a tetraploid (4N) amount of DNA until mitosis (M-phase) when they divide and return to the diploid state. In contrast cancer cells often contain abnormal amounts of DNA reflecting genetic instability, and are therefore considered aneuploid.

The hypo diploid sub-G_1 DNA fraction (<2n DNA) of the cells treated with 1, 5, 10, 30 and 50 µg/ml of PKBA for 24 h was found to be 2.91, 3.93, 57.13, 78.16, 80.10 & 95.81% respectively (Fig. 4.9), while in untreated cells it was only 3.3 %. PKBA showed an increase in sub-G_1 DNA fraction in a concentration dependent manner. PKBA was also found to arrest the G_2/M phase of cell cycle in a concentration dependent manner.

4.3.8. Determination of mitochondrial membrane potential

The loss of mitochondrial membrane potential is related to the opening of the mitochondrial permeability transition pores (PTP), which conduct the leakage of cytochrome c and pro apoptotic proteins from the mitochondria to the cytosol.

HL-60 cells exposed to PKBA for 24 h when analyzed for mitochondrial membrane potential loss ($\Delta\Psi m$) employing Rh-123 uptake by flowcytometry revealed that almost all the cells were functionally active with high Rh-123 uptake fluorescence in untreated cells while the cells treated with PKBA caused mitochondrial damage resulting in the loss of mitochondrial membrane potential. As is evident from the results the mitochondrial membrane potential loss was found to be 7.51, 64.73 and 91.38 % respectively at a concentration of 5, 10 and 20 µg/ml (Fig. 4.10).

4.3.9. Determination of intracellular caspase activity

Both extrinsic and intrinsic apoptotic pathways are initiated by the recruitment and activation of apical caspases in the apoptosis signalling pathways. In death receptor-mediated apoptosis, engagement of death receptors leads to the formation of the death-inducing signalling complex (DISC) containing death receptors, adaptor proteins, caspase-8 and caspase-10, while in mitochondrion-dependent apoptosis the release of cytochrome c into the cytosol results in the formation of apoptosome containing cytochrome c, Apaf-1 and caspase-9. The activated caspases from the DISC and the apoptosome then cleave downstream caspases and other target proteins to induce apoptosis. Caspase-1 and -2 can also induce the release of cytochrome c.

PKBA treated HL-60 cells at 10 & 20 µg/ml after 3 6, 12 and 24 h were studied for different caspase activities. Results revealed that cells treated with 20 µg/ml PKBA for 24 h enhanced the activity of caspase-1, -9 and -6 by 2 fold (Fig. 4.11) and that of caspase-2 and -3 by 10 fold (Fig. 4.11) while caspase -8 and -10 were activated to a lesser extent (Fig. 4.11). Activation of caspase-3 and -9 revealed PKBA to induce apoptosis through intrinsic pathway but the activation of caspase-6, -8 and -10 revealed that it can also undergo apoptosis *via* extrinsic pathway. Further it might be possible that the activation of one pathway may be responsible for the activation of the other.

4.3.10. Determination of cytochrome c release and PARP cleavage by Western blotting

PKBA induced loss of mitochondrial membrane potential has its relevance towards the opening of PTP and release of cytochrome c from mitochondria. The passage of cytochrome c through the outer membrane is considered a critical step for mitochondrial dependent caspase activation. Initially cytochrome c is known to activate caspase-9 and then executioner caspase-3. Our results demonstrated PKBA to induce the release of cytochrome c from mitochondria into the cytosol in a concentration dependent manner (Fig.4.12).

Several reports have shown that an elevation in the level of caspases especially caspase-3 results in the cleavage of a DNA repair enzyme Poly-ADP-ribose polymerase (PARP). The sequence at which caspase-3 cleaves PARP is very well conserved in the PARP protein indicating the potential importance of PARP cleavage in apoptosis. Full length PARP is a 116 KDa protein involved in the repair of DNA, in differentiation and in chromatin structure formation. During apoptosis the protein is cleaved by caspase-3, and possibly other caspases into a 89 KDa fragment, thus preventing any unwanted DNA repair. PKBA treated HL-60 cells showed cleavage of PARP in a concentration dependent manner (Fig.4.12) which later activates certain endonucleases, thus leading to the DNA fragmentation.

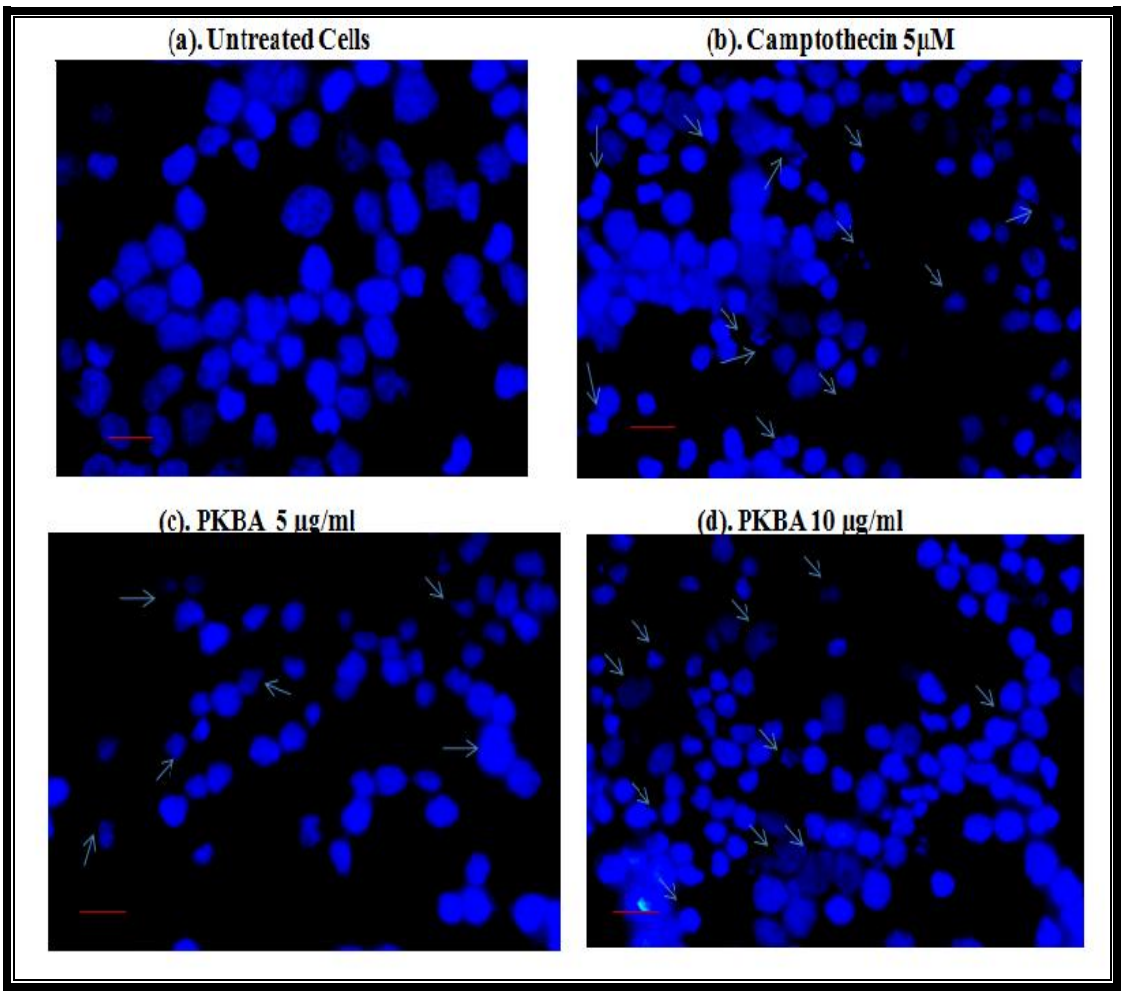


Fig.4.3. Influence of PKBA on nuclear morphology and apoptotic bodies formation in HL-60 cells. Cells (2×10^5/2 ml/6 well plate) were treated with indicated dose of PKBA for 18 h stained with DAPI and visualized for nuclear morphology and apoptotic bodies. (a) Untreated control cells showing large sized nuclei (b) Cells treated with 5 µM Camptothecin indicate condensation in nuclei and thus apoptosis (↑). (c) Cells treated with 5 µg/ml PKBA indicate apoptosis (↑). (d) Cells treated with 10 µg/ml PKBA showing increase in apoptotic population (↑). Magnifications 400X. Bar (—) 20 µm.

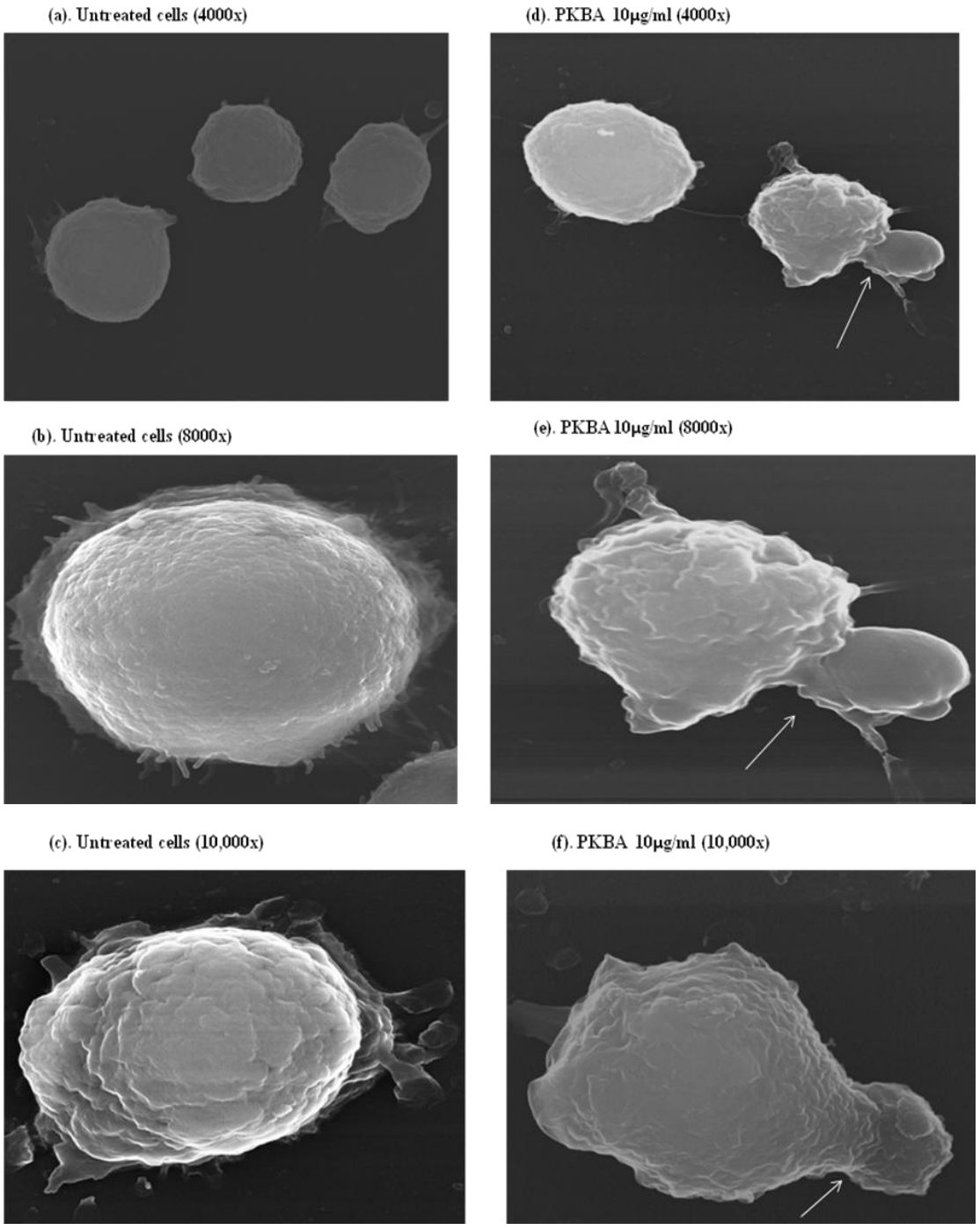


Fig. 4.4. SEM of control HL-60 cells (a, b, c) at 4000x, 8000x and 10,000x respectively and HL-60 cells treated with PKBA (10 µg/ml) for 24 h (d, e, f) at the respective magnifications. The untreated control cells (a, b, c) show rough surface and microvilli and the treatment, after 24 h causes reduction in cell size, smoothening of cell surface, blebbing (↑) of the plasma membrane and apoptosis (d, e, f).

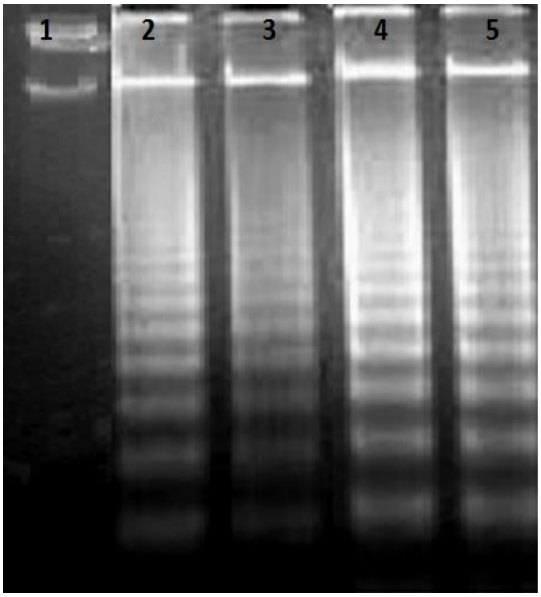


Fig. 4.5a. DNA fragmentation assay in HL-60 cells. Cells ($2x10^6$ /ml/well) were incubated with PKBA at various concentrations for 24 h. Lane-1 (Untreated cells), Lane-2 (HL-60 cells + 5 µM Camptothecin, 6 h), Lane -3, 4 & 5 (HL-60 cells +10 15 & 20 µg/ml PKBA).

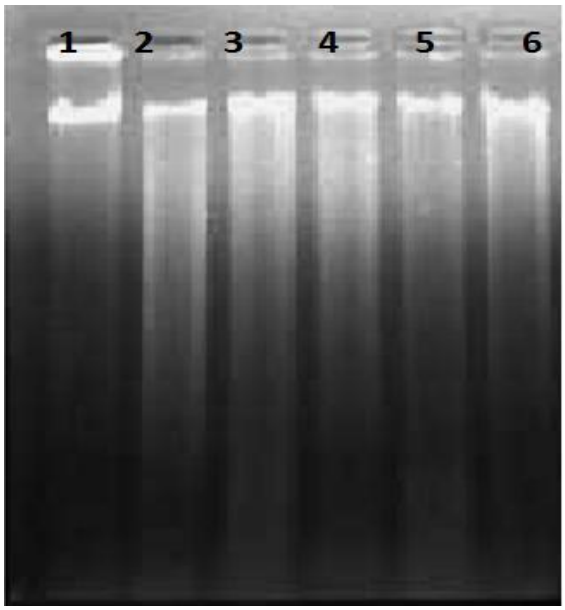


Fig. 4.5b. DNA fragmentation assay in CV-1 cells. Cells ($2x10^6$ /ml/well) were incubated with PKBA at various concentrations for 24 h. Lane-1(Untreated cells), Lane-2, 3, 4, 5 & 6 (PKBA 5, 10, 20, 30 and 50 µg/ml respectively)

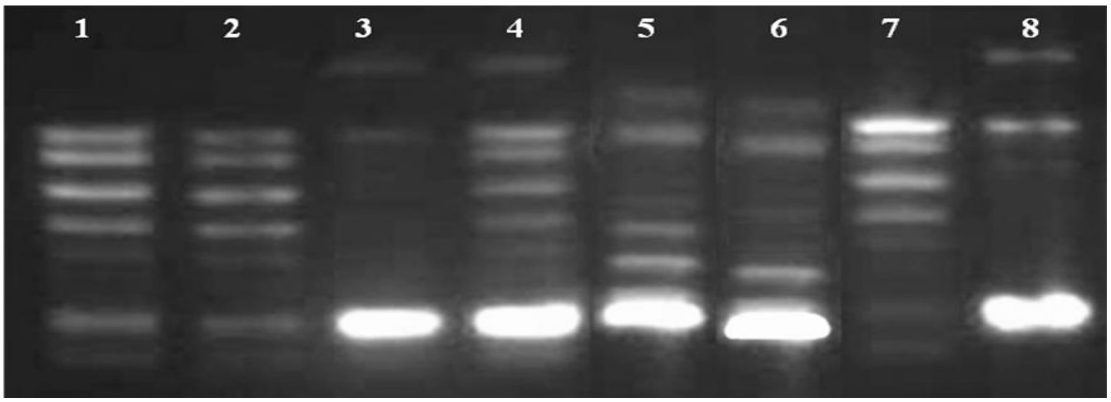


Fig. 4.6. PKBA inhibits topoisomerase I&II activity. Inhibitory assay of topoisomerase I&II was performed by using topoisomerase-I & II kits from Topogen. The assay was performed according to the instructions provided by the supplier. Lane 1 & 2 (Supercoiled DNA+Topo-I & Supercoiled DNA+Topo-II respectively). Lane 3, 4 (Suprecoiled DNA+Topo-I+ camptothecin 100 µM & Suprecoiled DNA+Topo-II+ Etoposide 100 µM respectively). Lane 5 & 6 (Suprecoiled DNA + Topo-I + PKBA 20 µg/ml & Suprecoiled DNA + Topo-II+ PKBA 20 µg/ml respectively). Lane 7 (Relaxed DNA as Marker). Lane 8 (Supercoiled DNA). Data is representative of one of the two similar experiments.

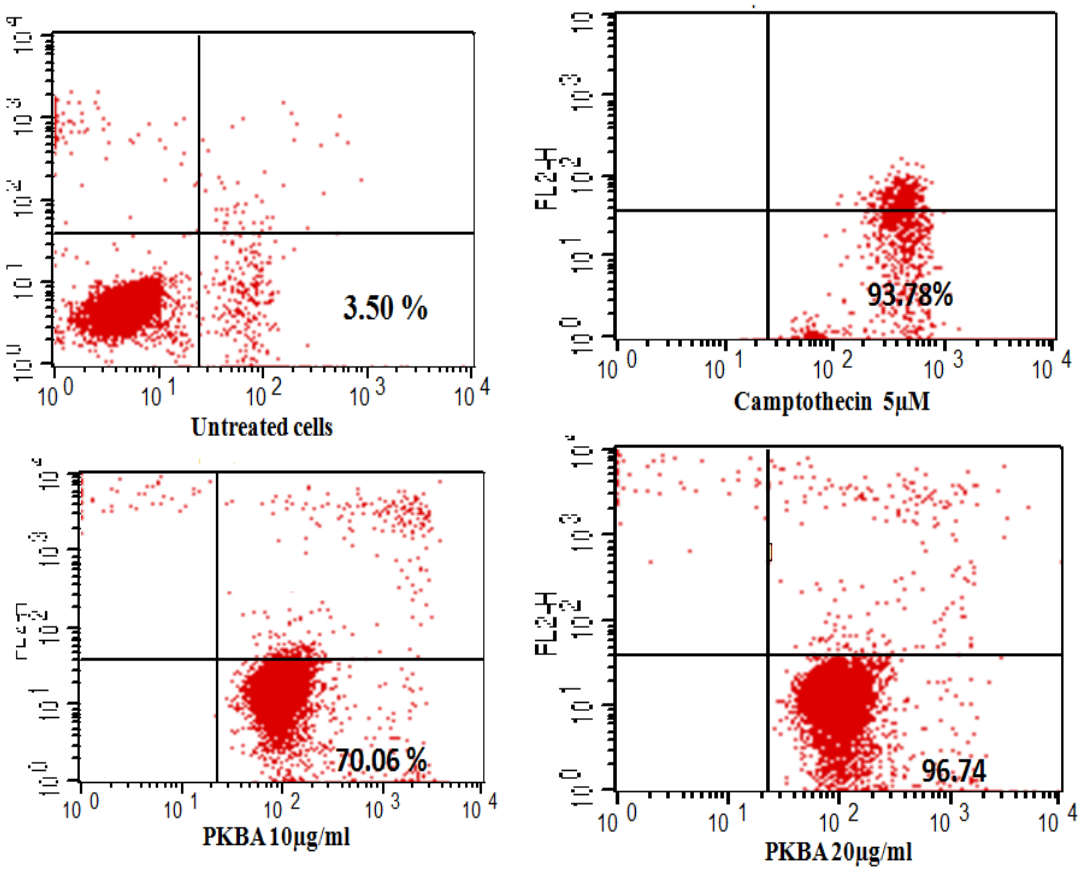


Fig. 4.7. PKBA induced apoptosis in HL-60 cells using Annexin V-FITC/PI. HL-60 Cells were treated with indicated concentrations of PKBA for 24 h and stained with annexin V-FITC/PI to analyze apoptotic and necrotic cell population. Apoptotic population was found to be 70.06 and 96.74 % at 10 & 20 µg/ml of PKBA respectively. Data are representative of one of the two similar experiments.

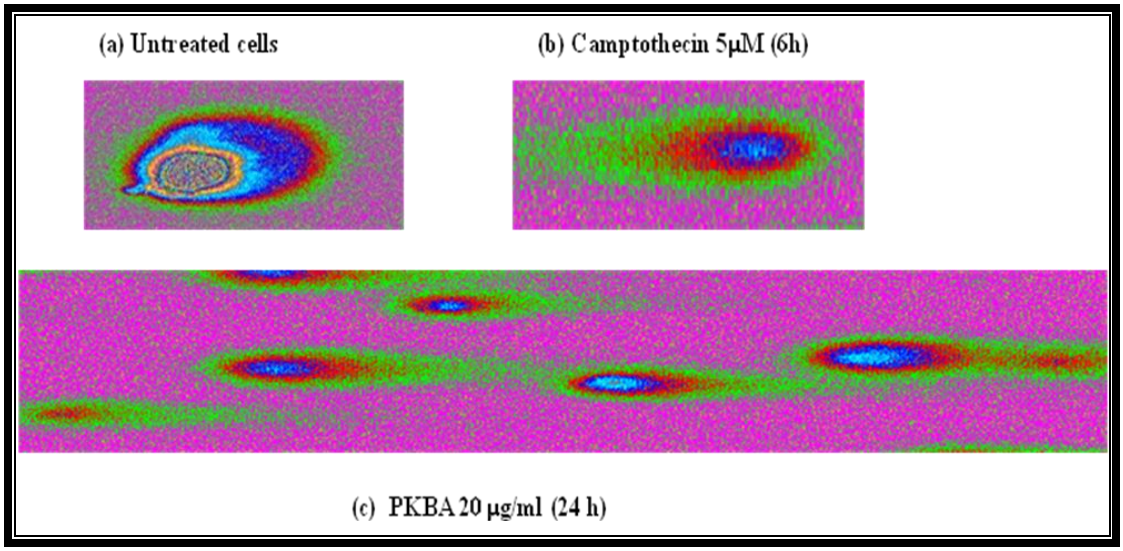


Fig. 4.8. Comet assay analysis. (a) Untreated cells, (b) Camptothecin treated HL-60 cells for 6h at 5µM (c) PKBA treated HL-60 cells for 24 h at 20 µg/ml. The untreated control cells (a) showed no comet formation and the treatment with PKBA after 24 h induced the formation of actual comets in HL-60 cells (c).

	Head DNA	Tail DNA	Olive Tail Movement	Tail Length
Untreated Cells	91.08±1.02	8.92±1.02	0.440±0.03	1.22±0.238
Camptothecin 5µM	66.66±0.02	33.34± 0.02	7.85± 0.05	33.25± 0.042
PKBA 10µg/ml	70.15 ±0.03	29.85 ± 0.02	2.83 ± 0.04	10.06 ± 0.03
PKBA 20µg/ml	43.21 ± 0.04	56.79 ± 0.03	4.46 ± 0.05	27.07 ± 0.04

Table 4.8. The single cell gel electrophoresis of PKBA treated HL-60 cells after 24 h of incubation revealed a head DNA of 70.15 % and 43.21 % , Tail DNA of 29.85 % and 56.79% at 10 and 20 µg/ml respectively which signifies the DNA damage. The olive tail movement was 2.83% and 4.46 % while a Tail length of 10.06 % and 27.07 % was observed at the respective concentrations. Camptothecin taken as a positive control showed a Head DNA of 66.66 % and Tail DNA of 33.34% at 5 µM concentration. Data are mean ± S.D.from three similar experiments.

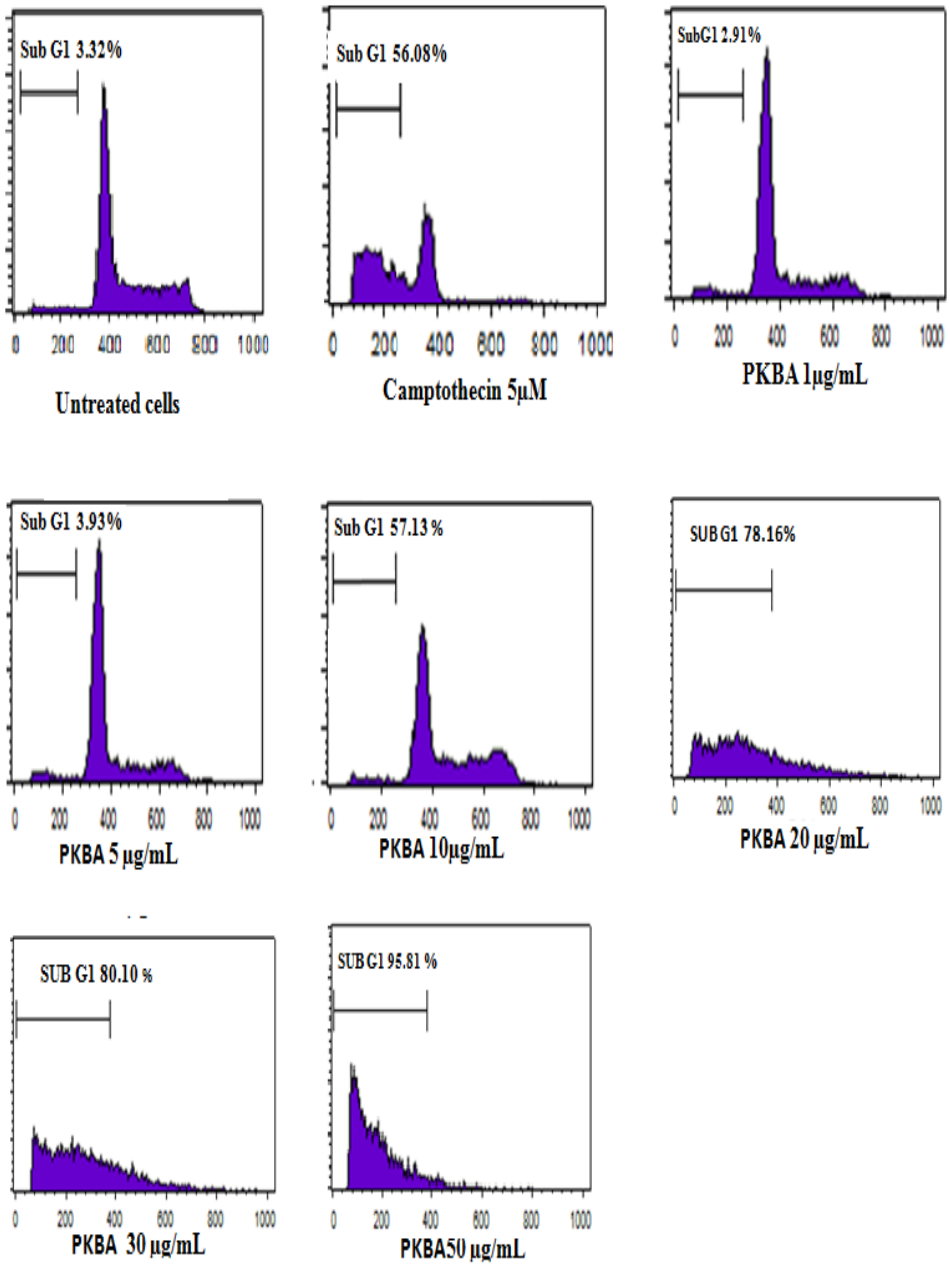


Fig. 4.9. Flow cytometric analysis of DNA content in PKBA treated HL-60 cells. HL-60 cells ($2x10^6$ cells/ml/well) were exposed to indicated concentrations of PKBA for 24 h and stained with PI to determine DNA fluorescence and cell cycle phase distribution as described in materials and methods. Fraction of cells for sub-G1 population analyzed from FL2-A vs. cell counts is shown (%). Data are representative of one of three similar experiments

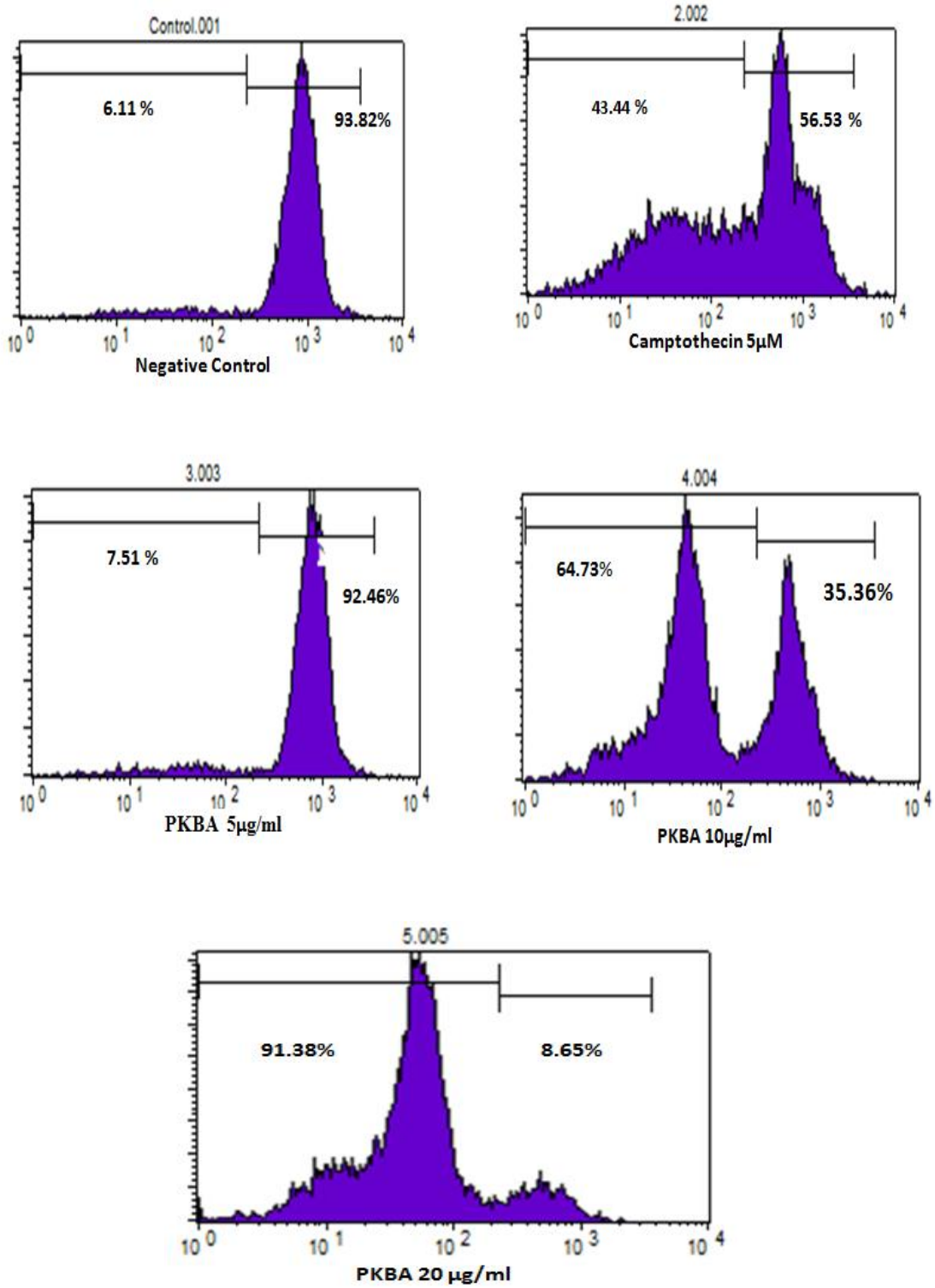
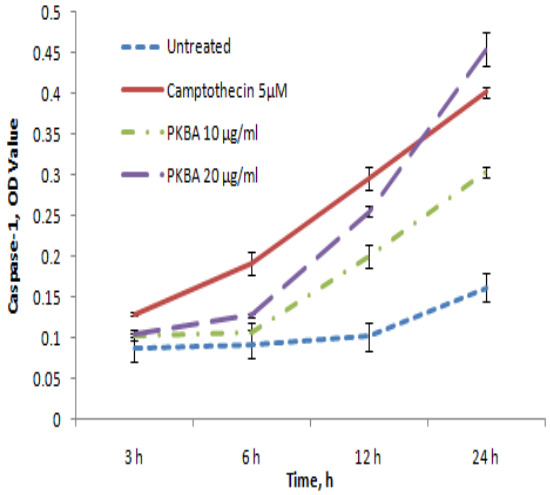


Fig. 4.10. PKBA-induced loss of mitochondrial membrane potential ($\Delta\Psi m$) in HL-60 cells. Cells (1×10^6/ml/6well plates) were incubated with indicated doses of PKBA for 24 h. Thereafter, cells were stained with Rhodamine-123 (10 nM) for 1h and analyzed in FL-1 vs. FL-2 channels of flow cytometer. Data are representative of one of three similar experiments.


Caspase-1, OD Value
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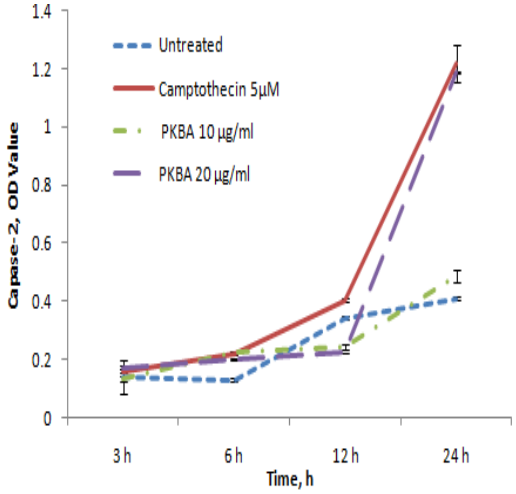
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Untreated
Camptothecin 5μM
PKBA 10 μg/ml
PKBA 20 μg/ml
3 h
6 h
12 h
24 h
Time, h


Capase-2, OD Value
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1
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0.6
0.4
0.2
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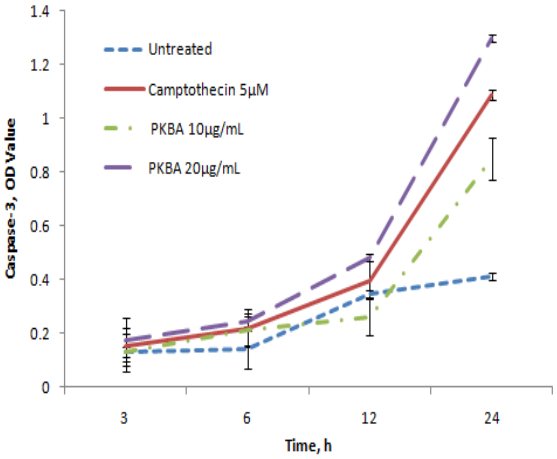
Untreated
Camptothecin 5μM
PKBA 10 μg/ml
PKBA 20 μg/ml
3 h
6 h
12 h
24 h
Time, h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0.6
0.4
0.2
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Untreated
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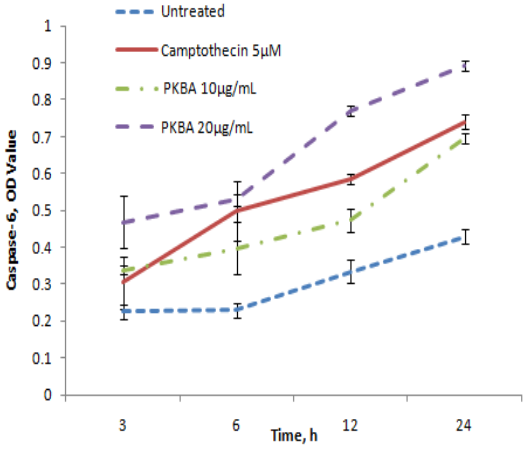
PKBA 10μg/mL
PKBA 20μg/mL
3
6
12
24
Time, h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0.6
0.5
0.4
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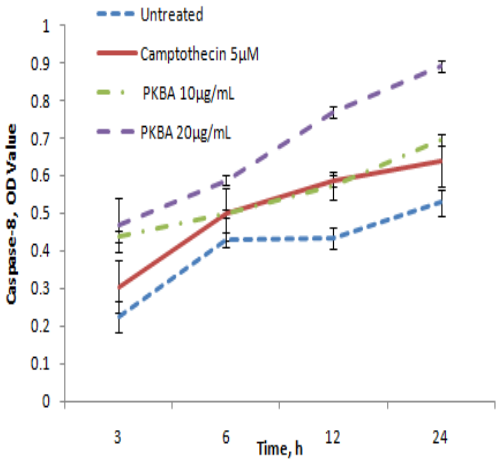
Untreated
Camptothecin 5μM
PKBA 10μg/mL
PKBA 20μg/mL
3
6
12
24
Time, h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0.4
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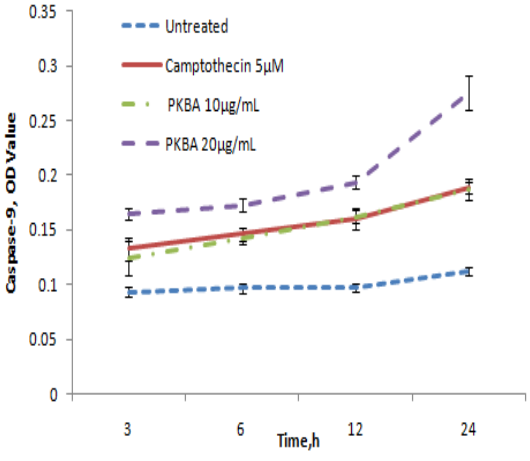
Untreated
Camptothecin 5μM
PKBA 10μg/mL
PKBA 20μg/mL
3
6
12
24
Time, h


Caspase-9, OD Value
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0.25
0.2
0.15
0.1
0.05
0
Untreated
Camptothecin 5μM
PKBA 10μg/mL
PKBA 20μg/mL
3
6
12
24
Time,h

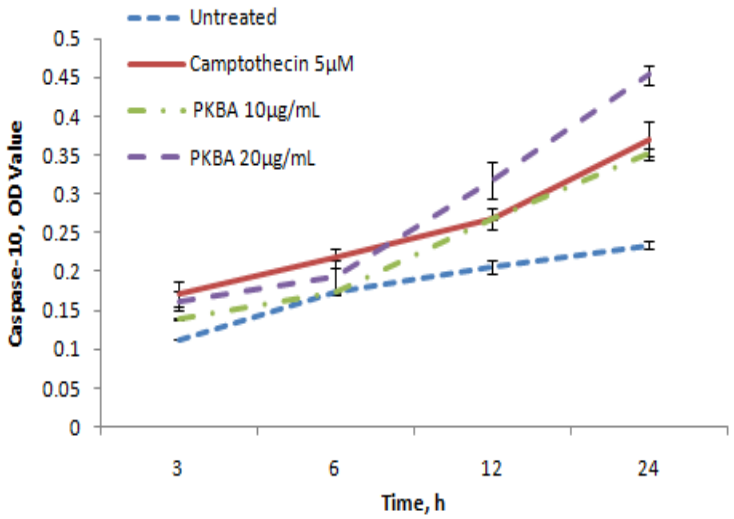


Fig.4.11. PKBA Induces the activation of Initiator caspases-1, -8, -9 &10 and effector caspases -2,-3 & -6. HL-60 cells (2×10^6/ 2ml/6-well plates) in culture were exposed to 10 and 20 µg/ml of PKBA for indicated time periods. The caspases activities were determined colorimetrically by using caspase-colorimetric kits from R&Dsystem as described in methodology. All assays were performed according to the instructions provided by the supplier. Data are Mean±S.D. from three similar experiments.

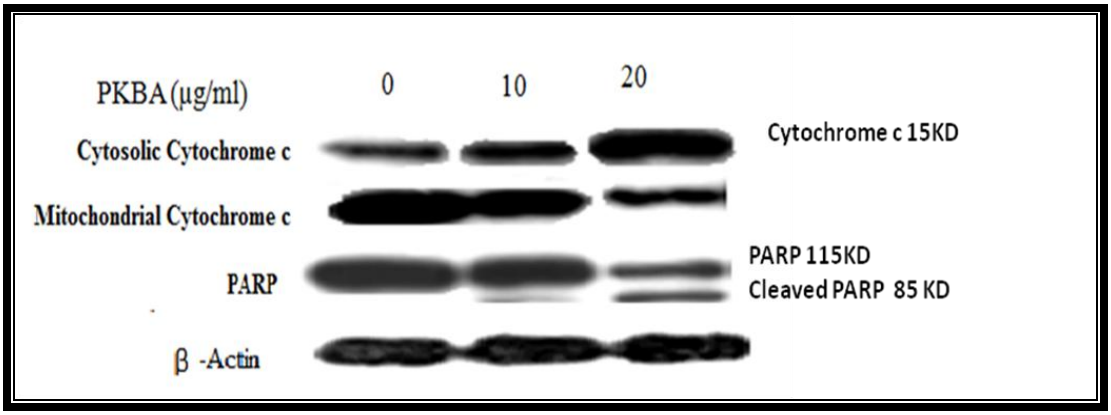


Fig.4.12. Immunoblot analysis of cytochrome c and PARP of PKBA. HL-60 cells (2×10^6/3 ml/6-well plates) were treated with indicated doses of PKBA for 24 h and different fractions were prepared and immunoblotted as described in Section 2. β -Actin used as internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of cytochrome c and PARP. Data are representative of one of three similar experiments.

4.4. Angiogenesis assays

4.4.1. Determination of inhibitory activity of pro-angiogenic factors Ang-2 & MMP-2

Angiopoietins play an important role in angiogenesis and tumor progression. Both Ang-2 and Ang-1 act as ligands of the endothelial cell (EC)-specific tyrosine kinase receptor, Tie2 to stabilize the established vasculature. Ang-2 modulates Ang-1-mediated vessel stabilization by competitively inhibiting the binding of Ang-1 to Tie2. In addition to expression of Ang-2 in ECs, up-regulated Ang2 protein is found in tumor cells. *In vitro* invasion analysis has shown that Ang-2 promotes tumor cell invasion and stimulates the activation of MMP-2.

Inhibition of proangiogenic agents Ang-2 and MMP-2 by PKBA was measured using commercial kits from R&D systems, USA. HL-60 cells treated with the test compound at 10 & 20 µg/ml for 3, 6, 12 & 24 h showed both dose and time dependent inhibition of pro- angiogenic factors Ang-2 (Fig. 4.13 A) and MMP-2 (Fig. 4.13 B). These results further support the anticancer potential of PKBA

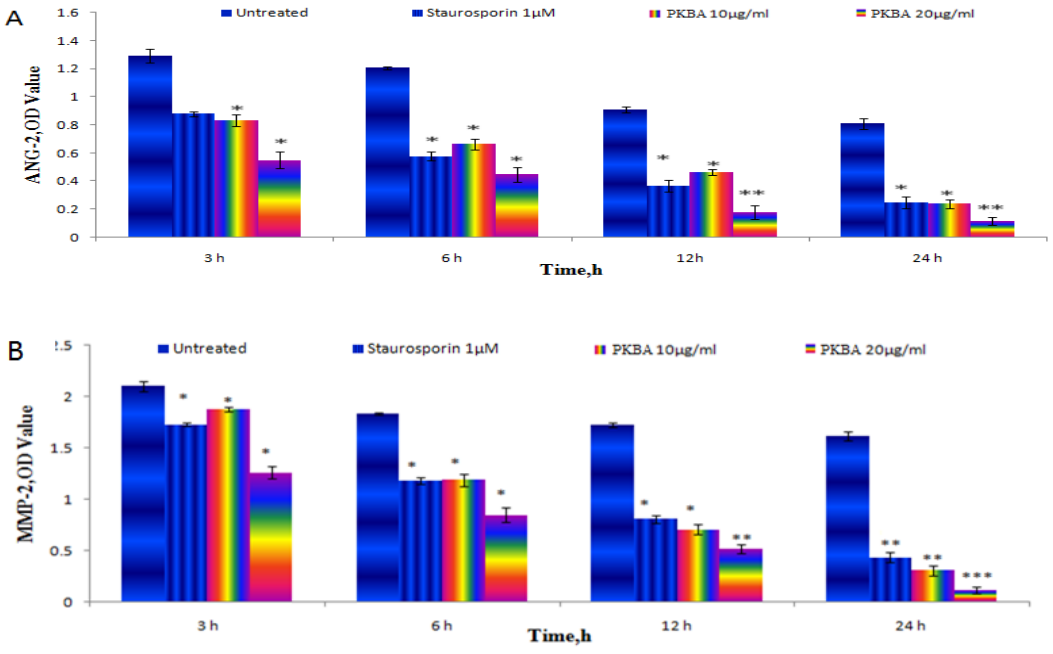


Fig. 4.13. PKBA induces *in-vitro* inhibition of pro-angiogenic factors, ANG-2 (A) and MMP-2 (B). HL-60 cells (2×10^6/ 3ml/6-well plates) in culture were exposed to 10 and 20 µg/ml of PKBA for indicated time periods. ANG-2 and MMP-2 activities were determined colorimetrically by using ANG-2 and MMP-9 kits from R &D system as described in methodology. All assays were performed according to the instructions provided by the supplier. Data are Mean ±S.D. from three similar experiments. P values: *<0.05, **< 0.01, ***0.001

4.5. Pharmacokinetic Studies

4.5.1. Determination of Plasma & Brain Pharmacokinetics

The science of pharmacokinetics (PK) deals with the important aspects of selecting a drug, dosing regimen and monitoring the dosing for appropriate therapeutic or toxic effects for an individual. PK explores what the body does to the drug, and essentially describes the fate of a drug after administration to a living being and involves several features like the extent and rate of absorption, distribution, metabolism and excretion referred to as the ADME. These four criteria influence the drug levels and kinetics of the drug exposure to the tissues and hence influence the performance and pharmacological action of a drug. Pharmacokinetic studies (PK) of PKBA were carried out in Swiss albino mice. Both blood plasma and brain homogenate were evaluated for determining the ADME profile of PKBA. The PK studies in plasma showed a maximum concentration 'C_{max}' of 10 µg/ml and the time to reach the maximum concentration 'T_{max}' was found to be 1 hr. The half-life of PKBA in the plasma '$T_{½}$' was found to be 4.38 hrs and the area under the curve AUC was found to be 50.9 µg/hr/ml. Further, the volume of distribution 'Vd' and clearance 'Cl' from the plasma were found to be 8071.3 ml/kg and 1965.4 ml/hr/kg respectively (Fig 4.14).

The PK studies in brain showed that PKBA was not able to cross the blood brain barrier as no drug was obtained from the brain samples.

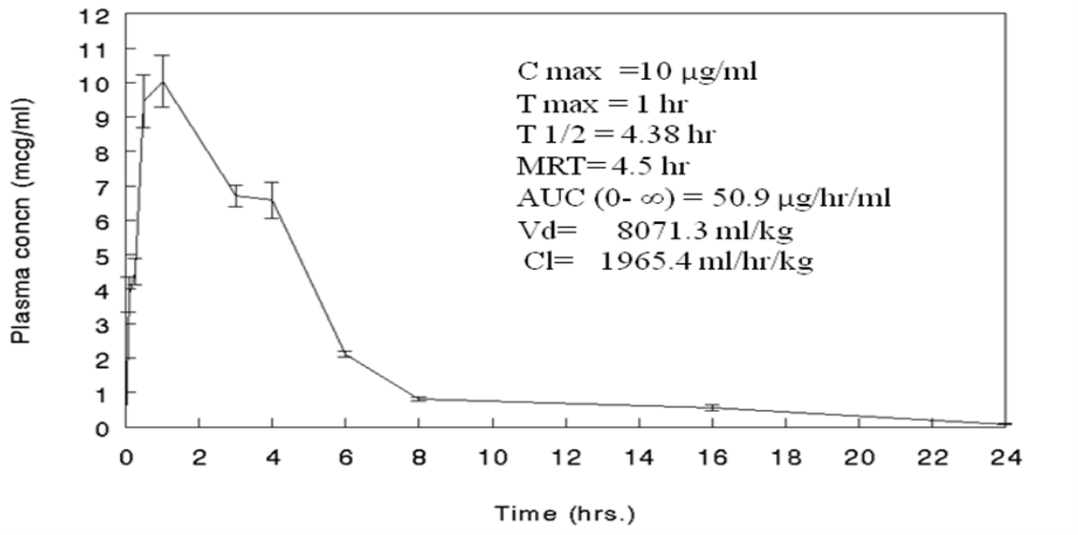


Fig. 4.14. Plasma PK studies of PKBA in Swiss albino mice.

4.6. Nanoparticle based drug delivery system of PKBA

4.6.1. Determination of Particle size, zeta potential, entrapment efficiency and yield of PKBA Nanoparticles (PKBA NPs)

Drug loaded PEG-PLGA copolymer NPs were formulated by oil-in-water single emulsion-solvent evaporation technique and were further appended with transferrin ligands. Particle size and size distribution (polydispersity index) of the formulations were determined, based on dynamic light scattering (DLS), using a Zetasizer (Nano ZS, ZEN3600, Malvern Instrument) with a wavelength of 532 nm at 25 ^{o}C with an angle detection of 90^{o}. The encapsulation efficiency of the particles was determined by the reverse phase isocratic mode of high performance liquid chromatography (RPHPLC) method. The PKBA NPs showed an average particle size of 268 ± 5.1 nm (Fig 4.15).

Zeta potential value is defined by the charge of different components of the NPs. The measurement of zeta potential is a useful method for assessing the extent of PEG coating on the surface of PEGylated NPs and to check whether the drug is truly encapsulated within the NPs or simply adsorbed onto the surface. The nanoparticles prepared from the copolymer of PEG-PLGA have a low negative zeta potential value -13.84 ± 2.3 mv because the carboxylic acid end groups of PLGA are capped by the PEG segments. The protective (stealth) action of PEG is mainly due to the formation of a dense, hydrophilic cloud of flexible chain on the surface of the particle that reduces the hydrophobic interaction with the RES. The tethered and/or chemically anchored PEG chains can undergo spatial conformations, thus preventing the opsonization of particles by the macrophages of the RES, which leads to preferential accumulation. PEG surface modification therefore enhances the circulation time of the molecules and colloidal particle in the blood (Gref et al., 1994).

The transferrin conjugated PEG-PLGA co-polymer based nanoparticles of PKBA showed a yield of 83 %. The indirect entrapment efficiency was found to be 96 % and the direct entrapment efficiency was 68.71 % (Table 4.9).

4.6.2. Determination of *in vitro* release kinetics of drug from nanoparticles

Nanoparticles exhibit their special drug delivery effects in most cases by direct interaction with their environment i.e., their biological environment. Drug release may

occur by: desorption of surface bound drug and diffusion through the nanoparticles matrix. The *in vitro* release kinetics of nanoparticles was performed by dispersing the drug loaded nanoparticles in PBS pH 7.4. The *in-vitro* drug release behaviour of Transferrin coupled PEG-PLGA NPs shows biphasic pattern which is characterized by an initial fast release, followed by a slow sustained release. Results revealed that almost 90 % of drug gets released within 48 h (Fig. 4.16). Biodegradable materials are intended to degrade within the body after the release of the active agent. This eliminates the need for removal of the material after drug release.

4.6.3. Determination of surface morphology and shape

The shape & surface morphology of NPs were evaluated by scanning and transmission electron microscopy (SEM). Most of the particles were spherical in shape although some particles were found to be elliptical (Fig. 4.17, 4.18).

4.6.4. Determination of *in vitro* cytotoxicity

In vitro cytotoxicity of PKBA NPs was evaluated against the same twelve human cancer cell lines as used for PKBA alone namely, IMR-32, SF-295 (both neuroblastoma), PC-3(Prostate), Colo-205 & HCT-15 (both Colon), MCF-7(Breast), OVCAR-5(Ovarian), Hep-2 (Liver), A549 (Lung), THP-1, HL-60 & MOLT-4 (all three Leukemia). Compared to PKBA, PKBA NPs were found to induce more potent cytotoxicity in all the twelve cancer cell lines. In most of the cell lines the growth inhibition of PKBA NPs was 39-94 % at 10 µg/ml which increased to 87-100% at 100 µg/ml (Table 4.10). The growth inhibitory effect against adherent cell line was evaluated by SRB assay (Table 4.10); however the inhibitory effect against suspension cell lines HL-60 (Table 4.11) & MOLT-4 (Table 4.12) was evaluated using MTT assay. The efficacy of PKBA was compared with known anticancer drugs including Adriamycin, Paclitaxel, Mitomicin-C and 5-Fluorouracil.

4.6.5. Determination of IC_{50} values

The *in vitro* cytotoxicity results showed PKBA NPs to be active against all the twelve human cancer cell lines. The IC_{50} values were found to be in the range of 3.5-11 µg/ml with THP-1 showing the lowest value (3.5 µg/ml) and HCT-15 showing the highest value (11 µg/ml) (Table 4.13). The IC_{50} value in HL-60 was 5 µg/ml (Table4.13 & Fig. 4.19) which was less than that of MOLT-4 (6 µg/ml) (Fig.4.20)

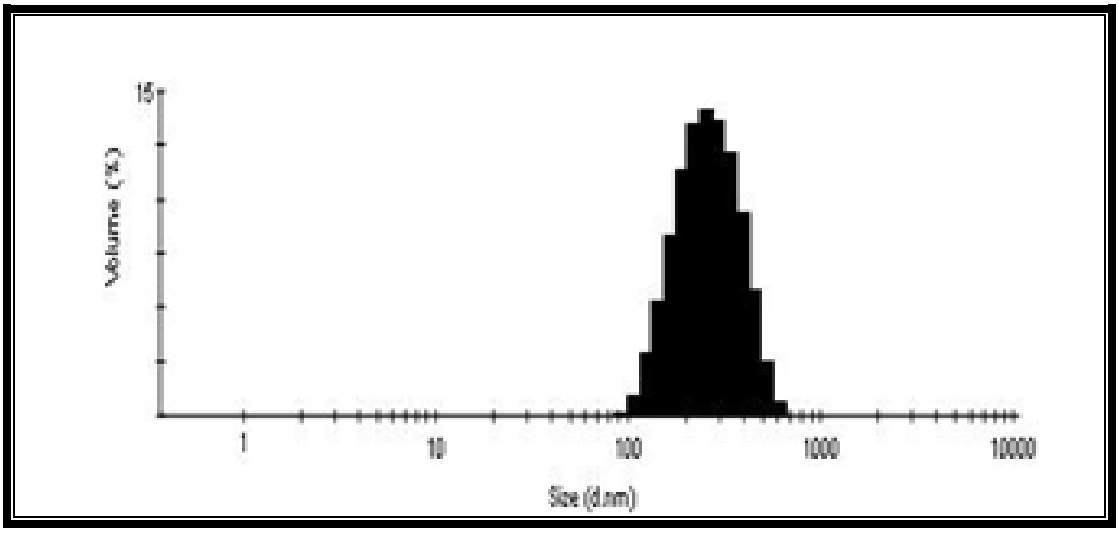


Fig .4.15. Particle size distribution of PKBA NPs

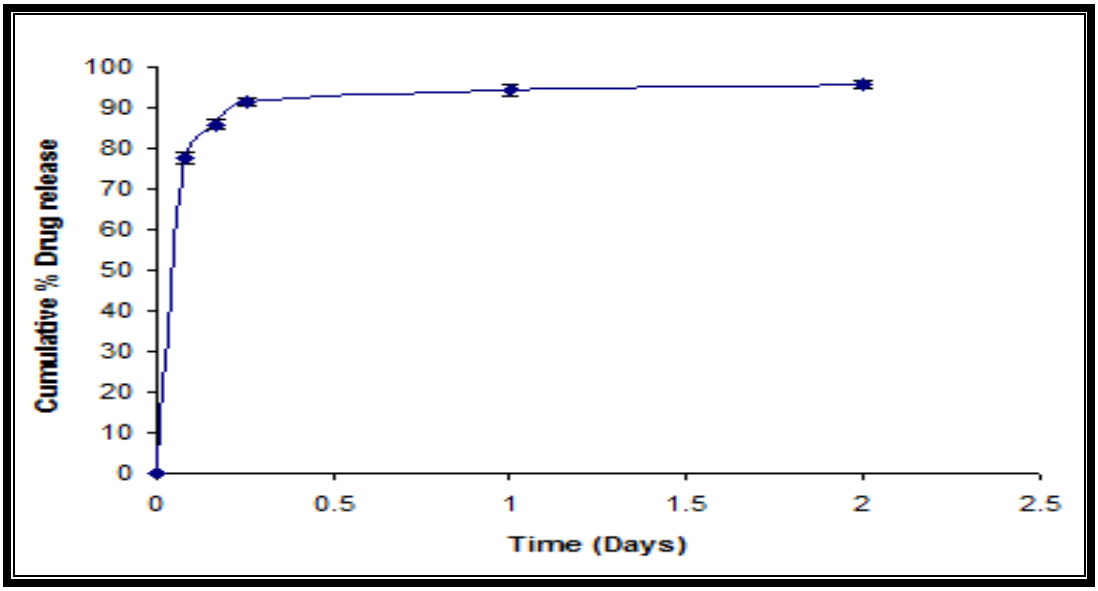


Fig.4.16. *In vitro* release kinetics of PKBA NPs

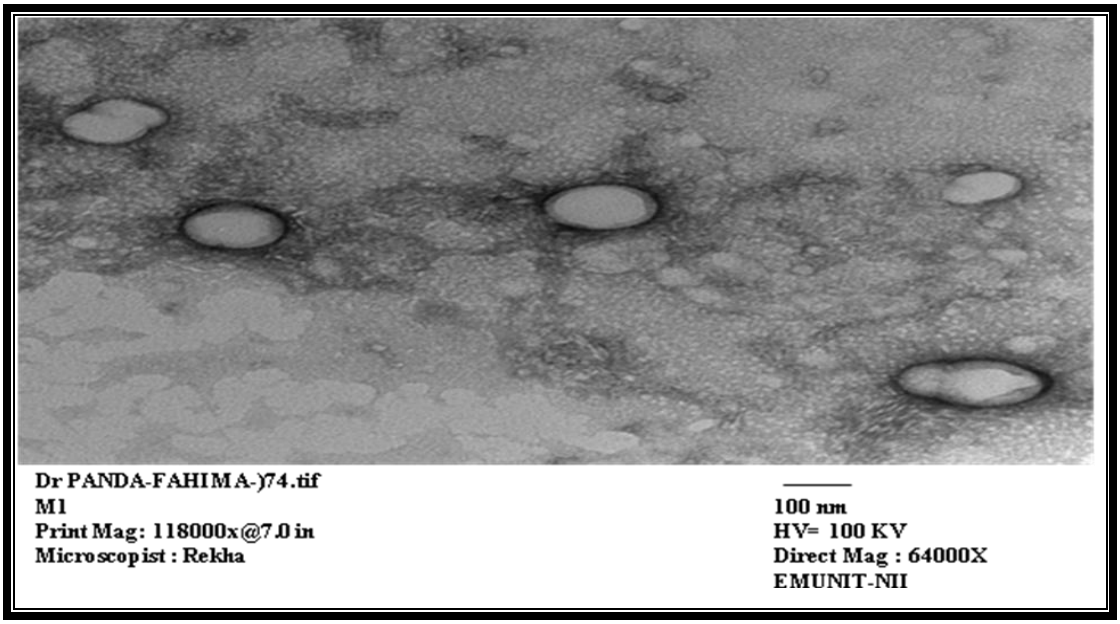


Fig 4.17. Transmission Electron microscopy of PKBA NPs

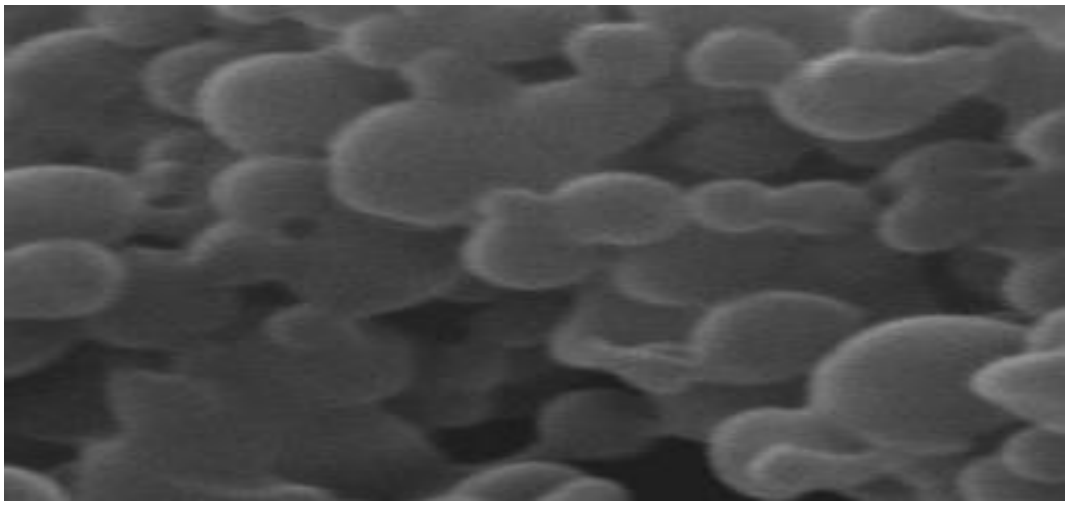


Fig 4.18. Scanning Electron microscopy of PKBA NPs

Sample	Loading (%)	Yield	Indirect Entrapment Efficiency (%)	Direct Entrapment Efficiency (%)	Size (nm)	Zeta Potential	PDI
PKBA NPs	10	83	96	68.71	268 ± 5.1	-13.84 ± 2.3	0.14 ± 0.008

Table 4.9. Physicochemical characterisation of drug loaded PKBA NPs.

Tissue		Neuroblastoma		Prostate	Colon		Breast	Ovary	Liver	Lung	Leukemia
Cell Line		IMR-32	SF-295	PC-3	Colo-205	HCT-15	MCF-7	OVCAR-5	Hep-2	A549	THP-1
Code	Conc. µg/ml	% Growth Inhibition									
PKBA NPs	1	35	31	25	31	16	32	29	32	20	31
	5	50	51	49	40	42	39	41	38	31	56
	10	70	69	53	63	63	49	49	42	39	94
	30	78	80	77	78	75	76	65	55	68	95
	50	82	88	84	89	93	81	77	75	84	96
	70	93	88	91	92	95	86	86	85	86	96
	100	99	97	96	99	97	95	93	92	87	100
Adriamycin	1µM	65	67	-	-	-	68	-	--	-	-
Paclitaxel	1 µM	-	-	-	-	-	-	58	-	-	-
Mitomycin	1 µM	-	-	50	-	-	-	-	58	-	51
5-Fluorouracil	20 µM	-	-	-	50	62	-	-		52	70

Table 4.10. *In vitro* cytotoxicity (percent growth inhibition) of PKBA NPs. Cells were grown in 96 well plates and treated with various concentrations 1,5 10, 30,50, 70 and 100 µg/ml of PKBA NPs for 48 h. The SRB assay was performed as described in material & method section.

Cell Line	HL-60 (Leukemia)			
Conc. µg/ml	**Average O.D**	**SD**	**%Viability**	**% Inhibition**
0	0.991	0.04525	100	0
10	0.2186	0.0757	20	80
30	0.159	0.037321	15	85
50	0.1146	0.019703	11	89
70	0.0925	0.055417	9.29	90.71
100	0.054	0.024075	5	95

Table 4.11 *In vitro* proliferation and percent growth inhibition of PKBA NPs in HL-60 cells. Cells were treated with PKBA NPs for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Cell Line	MOLT-4 (Leukemia)			
Conc. µg/ml	**Average O.D**	**SD**	**%Viability**	**% Inhibition**
0	0.986	0.005783	100	0
10	0.29075	0.051602	30	70
30	0.19775	0.038743	20	80
50	0.15225	0.057823	15	85
70	0.07425	0.054012	7	93
100	0.05	0.069609	5	95

Table 4.12 *In vitro* proliferation and percent growth inhibition of PKBA NPs in MOLT-4 cells. Cells were treated with PKBA NPs for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Tissue	**Cell line**	**PKBA NPs**
		Conc. µg/ml
Neuroblastoma	**IMR-32**	4.2
	SF-295	5.0
Prostate	**PC-3**	8.3
Breast	**MCF-7**	6.6
Ovarian	**OVCAR-5**	8.6
Liver	**Hep-2**	9.9
Lung	**A549**	10.8
Colon	**Colo-205**	6.7
	HCT-15	11
Leukemia	**THP-1**	3.5
	HL-60	5
	Molt-4	6

Table 4.13 IC_{50} values of PKBA NPs against twelve human cancer cell lines

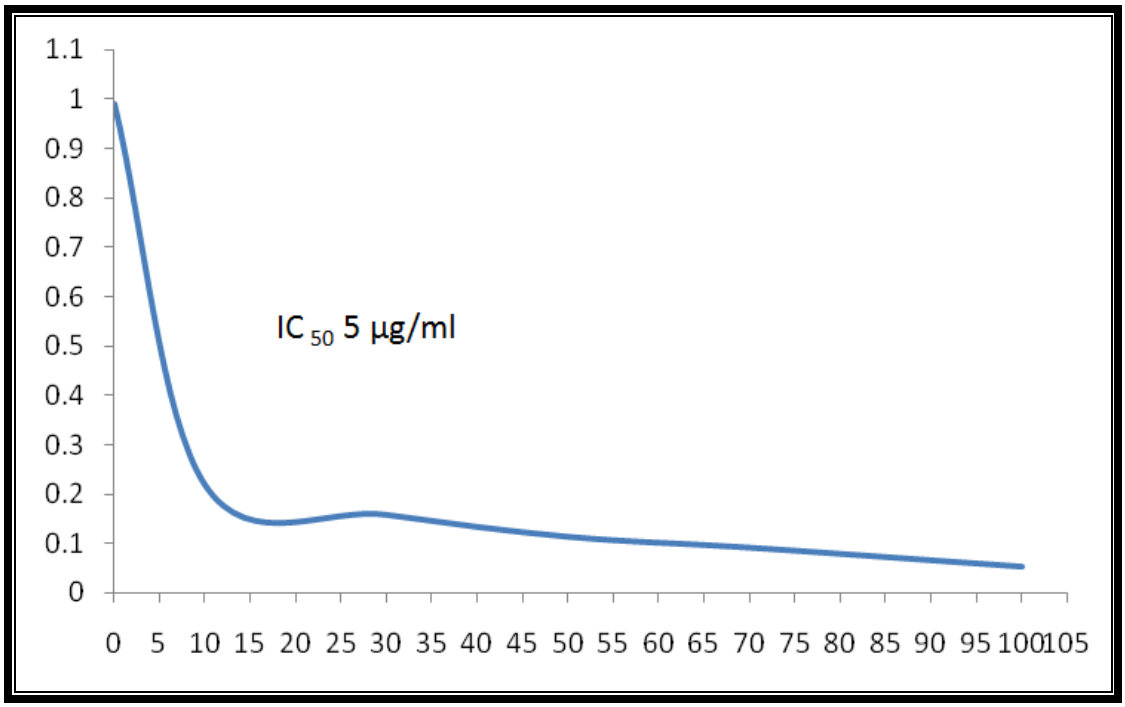


Fig. 4.19 IC_{50} value of PKBA NPs against HL-60 cell line by MTT assay

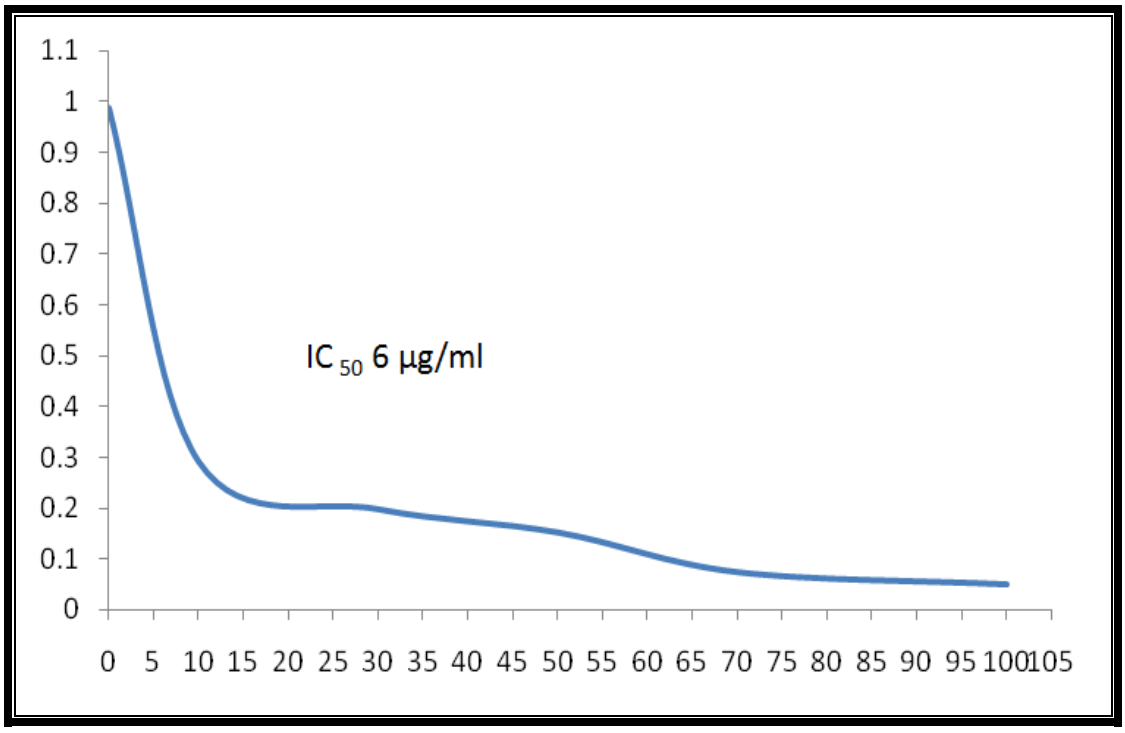


Fig. 4.20 IC_{50} value of PKBA NPs against MOLT-4 cell line by MTT assay

4.6.6. Determination of plasma and brain pharmacokinetics

The Pharmacokinetic studies of PKBA NPs were carried at 1 h. The plasma PK studies of PKBA NPs revealed an increase in the C_{max} (13.624 µg/ml) in 1 h compared to PKBA alone. Further, it was observed that, compared to PKBA, PKBA NPs were able to penetrate the blood brain barrier showing a C_{max} of 1.003 µg/100 mg Brain (Table 4.14).

	1 h	
Animal No.	**PKBA NPs (µg/ml)Plasma**	**PKBA NPs (µg/100 mg)Brain**
1	14.795	1.043
2	13.585	0.963
3	11.830	1.041
4	14.740	0.962
5	13.170	1.044
Mean	**13.624**	**1.003**
SD	1.229	0.0565

Table 4.14. PK studies of PKBA NPs in Plasma and Brain of Swiss albino mice

4.7. Discussion

The gum resin of *Boswellia serrata*, a kind of deciduous tree grown in the dry parts of China and India, has traditionally been used for the treatment of inflammatory and arthritic diseases (Han, 1994). The gum resin has been found to contain four major boswellic acids which include β-boswellic acid, 11-keto-β-boswellic acid, acetyl-β-boswellic acid and acetyl-11-keto-β-boswellic acid. These boswellic acids have repeatedly been reported for their anti-inflammatory, antiproliferative and anticancer activities (Safayhi et al., 1992; Hoernlein et al 1999). Structure activity relationship indicated that the pentacyclic ring skeleton of boswellic acid is responsible for its anti-topoisomerase activity. The anticancer potential of boswellic acids (BAs) has been found to be of the order of acetyl -11-keto-β-boswellic acid (AKBA) as the most active, followed by 11-keto-β-boswellic acid (KBA), acetyl-β-boswellic acid (ABA) and boswellic acid (BA) respectively (Bhawal et al., 2005). Acetyl-11-keto-β-boswellic acid has been shown to induce apoptosis in HL-60 cells by inhibiting Topoisomerase I (Hoernlein et al., 1999). In this regard we have synthesized an acyl derivative of KBA having propionyloxy substitution at 3-hydroxyl functionality. The propionyloxy derivative of 11-keto-β-boswellic acid (PKBA) was found to have a lower IC_{50} value as compared to AKBA (Hoernlein et al., 1999) and was evaluated further for its anticancer and apoptotic potential. The *in vitro* cytotoxicity of PKBA was evaluated in twelve human cancer cell lines of six different tissues. Although PKBA was found to be active against most of these cell lines, however the more potent activity was observed against neuroblastoma and leukemia cell lines. In case of normal Monkey Kidney CV-1 cell line, PKBA did not show any cytotoxicity up to a dose of 100 µg/ml, thus may be considered to be non-toxic to the normal cells. The variation in cytotoxicity from cell line to cell line can be explained due to variation in their molecular characteristics. Since in the present study the "end point method" was used, therefore it was not possible to derive any conclusion about the mode of cytotoxic action. In the method used, only the cell growth or cell numbers in other words, was measured after definite period of incubation in presence of test material and in turn, cell growth was calculated. The reduction of the cell growth can be by cell death, inhibition of cell proliferation or else. Therefore, in the later part, the mode

of anticancer action was studied. Further, the test samples which are found to be cytotoxic *in vitro* need to be evaluated for their *vivo* anticancer potential to characterise them as good therapeutic molecules. It has been emphasised that many test compounds which are active *in vitro* do not show any activity *in vivo*. In fact, many of the agents which are active *in vitro* either fail to show the activity *in vivo* or their activity potential does not communicate with their *in vitro* activity. It is quite obvious also because *in vitro* conditions, the agents directly interact with the cell whereas *in vivo* numbers of barriers are involved in the action of an agent including bioavailability, pharmacokinetic profile, various transport and efflux pumps and many more. Therefore, it was important to evaluate the *in vivo* anticancer potential of PKBA and same has been done. The *in vivo* anti-cancer activity was evaluated using murine tumor models. Although, different murine models are available to determine the *in vivo* anti-cancer activity but murine ascitic model is used very widely because it provides the insight into the anti-cancer potential in general. Studies with ascitic tumor models are followed by studies with solid tumor models. Even though the use of specific tumor models could be more valuable for the study, but it was beyond the scope of the present study.

PKBA showed promising anticancer activity against the entire three tumor models used in the current study, namely, Ehrlich Ascites Carcinoma (EAC), Ehrlich Tumor (ET) and Sarcoma-180 (S-180). The maximum activity was observed in Ehrlich ascitic carcinoma, it may be because the test material and the cancer cells are present at the same site. In case of solid tumors, the effect was less as the drug has to pass through systemic circulation. The statistically significant results at 150 mg/kg of PKBA in case of Sarcoma-180 and Ehrlich tumor showed that it has anti-cancer potential and can pass through the systemic circulation. The *in vivo* anticancer potential of PKBA raised our interest to undertake detailed studies, to determine the mechanism of cell death. Since apoptosis is a key defence strategy against the emergence of cancer, the anticancer agents that activate apoptosis in cancer cells could be valuable anticancer therapeutics (Debatin, 2000). Mechanism of apoptosis is highly complex and sophisticated. Much of the contemporary research in the development of anticancer therapeutics from plants has been focused on investigating

the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells. Anticancer drugs inducing apoptosis, having low side effects and target specific cytotoxicity to the cancer cells are the drugs of choice. Keeping this in mind, an attempt was made to investigate the molecular mechanism of cell death/apoptosis by PKBA in human leukemia HL-60 cells.

Cells undergoing apoptosis are characterized by a series of stereotypic morphological changes such as formation of apoptotic bodies, chromatin condensation, shrinkage of cells, plasma membrane blebbing, chromosomal DNA fragmentation and formation of apoptotic bodies. PKBA showed typical apoptotic morphological changes such as formation of apoptotic bodies and shrinkage of cells which was studied through Fluorescence and Scanning Electron Microscopy. The DNA fragmentation in HL-60 cells was also confirmed by agarose gel electrophoresis, where a discrete ladder pattern was observed in PKBA treated HL-60 cells. Moreover The DNA fragmentation analysis in monkey kidney normal cell line CV- 1 revealed that PKBA was not able to induce fragmentation in normal cells up to the tone of 100 µg/ml which indeed increased its therapeutic value, showing its apoptotic action selectively against cancer cell lines.

The DNA damage induced by PKBA in HL-60 cells was also determined by comet assay. The intensity of staining in the comet's tail region was presumed to be related to the DNA content and DNA damage was estimated from measurement of tail length, tail moment or tail ratio. The differential migration of DNA fragments away from the nuclear head, form a fluorescent tail, producing DNA images resembling comets. The non-degraded DNA in the nucleus of HL-60 cells remaining intact when subjected to electrophoresis, illustrated features to same as untreated cells. HL-60 cells were exposed to PKBA for 24 h, the moderately degraded DNA of apoptotic cells migrated away from the nuclear head as a function of the severity of damage showed comet shape.

Loss of plasma membrane integrity is an early event in apoptosis, independent of the cell type, resulting in the exposure of phosphatidylserine (PS) residues at the outer plasma membrane (Manon et al., 2008). Annexin V in the presence of calcium interacts strongly and specifically with PS and can be used to detect apoptosis by

targeting for the loss of plasma membrane asymmetry. Propidium iodide (PI) stains the cellular DNA of the cells and identifies late apoptotic or necrotic cells, since it does not enter cells with intact membranes. Thus, Annexin V identifies early apoptotic cells where as PI identifies late apoptotic or necrotic cells. In the present study, the cells were stained with both Annexin V-FITC as well as PI. Concentration dependent increase in apoptotic cell population was observed in case of PKBA treated HL-60 cells confirming its apoptotic potential.

The sensitivity of proliferating cells to various death stimuli is usually cell cycle phase specific. Many anticancer agents and DNA damaging agents arrest cell cycle at the G_0/G_1, S, G_2/M phase and then induce apoptosis (Orren and Sancar, 1990). The effect of anti proliferative agent on cell cycle progression appears to depend on the concentration of the compound and also on the duration of the treatment (Loplay et al., 1996). In principle, the DNA of the cancer cell population is intercalated with fluorescent dye (like propidium iodide) and analyzed for its distribution in cell cycle and ploidy level as it flows through the cytometer. This technique is finding wide application especially with regard to discovery of anticancer leads. Loss of DNA is a typical feature of apoptotic cells that occurs as a result of diffusion of degraded DNA out of the cells after endonucleases cleavage. Cells that have lost DNA would take up less stain on staining with PI and appear left to the G_0/G_1 peak. PKBA showed a concentration dependent increase in the sub G_1 peak and progressive loss of G_1 phase as observed from the DNA content histogram analysis. DNA content histogram analysis obtained from PI stained cells showed a sub G_1 peak and a progressive loss of the normal G_0/G_1 peak and blockage of G_2/M phase indicating increase in the reduced or sub- diploid DNA content with concomitant fall in the proportion of the cells with diploid DNA. The gradual reduction in the promotion of cells in the G_0/G_1 phase with the concomitant appearance of apoptotic cells after treatment with PKBA suggest that cells arrested in the G_0/G_1 phase are preferentially undergoing apoptosis.

Mitochondria are supposed to act as central coordinators of cell death that get affected in the early apoptotic process (Guo et al., 2002; Green et al., 1998). The loss of mitochondrial membrane potential is largely due to the opening of PTP, which conduct the leakage of cytochrome c and pro-apoptotic proteins from mitochondria to

the cytosol (Henry et al., 2004). In the current study PKBA was found to induce loss of mitochondrial membrane potential and also lead to the release of cytochrome c from mitochondria into the cytosol in a concentration dependent manner. The review of literature has shown that the loss of mitochondrial membrane potential also results in the activation of caspases.

Caspases represent the family of cysteinyl endopeptidases, which cleave their substrate at specific aspartic acid residues. The initiator or apical caspases (-2,-8,-9,-10 and -11) activate the second group or effector caspases (-3,-6 and-7). The effector caspases have been reported to degrade multiple substrates including the structural and regulatory proteins in cell nucleus, cytoplasm and cytoskeleton leading to the deregulation of vital cell processes and, ultimately to cell death. Caspase-1 has been reported to promote the activation of effector caspase-3 and -7. Caspase-9 is necessary for the cytochrome c dependant activation of caspase -2,-3,-6,-7,-8 & -10 while caspase-3 is required for the activation of caspase-2,-6,-8 & -10 and subsequently the activation of caspase-9 which is cytochrome c dependent (Loss et al., 1995). Caspase-8 is able to activate caspases-1,-2,-3,-6,-7,-9 and -11 (Slee et al., 1999). It has been found that caspase-6 catalyze the activation of caspase-8 and -10 whereas, caspase-2,-7,-8 and -10 can cleave protein substrates directly (Wang et al., 2002). Caspases and their regulators are potentially attractive targets towards the development of new anticancer therapeutics. The present study demonstrates PKBA to induce the activation of caspases -1, -2, -3 -8, -6 -9, & -10 in a dose and time dependant manner.

Several reports have shown that an elevation in the level of caspases especially caspase-3 results in the cleavage of a DNA repair enzyme, Poly-ADP-ribose polymerase (PARP), thus preventing any unwanted DNA repair. The sequence at which caspase-3 cleaves PARP is very well conserved in the PARP protein indicating the potential importance of PARP cleavage in apoptosis. Full length PARP is a 116 KDa protein involved in the repair of DNA, in differentiation and in chromatin structure formation. During apoptosis the protein is cleaved by caspase-3, and possibly other caspases into an 89 KDa fragment, thus preventing any unwanted DNA repair. PKBA treated HL-60 cells showed cleavage of PARP in a concentration

dependent manner which later activates certain endonucleases, thus leading to the DNA fragmentation as discussed earlier.

The existing literature indicates that boswellic acids induce apoptosis in cancer cells by inhibiting topoisomerases. Topoisomerases are the essential enzymes that control and modify the topological state of DNA. Topoisomerse I and II act by sequential breakage and reunion of single and double strands of DNA respectively. The topoisomerase targeting drugs can either be classified as topo poisons or topo catalytic inhibitors. The former acts by stabilizing DNA cleavable enzyme complexes leading to DNA break and latter acts by stabilizing enzyme where both DNA strands remain intact and no DNA breaks occur, resulting in apoptosis. PKBA when subjected to topoisomerase inhibition studies inhibited the enzymatic activity of both topoisomerases I & II strongly and at lower concentration than AKBA (Hoernlein et al., 1999) The dual inhibitory activity of PKBA may be due to the recognition of structural motifs present on both topoisomerase I & II, however, there is as yet no detailed understanding of the factor that result in selective or dual inhibition, but the structure-activity studies in several classes have shown that the changes in the structure can influence topo I/II selectivity.

These observations therefore reveal PKBA to induce apoptosis as characterized by various biological parameters such as membrane blebbing, chromatin condensation, nuclear fragmentation, PS exposure to outer membrane, increased sub G_0 population, loss of mitochondrial membrane potential, release of cytochrome c from mitochondria, intracellular caspase activation, PARP cleavage, DNA fragmentation and topoisomerase inhibition. The above studies lead to a conclusion that, PKBA induced apoptosis in HL-60 cells is *via* mitochondrial-dependent pathway and topoisomerases I & II are the targets responsible for apoptosis.

The anticancer therapeutic potential of PKBA was further supported by its inhibitory effect on tumor angiogenesis. Angiogenesis is a pre-requisite for the growth of solid tumors and vascular targeting has been explored as a potential strategy to suppress tumor growth and metastasis. Matrix metalloproteinase's (MMPs) and angiopoietins play important role in angiogenesis process for tumor progression and metastasis. Endothelial and tumor cells show increased expression of MMPs which are correlated

with the tumor grade. Accumulated evidences have indicated that production of Ang-2 is implicated in tumor progression. Ang2 promotes the tumor cell invasion and stimulates the activation of MMP-2. Therefore Pro-angiogenesis factors like Ang-2 and MMP-2 could be important targets to suppress tumor growth and metastasis. The present study demonstrates PKBA to inhibit both ANG-2 and MMP-2 in HL-60 cells in a concentration as well as time dependant manner.

After confirmation of with the anticancer therapeutic potential of PKBA, it was essential to determine the fate of this analogue in the body i.e. its ADME profile *via* pharmacokinetic studies. The science of pharmacokinetics (PK) deals with the important aspects of selecting a drug, dosing regimen and monitoring the dosing for appropriate therapeutic or toxic effects for an individual. PK explores what the body does to the drug, and essentially describes the fate of a drug after administration to a living being and involves several features like the extent and rate of absorption, distribution, metabolism and excretion referred to as the ADME. These four criteria influence the drug levels and kinetics of the drug exposure to the tissues and hence influence the performance and pharmacological action of a drug. The PK studies of PKBA were conducted in Swiss albino mice, taking plasma and brain into consideration. The assessment of bioavailability from plasma conc.-time data usually involves determining the maximum (Peak) plasma drug conc. (C_{max}) and the area under the plasma conc.-time curve (AUC). The plasma drug concentration increases with the rate of absorption; therefore the most widely used general index of absorption is C_{max} . AUC is another reliable measure of bioavailability. It is directly proportional to the total amount of unchanged drug that reaches systemic circulation. The PK studies of PKBA in plasma revealed a C_{max} 10 µg/ml and the time taken to reach C_{max} was 1 h (T_{max}) with a half life of 4.43 h. The other parameters being evaluated also revealed the good plasma PK profile of PKBA. However, the PK studies in brain showed that PKBA could not penetrate the blood brain barrier as no drug was recovered from brain samples.

In order to improve its therapeutic efficacy and to utilize it for brain targeting, a nanoparticle based drug delivery system of PKBA was developed. Biodegradable co-polymer, PEG-PLGA was used for the preparation of nanoparticles. Further the

particles were appended with transferrin proteins to make them better for brain targeting. Nanoparticles of PKBA (PKBA NPs) when subjected to *in vitro* cytotoxicity studies showed more cytotoxic effect with much lower IC_{50} values than PKBA alone. The comparative pharmacokinetic studies of PKBA NPs after 1 h revealed a C_{max} of 13.624 µg/ml in plasma and were also able to penetrate the blood brain barrier showing a C_{max} of 1.003 µg/100 mg brain after 1 h.

These studies indicate that PKBA has a potential to be developed as an anticancer therapeutic agent and its therapeutic potential is further improved by designing a nanoparitcle based drug delivery system.

4.8. Antiproliferative assays of HKBA

4.8.1. Determination of *in vitro* cytotoxicty in human cancer cell lines

HKBA showed growth inhibition between 75-100 % against most of the cell lines at 100 µg/ml. At lower concentration i.e. 70, 50, 30 10, 5 and 1 µg/ml activities the same was found to be 67-94, 63-91, 56-91, 51-94, 26-66 and 11-39 % respectively. 5-Flurouracil, Adriamycin, Mitomycin c and Paclitaxel were used as reference and various degree of growth inhibition was observed. Thus, HKBA showed concentration dependent cytotoxicity depending on cell lines (Table 4.15, 4.16, 4.17).

4.8.2. Determination of IC_{50} values

The IC_{50} values of HKBA were found to be in the range of 2.4 to 13.9 µg/ml with OVCAR-5 showing the lowest value (2.4 µg/ml) and A549 showing the highest value (13.9 µg/ml). The IC_{50} values for adherent cell lines, IMR-32, SF-295, PC-3, Colo-205, HCT-15, MCF-7, OVCAR-5, Hep-2, A549 and THP-1 was calculated by SRB (Table 4.18) method and that of suspension cell lines HL-60 (Fig 4.21) and MOLT-4 (Fig 4.22) was calculated via MTT assay. The IC_{50} value of HL-60 (4.5 µg/ml) was less than that of MOLT-4 (6 µg/ml).

Tissue		Neuroblastoma		Prostate	Colon	Breast	Ovary	Liver	Lung		Leukemia
Cell Line		IMR-32	SF-295	PC-3	Colo-205	MCF-7	OVCAR-5	Hep-2	A549	HCT-15	THP-1
Code	Conc. µg/ml	% Growth Inhibition									
HKBA	1	26	28	38	33	39	39	20	13	11	19
	5	43	45	65	52	55	66	26	24	35	32
	10	69	68	78	71	62	77	51	68	90	94
	30	81	73	82	75	65	81	56	78	91	89
	50	89	78	89	79	74	86	63	82	97	91
	70	94	80	92	82	82	91	67	84	97	94
	100	94	99	100	88	93	100	75	86	98	97
Adriamycin	1µM	65	67	-	-	68	-	--	-	-	-
Paclitaxel	1 µM	-	-	-	-	-	58	-	-	-	-
Mitomycin	1 µM	-	-	50	-	-	-	58	-	-	51
5-Flurouracil	20 µM	-	-	-	50	-	-		52	62	70

Table 4.15. *In vitro* cytotoxicity (percent growth inhibition) of HKBA. Cells were grown in 96 well plates and treated with various concentrations 1,5 10, 30,50, 70 and 100 µg/ml of HKBA for 48 h. The SRB assay was performed as described in material & method section.

Cell Line	HL-60 (Leukemia)			
Conc. µg/ml	**Average O.D**	**SD**	**%Viability**	**% Inhibition**
0	0.941	0.03528	100	0
10	0.33525	0.020903	33.3	66.7
30	0.2005	0.103313	22.2	77.8
50	0.2235	0.053805	22.2	77.8
70	0.1725	0.007047	18.8	81.2
100	0.14075	0.043828	15	85

Table 4.16 *In vitro* proliferation and percent growth inhibition of HKBA in HL-60 cells. Cells were treated with PKBA for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Cell Line	MOLT-4 (Leukemia)			
Conc. µg/ml	**Average O.D**	**SD**	**%Viability**	**% Inhibition**
0	0.976	0.006782	100	0
10	0.36825	0.022157	37.1	62.9
30	0.23	0.027785	23.7	76.3
50	0.20975	0.009106	21.5	78.5
70	0.204	0.06121	21	79
100	0.0575	0.001732	5.1	94.9

Table 4.17 *In vitro* proliferation and percent growth inhibition of HKBA in MOLT-4 cells. Cells were treated with HKBA for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Tissue	Cell line	IC_{50} (µg/ml) of HKBA
Neuroblastoma	IMR-32	6.7
	SF-295	6.5
Prostate	PC-3	2.7
Breast	MCF-7	4.2
Ovarian	OVCAR-5	2.4
Liver	Hep-2	19
Lung	A549	13.9
	Colo-205	5
Colon	HCT-15	18
	THP-1	7.9
Leukemia	HL-60	4.5
	Molt-4	6
Normal Monkey Kidney	CV-1	> 50

Table 4.18 IC_{50} values of HKBA against twelve human cancer cell lines

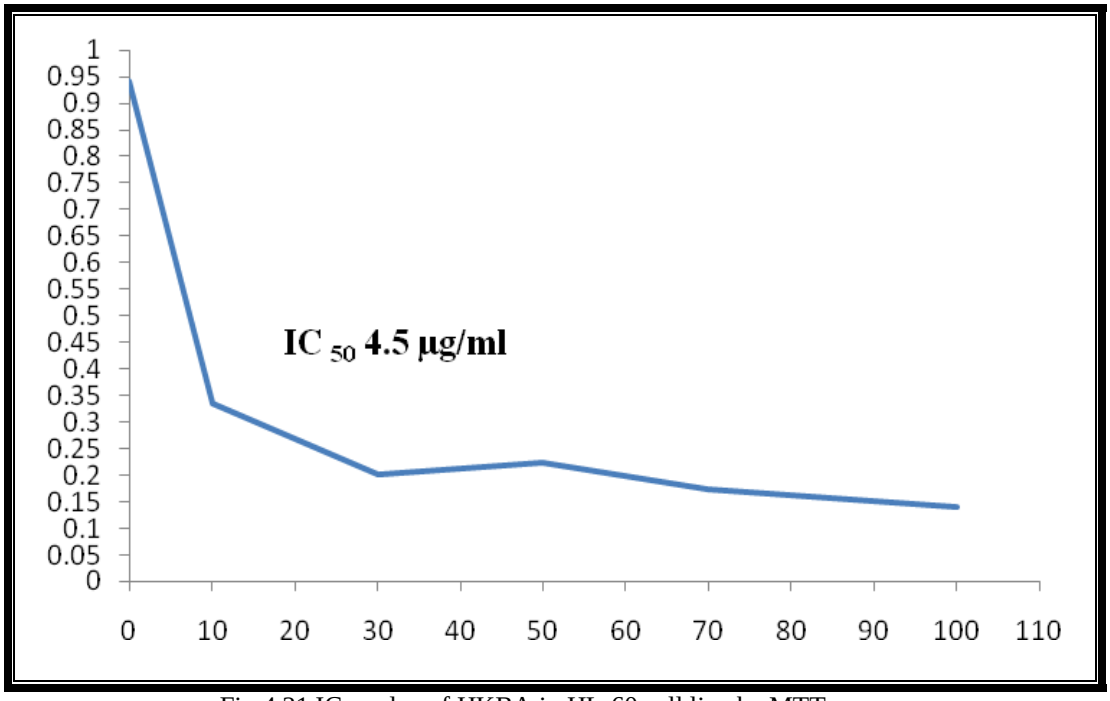


Fig.4.21 IC_{50} value of HKBA in HL-60 cell line by MTT assay

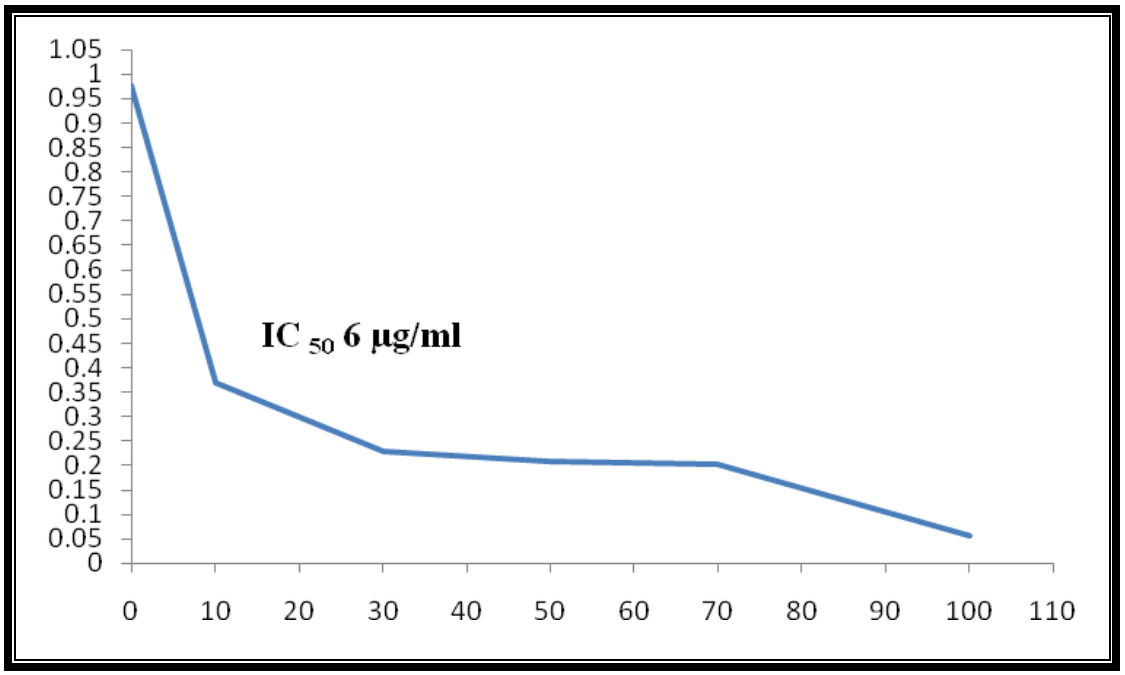


Fig.4.22 IC_{50} value of HKBA in MOLT-4 cell line by MTT assay

4.9. *In vivo* Anti cancer activity

4.9.1. Determination of *In vivo* anti cancer activity against Ehrlich ascites carcinoma

In vivo anticancer activity of HKBA against Ehrlich ascites carcinoma (EAC) is shown in Table 4.19. HKBA showed 47.54 ±0.34 % and 67.29 ±0.48 % at 50 and 60 mg/kg i.p. dose level respectively. 5-FU (Positive control) at 20 mg/kg, i.p. showed 94.66 ±0.32 % tumor growth inhibition ($p = < 0.01$).

4.9.2. Determination of *In vivo* Anti cancer activity against Ehrlich tumor

The *in vivo* anticancer activity of HKBA against Ehrlich tumor (solid) is depicted in Table 4.20. HKBA showed 35.65 ±0.32 % and 50.64 ±0.42 % at 50 and 60 mg/kg, i.p. respectively. The positive control 5-FU showed 48.11 ± 0.31 % ($p = < 0.05$) tumor growth inhibition.

4.9.3. Determination of *In vivo* Anti cancer activity against sarcoma-180

In vivo anti cancer activity of HKBA against sarcoma-180 (solid) was found to be 38.35 ±0.62 % and 54.36 ±0.61 % at 50 and 60 mg/kg, i.p. dose levels (Table 4.21). 5 FU (positive control) at 22 mg/kg, i.p. dose level inhibited tumor growth by 46.75 ±1.12 % ($p = < 0.05$).

Sample	Dose (mg/ kg i.p.)	Animals/ Mortality	Tumor volume (ml)	Cell count (10^7)	% Tumor growth inhibition
Control	**NS**	10/0	9.05 ±0.15	286.95 ±3.26	-
HKBA	**50**	7/0	4.79 ± 0.22*	150.52 ± 5.60*	47.54±0.34*
	60	7/0	3.14± 0.14**	93.84± 0.51	67.29±0.48**
5 FU	**20**	7/0	0.92 ± 1.02**	15.32 ± 1.3	94.66 ±0.32**

Table 4.19. *In vivo* anticancer activity of HKBA against Ehrlich Ascities Carcinoma. * represent significant (p= < 0.05), ** highly significant (p= < 0.01). Data are Mean ± S.D.

Sample	Dose (mg/ kg i.p.)	Animals/ Mortality	Body Weight (g)	Tumor Weight (mg)	% Tumor growth inhibition
Control	**NS**	10/0	21.21 ± 0.35	1266.05 ± 81.22	-
HKBA	**50**	7/0	20.21 ± 0.40	814.66 ± 49.30	35.65 ±0.32
	60	7/0	20.02 ± 0.61	713.45 ±35.82**	50.64 ±0.42 **
5 FU	**22**	7/0	18.85 ± 0.50	722.14 ± 50.1*	48.11 ± 0.31*

Table 4.20. in vivo anticancer activity of HKBA against Ehrlich Tumor (solid) * represents significant (p= < 0.05), ** highly significant (p= < 0.01) Data are Mean ± S.D.

Sample	Dose (mg/ kg i.p.)	Animals/ Mortality	Body Weight (g)	Tumor Weight (mg)	% Tumor growth inhibition
Control	NS	10/0	21.33 ± 0.45	1136.05±73.31	-
HKBA	50	7/0	21.25 ± 0.50	723.30 ± 52.91	38.35 ±0.62
	60	7/0	19.28 ± 0.63*	659.76±50.01**	54.36 ±0.61**
5 FU	22	7/0	18.72 ± 0.49**	711.22±52.11*	46.75 ± 1.12*

Table 4.21. *in vivo* anticancer activity of HKBA against Sarcoma 180 (solid) * represents significant (p= < 0.05), ** highly significant (p= < 0.01) Data are Mean ± S.D

4.10. Apoptotic assays

4.10.1. Determination of DNA fragmentation in HL-60 cell line

Induction of DNA fragmentation by HKBA treatment was investigated by isolating genomic DNA from HL-60 cells. Cells treated with test compound exhibited typical DNA ladder formation at 5, 10 and 20 µg/ml after 24 h (Fig 4.23A). Camptothecin taken as a positive control showed DNA fragmentation after 6 h treatment at 5 µM. However, no ladder formation was observed in untreated cells. DNA fragmentation assay with normal monkey kidney CV-1 cells did not show any ladder formation in both treated and untreated cells (Fig 4.23B).

4.10.2. Determination of nuclear morphologic features

After treatment with different concentrations of HKBA (1 & 5 µg/ml) for 18 h, HL-60 cells when observed under fluorescence microscope showed marked morphologic changes compared to control. Nuclei of untreated cells appeared round in shape (Fig 4.24a), while treatment with HKBA resulted in nuclear condensation and formation of apoptotic bodies (Fig. 4.24 c, d). The morphological changes were accompanied by increase in number of scattered apoptotic bodies. Camptothecin taken as positive control also showed clear apoptosis (Fig 4.24 b).

4.10.3.Determination of morphological changes by Scanning electron microscopy

HL-60 cells were treated with 5 µg/ml of HKBA for 18 h and analyzed by scanning electron microscope. SEM examination revealed that untreated cells were spherical in shape with a few surface projections (Fig. 4.25 a, b & c), however cells treated with test compound showed smoothening of cell surface, decrease in cell size and a clear apoptosis (Fig. 4.25 d, e, f).

4.10.4. Determination of phosphatidylserine externalization by Annexin V-FITC

The annexin V-FITC (stains phosphatidylserine residues) /PI (stains DNA) dual staining assay was used to detect apoptotic cells. Positive staining with annexin V-FITC correlates with loss of membrane polarity, and the complete loss of membrane integrity will lead to apoptosis or necrosis. In contrast, PI can only enter cells after loss of membrane integrity. Thus, dual staining with annexin V and PI allows clear discrimination between unaffected cells, early apoptotic cells and late apoptotic cells. After HKBA treatment, the cells were analyzed by flowcytometer. The results showed

that, the basal apoptotic population in the untreated culture was 3.5 %. After treatment with 5 & 10 µg/ml of HKBA for 24 h, the apoptotic cell population was found to be to about 80.78 % & 73.44 % respectively, the necrotic population at the respective concentrations was found to be 19.22 % and 26.56 %. Camptothecin, used as a positive control, produced about 93.78 % apoptotic population during the same exposure period (Fig 4.26).

4.10.5. Determination of DNA damage by comet assay

Comet assay under neutral electrophoresis conditions was performed to examine nuclear DNA integrities, so that DNA double-strand breaks could be detected. DNA strand breakages in HL-60 cells were analyzed after exposure to 5 &10 µg/ml of HKBA for 24 h. The image (Fig 4.27c) of representative nuclei after electrophoresis of HKBA (10 µg/ml) treated cells showed formation of typical comets, with Head DNA of 65.86 % and 31.23 % at 5 and 10 µg/ml respectively. Tail DNA which reveals the actual DNA damage was found to be 34.14 % and 68.77 % at the respective concentrations. The Olive Tail movement was 3.05 % and 7.23 % and Tail length was found to be 12.94 % and 28.42 % respectively (Table.4.22). Camptothecin (5 µM) (Fig 4.22 b) taken as a positive control showed a head DNA of 60.06 % and a Tail DNA of 39.94 %.

4.10.6. Determination of sub G_1, G_0/G_1 & G_2/M phases of cell cycle

An effective strategy to inhibit tumor growth is deregulated cell cycle progression in cancer cells. Effect of HKBA on cell cycle progression in HL-60 cells was examined. Cells were treated with test compound at a dosage of 1, 5, and 10 µg/ml for 24 h and FACS analysis was followed. The DNA histogram showed that HKBA induces concentration dependent increase in hypo diploid sub-G1 DNA fraction (<2n DNA) (Fig.4.28).The sub-G1 DNA fraction was 3.76 % in untreated cells however after treatment with test compound (10 µg/ml) it increased to about 88.13 %.

4.10.7. Determination of loss of mitochondrial membrane potential

Upon HKBA treatment the fluorescent intensity, an indicator for mitochondrial membrane potential, was reduced from 93.60 % to 0.26 % after 24 h of treatment at 10 µg/ml, consistent with the results that HL-60 cell apoptosis occurred 24 h after treatment with HKBA at the same concentration (Fig 4.29).

4.10.8. Determination of intracellular caspase activity

Despite the variety of apoptosis-initiating events available, programmed cell death signaling pathways finally converge on common effector cellular disassembly machinery mediated by a family of cysteinyl endopeptidases known as caspases. The caspase proteolytic signaling cascades are interconnected and due to overlapping substrate specificity they are also partially redundant. Caspase-9 is necessary for the cytochrome c-dependant activation of caspase-2, -3, -6, -7, -8 and -10, caspase-3 is required for activating caspase-2, -6, -8 and -10 and subsequently for the cytochrome c-dependent activation of caspase-9. Caspase-8 is able to activate *in vitro* seven zymogens of other caspases-1,-2,-3,-6,-7,-9 and -11, caspase-6 catalyzes the activation of caspase-8 and -10 and caspase-2,-7,-8 and -10 may directly cleave protein substrates. In the present study, it was found that HKBA treatment at 5 & 10 µg/ml for 24 h resulted in enhancement in the activity of different caspases in HL-60 cells. Caspase activities were measured using commercial kits from R&D. Results revealed that HL-60 cells treated with 10 µg/ml of test compound for 24 h enhanced the activation of caspase-8,-9 and -10 by 2 fold (Fig. 4.30) and that of caspase-1, -2 , -3 and -6 by 10 fold (Fig. 4.30). These findings suggest that caspase activation is involved in HKBA-induced apoptosis in HL-60 cells.

4.10.9. Determination of cytochrome c & PARP by western blotting

HKBA induced loss of mitochondrial membrane potential has its relevence towards the opening of PTP and release of cytochrome c from mitochondria (Fig.4.31). Initially cytochrome c is known to activate caspase-9 and then executioner caspase-3 which used PARP as a substrate that inhibited the repair of damaged DNA. In PKBA treated HL-60 cells the cleavage of PARP occurs in a concentration dependent manner (Fig.4.31) thereby, confirming the involvement of intrinsic apoptotic pathway.

4.10.10. Determination of topoisomerase I & II inhibitory activity

Topoisomerases (I & II) are the essential enzymes for proliferation of eukaryotic cells (Nakagawa et al., 2006; Osheroff et al., 1983). Drugs that target topoisomerases are among the most effective anticancer drugs. In our previous studies we found that PKBA inhibited the enzymatic activity of both the topoisomerases I & II at 20 µg/ml.

The present study showed that HKBA was also able to inhibit the enzymatic activity of topoisomerases I & II, at lower concentration of 10 µg /ml (Fig.4.32). The inhibitory activity of HKBA was compared with the reference molecules camptothecin and etoposide for topoisomerase I & II respectively.

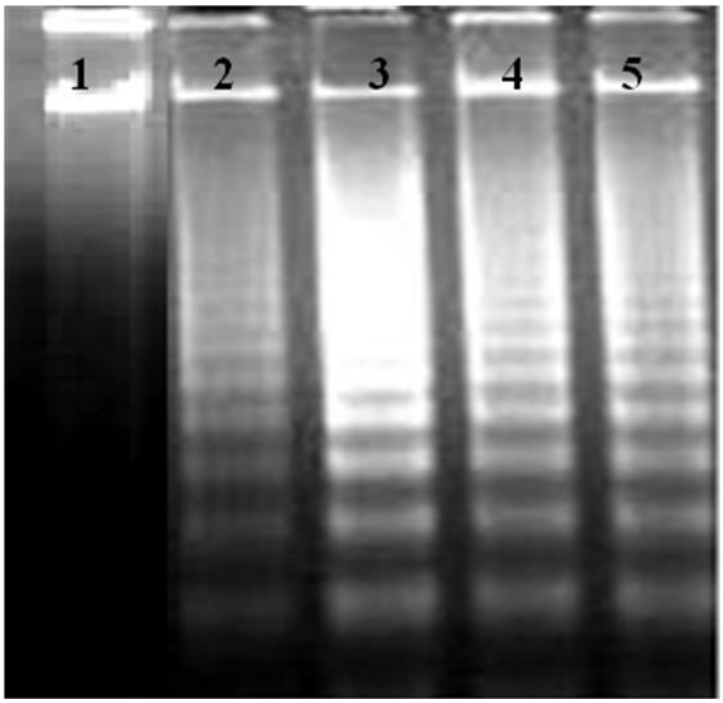


Fig.4.23A. HKBA induces DNA fragmentation in HL-60 cells. Cells ($2x10^6$ /ml/well) were incubated with HKBA at various concentrations for 24 h. Lane-1 (Untreated cells), Lane-2 (HL-60 cells+5 µM Camptothecin, 6h), Lane-3, 4 & 5 (HL-60 cells + 20, 10 & 5 µg/ml HKBA).

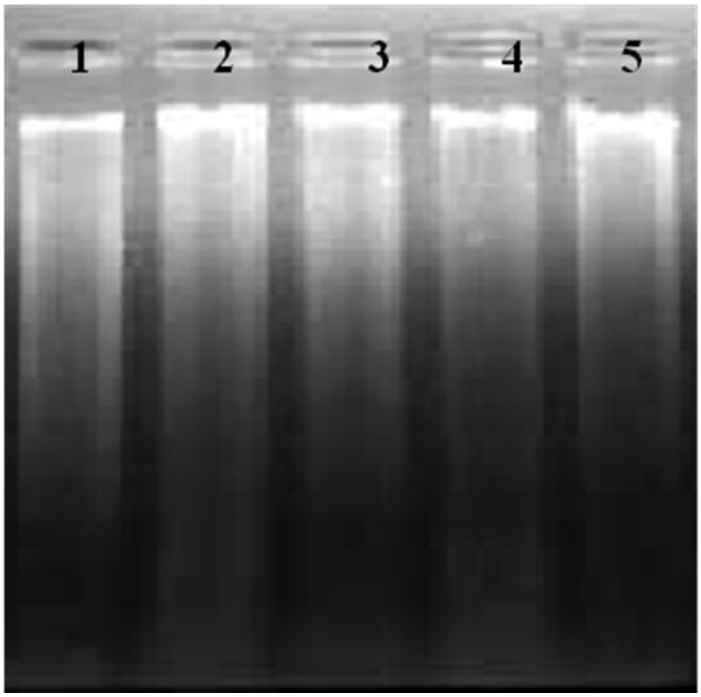


Fig. 4.23B. DNA fragmentation assay in Normal Monkey Kidney CV-1 cells. Cells ($2x10^6$ /ml/well) were incubated with HKBA at various concentrations for 24 h. Lane-1 (Untreated cells), Lane-2, 3, 4 & 5 (CV-1 cells + 5, 10, 30 & 50 µg/ml HKBA).

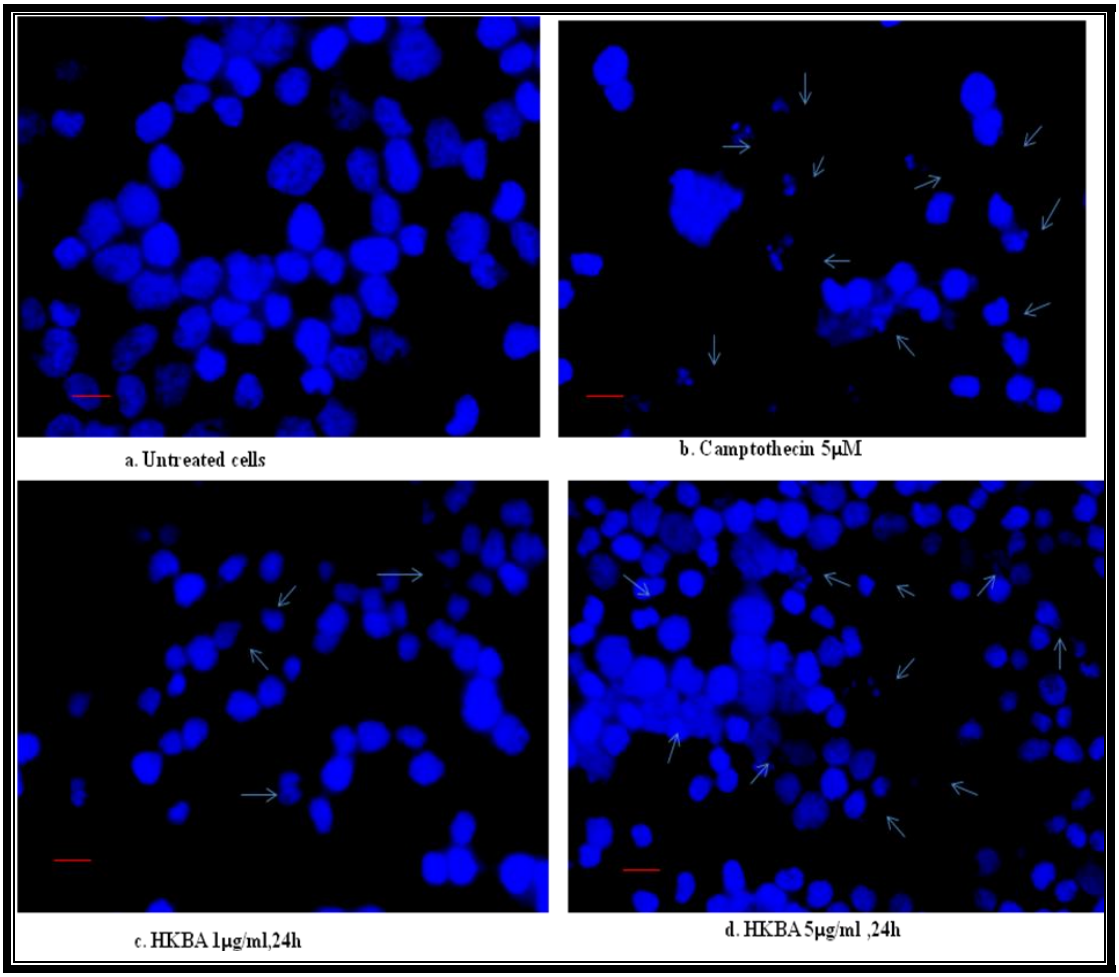


Fig. 4.24. HKBA influences the nuclear morphology and leads to the formation of apoptotic bodies in HL-60 cells. Cells (2×10^5/2 ml/6 well plate) were treated with 1 & 5 µg/ml of HKBA for 18 h, stained with DAPI and visualized for nuclear morphology and apoptotic bodies. (a) Untreated control cells showing large sized nuclei (b) Cells treated with 5 µM Camptothecin indicate condensation both in cytoplasm and nuclei and thus apoptosis (↑). (c) Cells treated with 1µg/ml HKBA for 18 h indicate apoptosis (↑). (d) Cells treated with 5µg/ml of HKBA for 18 h showing increase in apoptotic population (↑). Magnification 400X, bar (—) 20 µm.

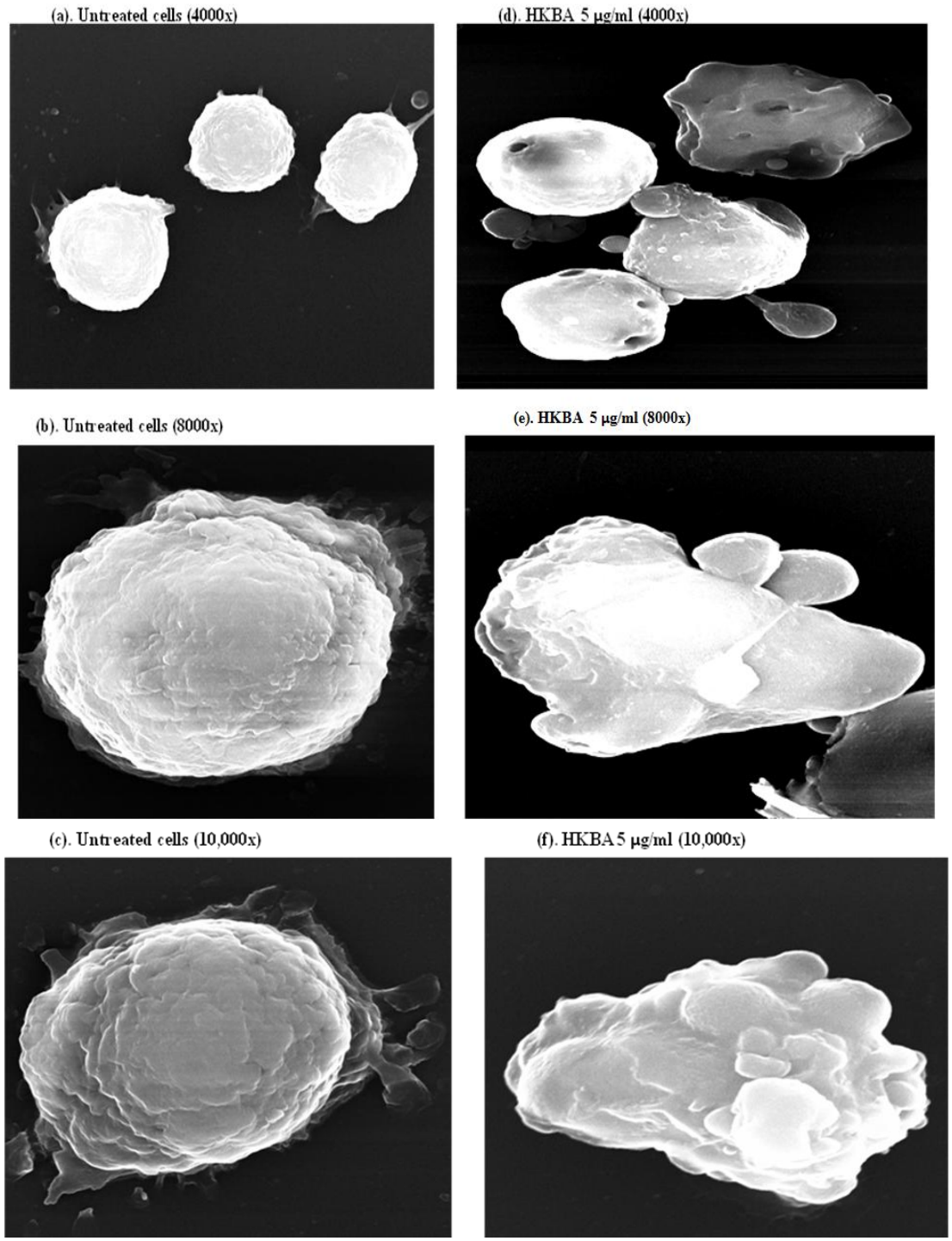


Fig. 4.25. Scanning Electron Microscopy of HKBA treated HL-60 cells. Untreated cells (a, b, c) at 4000x, 8000x and 10,000x respectively and cells treated with HKBA (5 µg/ml) for 24 h (d, e, f) at the respective magnifications. The untreated control cells (a, b, c) show rough surface and microvilli and the treatment, after 24 h causes reduction in cell size, smoothening of cell surface, blebbing (↑) of the plasma membrane and apoptosis (d, e, f).

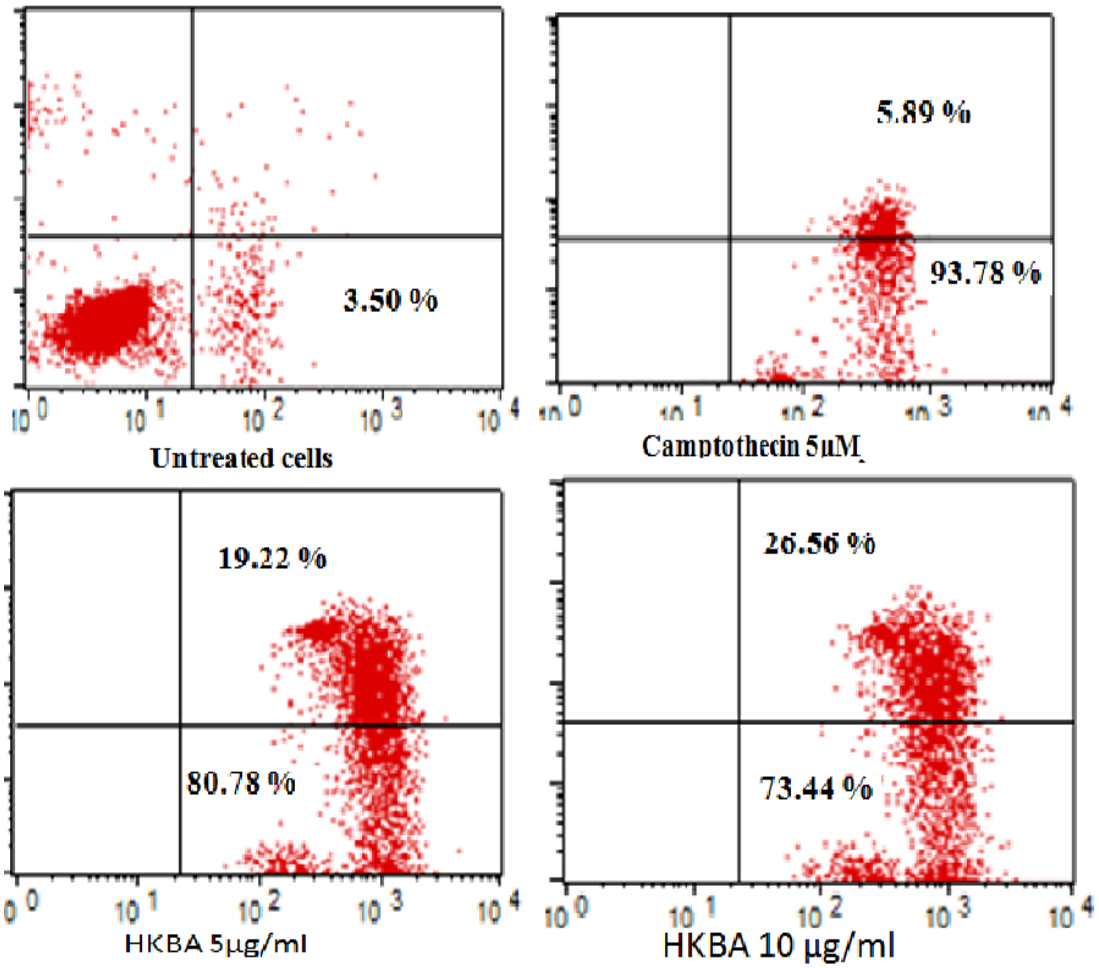


Fig. 4.26. Annexin V-FITC/PI staining of HKBA treated HL-60 cells. Cells were treated with indicated concentrations of HKBA for 24 h and stained with annexin V-FITC/PI to analyze apoptotic and necrotic cell population as described earlier. Apoptotic population was found to be 80.78 and 73.44% while as necrotic population was found to be 19.22 and 26.56% at 5 & 10 µg/ml respectively. Data are representative of one of the two similar experiments.

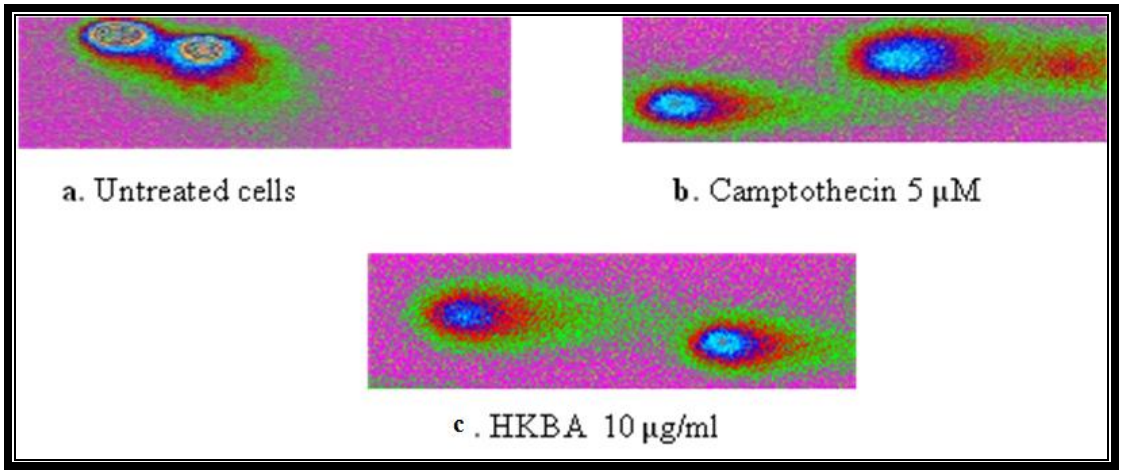


Fig. 4.27. Comet formation in HKBA treated HL-60 cells. (a) Untreated cells (b) Camptothecin taken as a positive control. (c) HKBA treated HL-60 cells after 24 h of incubation.

	Head DNA	Tail DNA	OliveTail Movement	Tail Length
Untreated Cells	90.91±0.01	9.09± 0.03	0.501±0.04	1.34±0.011
Camptothecin 5µM	60.06±0.02	39.94±0.01	8.84±0.022	35.13±0.042
HKBA 5 µg/ml	65.86±0.02	34.14±0.03	3.05±0.011	12.94±0.021
HKBA 10 µg/ml	31.23±0.04	68.77±0.03	7.23±0.042	28.42±0.036

Table 4.22. Comet assay analysis of HKBA treated HL-60 cells after 24 h of incubation revealed a head DNA of 65.86 % and 31.23 %, Tail DNA of 34.14 % and 68.77% at 5 and 10 µg/ml respectively which signifies the DNA damage. The olive tail movement was 3.05% and 7.23 % while a Tail length of 12.94 %. and 28.42 % was observed at the respective concentrations. Camptothecin taken as a positive control revealed a Head DNA of 60.06 % and a tail DNA of 39.94 %. Data are mean ± S.D. from three similar experiments.

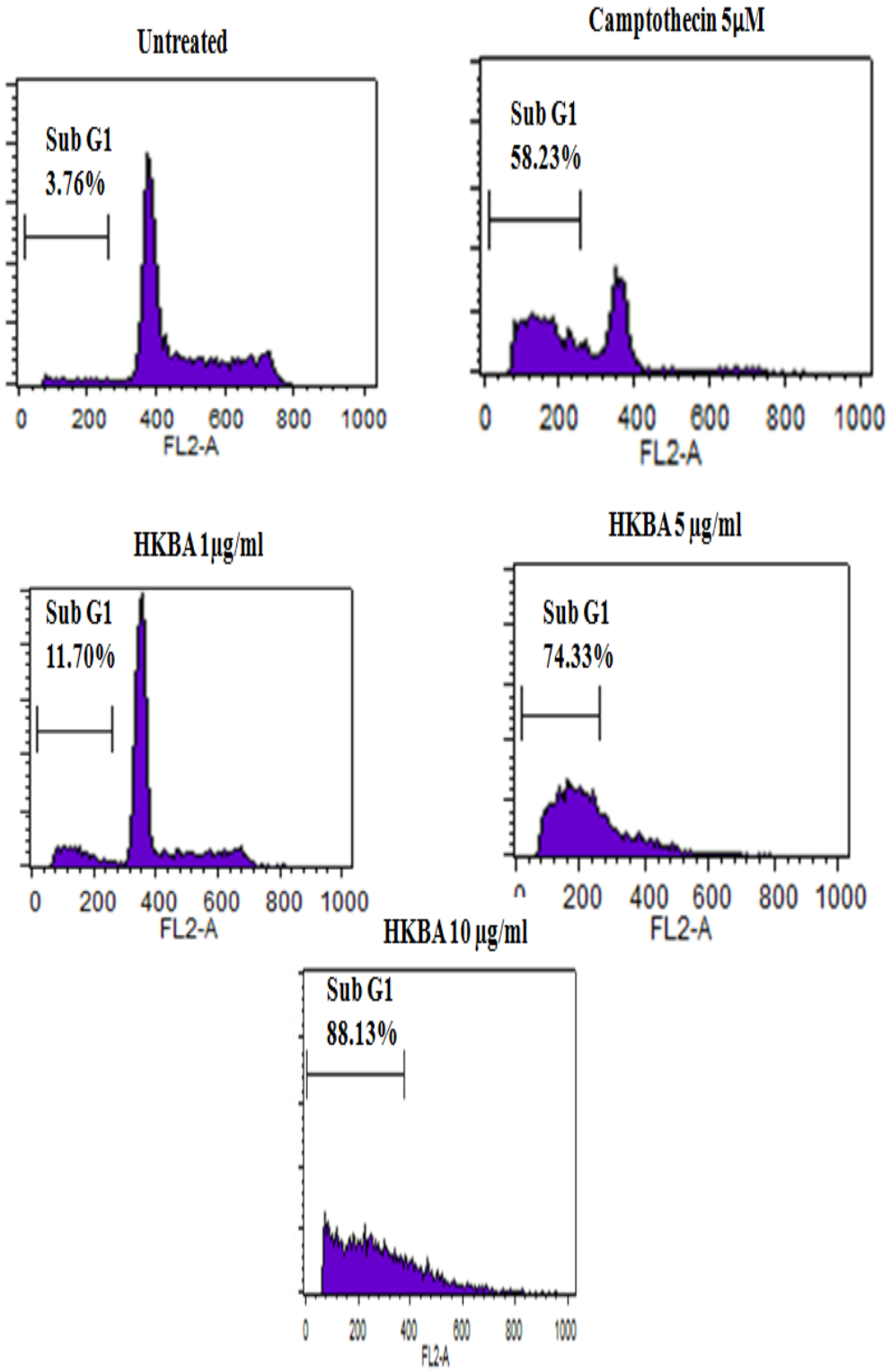


Fig. 4.28. Cell cycle analysis of HKBA treated HL-60 cells. HL-60 Cells ($2x10^6$ cells/ml/well) were exposed to indicated concentrations of HKBA for 24 h and stained with PI to determine DNA fluorescence and cell cycle phase distribution as described in materials and methods. Fraction of cells for sub-G1 population analyzed from FL2-A vs. cell counts is shown (%). Data are representative of one of three similar experiments.

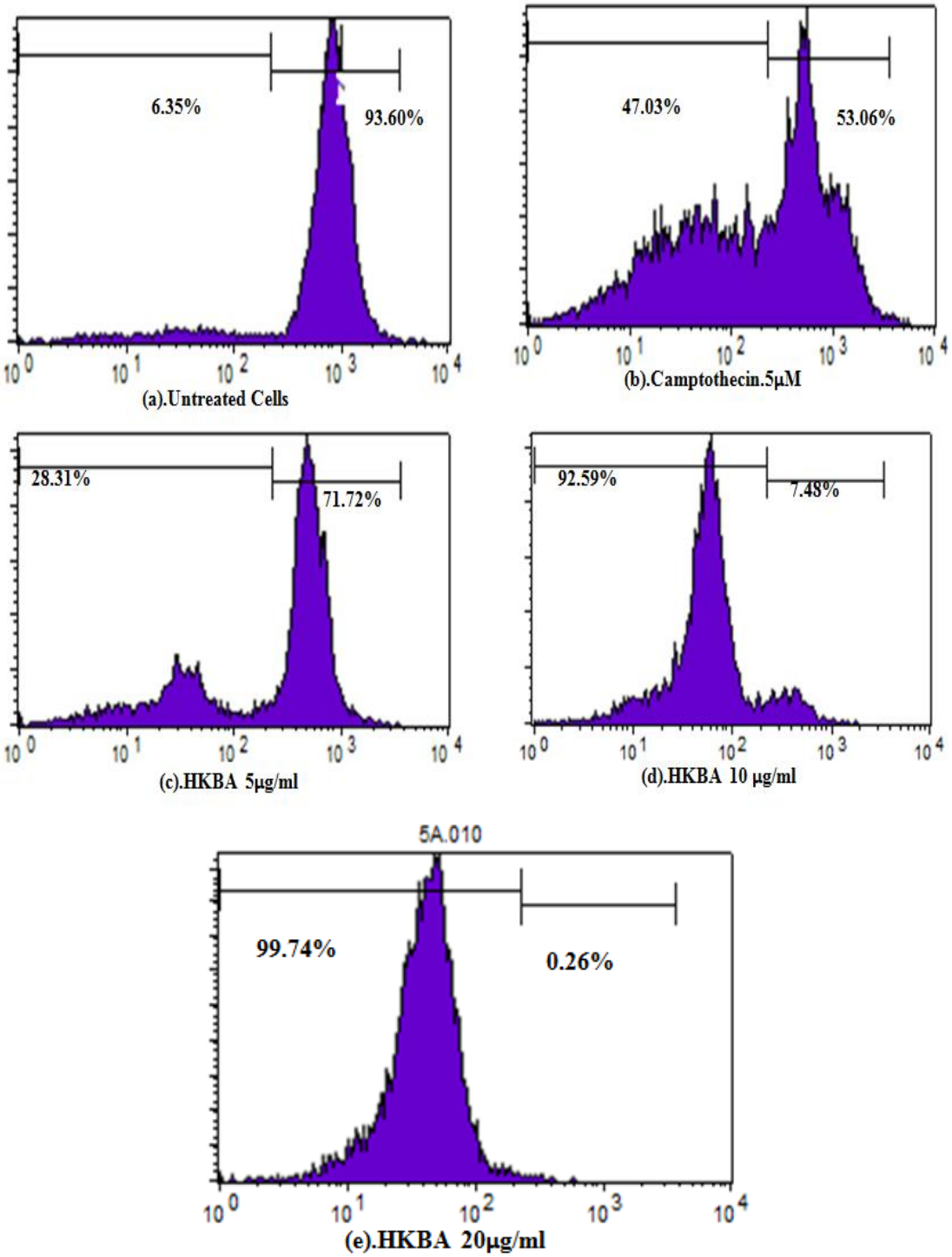
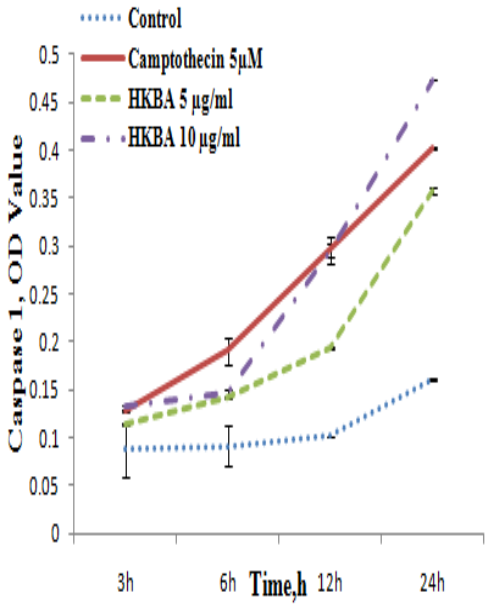
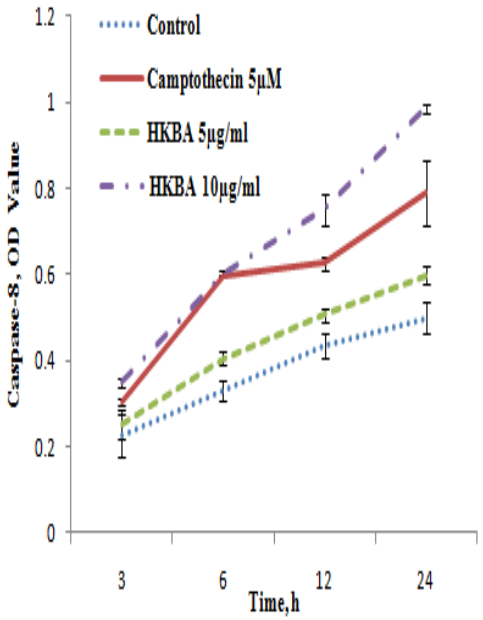
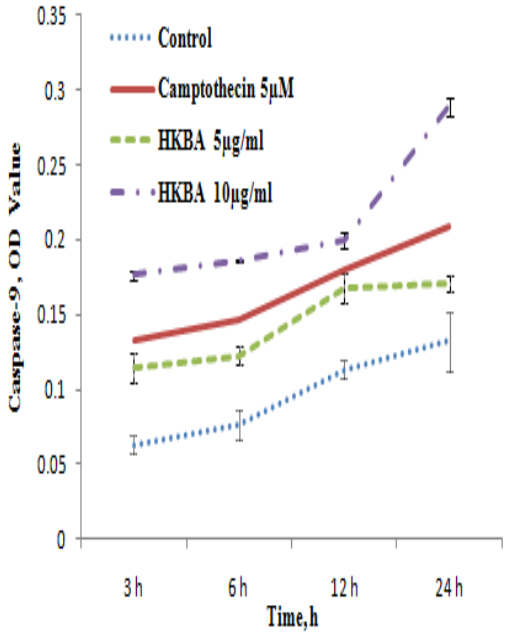
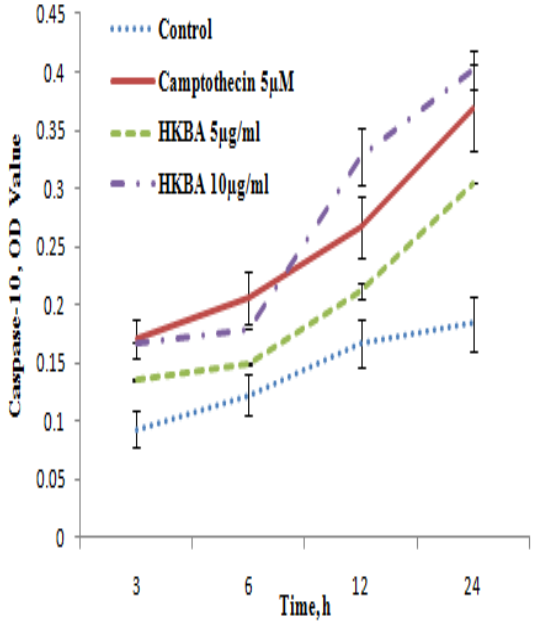


Fig. 4.29. Loss of mitochondrial membrane potential (ΔΨm) in HL-60 cells. Cells (1×10^6/ml/6well plates) were incubated with indicated doses of HKBA for 24 h. Thereafter, cells were stained with Rhodamine-123 (200 nM) for 1 h and analyzed in FL-1 vs. FL-2 channels of flow cytometer. Data are representative of one of three similar experiments.


Control
Camptothecin 5µM
HKBA 5 µg/ml
HKBA 10 µg/ml
Caspase 1, OD Value
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3h
6h
Time,h
12h
24h


Control
Camptothecin 5µM
HKBA 5µg/ml
HKBA 10µg/ml
Caspase-8, OD Value
1.2
1
0.8
0.6
0.4
0.2
0
3
6
12
24
Time,h


Control
Camptothecin 5µM
HKBA 5µg/ml
HKBA 10µg/ml
Caspase-9, OD Value
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3 h
6 h
12 h
24 h
Time,h


Control
Camptothecin 5µM
HKBA 5µg/ml
HKBA 10µg/ml
Caspase-10, OD Value
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3
6
12
24
Time,h

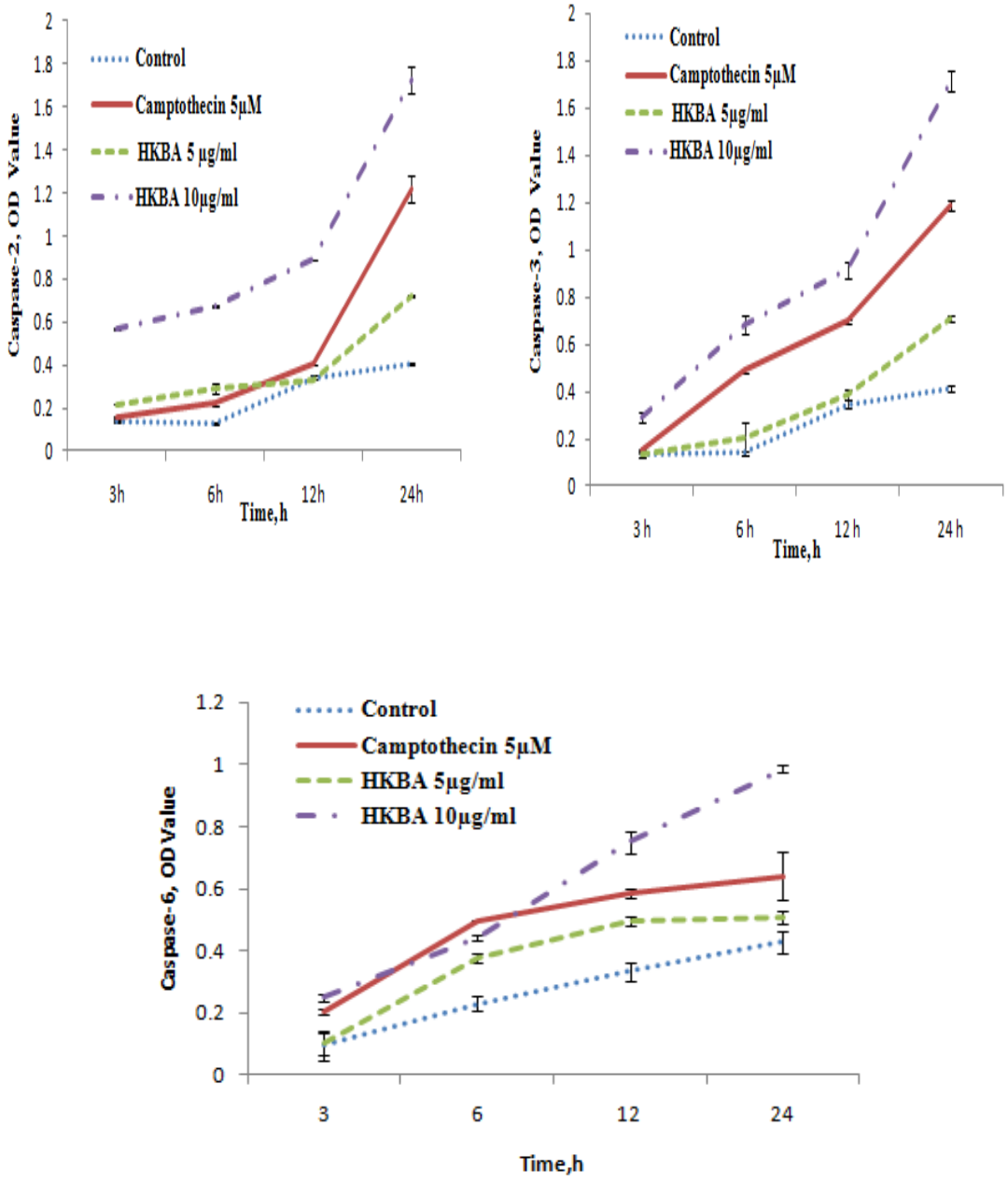


Fig.4.30. Caspase activity of HKBA. HKBA induces the activation of Initiator caspases-1, -8, -9 & -10 and effector caspases -2,-3 & -6. HL-60 cells (2×10^6/ 2ml/6-well plates) in culture were exposed to 5 and 10 µg/ml of HKBA for indicated time periods. The caspases activities were determined colorimetrically by using caspase-colorimetric kits from R&D system as described in methodology. All assays were performed according to the instructions provided by the supplier. Data are Mean ±S.D. from three similar experiments. P values: *< 0.05, **< 0.01 as compared to control.

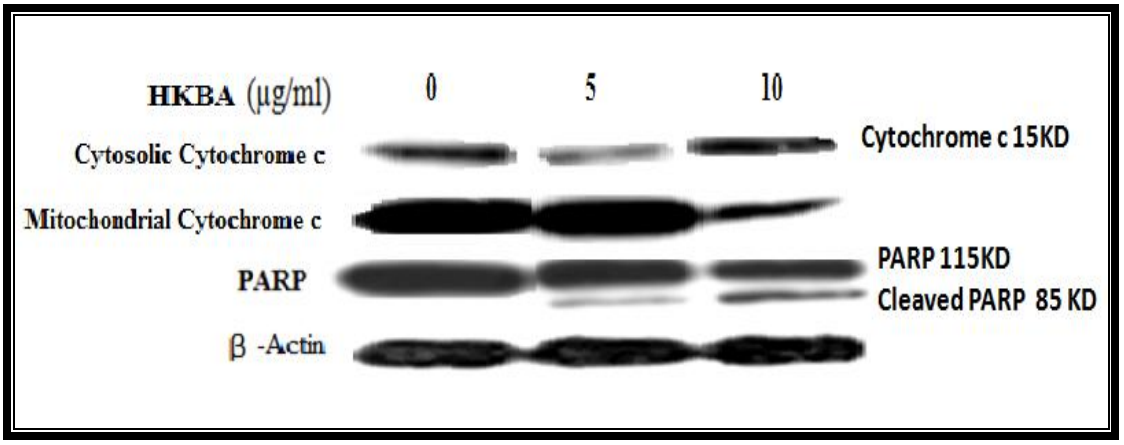


Fig.4.31. HKBA influences the expression of cytochrome c and PARP in HL-60 cells. Cells (2×10^6/3 ml/6-well plates) were treated with 5 and 10 µg/ml of HKBA for 24 h and different fractions were prepared and immunoblotted as described in material and methods. β-Actin used as internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of cytochrome c and PARP. Data are representative of one of three similar experiments.

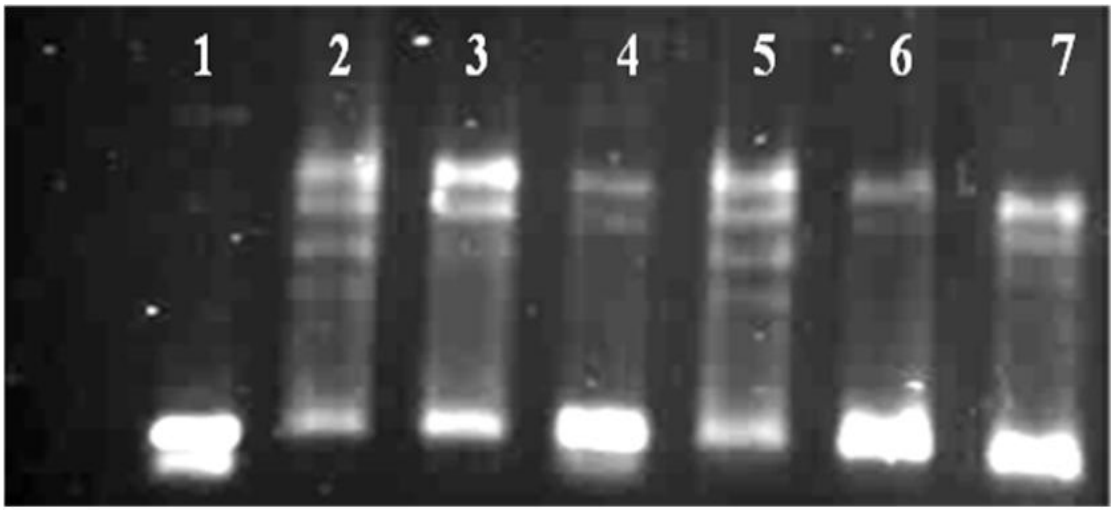


Fig. 4.32. HKBA inhibits topoisomerase–I & II activity. Inhibitory assay of topoisomerase-I & II was performed by using topoisomerase-I & II kits from Topogen. Lane 1 (pRYG DNA), Lane 2 (pRYG DNA + Topoisomerase-II), Lane 3(pRYG DNA + Topoisomerase-II + Etoposide 100 µM), Lane 4 (pRYG DNA + Topoisomerase-II +HKBA 10 µg/ml), Lane 5 (pRYG DNA + Topoisomerase-I), Lane 6 (pRYG DNA + Topoisomerase-I + Camptothecin 100 µM) & Lane 7 (pRYG DNA + Topoisomerase-I +HKBA 10 µg/ml).

4.11. Anti- angiogenesis studies

4.11.1. Determination of Angiopioetin-2 (Ang-2) and Matrix Metalloproteinase-2 (MMP-2) inhibitory activity

Likewise PKBA, HKBA was found to inhibit the activity of pro-angiogenic factors Ang-2 and MMP-2. Unlike PKBA, the inhibition was observed to be at lower concentration, including, 5 & 10 µg/ml. Inhibition of pro-angiogenic agents Ang-2 and MMP-2 by HKBA was measured using commercial R&D kits. HL-60 cells treated with the test compound at 5 & 10 µg/ml for 3, 6, 12 & 24 h showed both dose and time dependent inhibition of pro- angiogenic factors Ang-2(Fig. 4.33A) and MMP-2 (Fig. 4.33B). These results further support the low dose anticancer potential of HKBA.

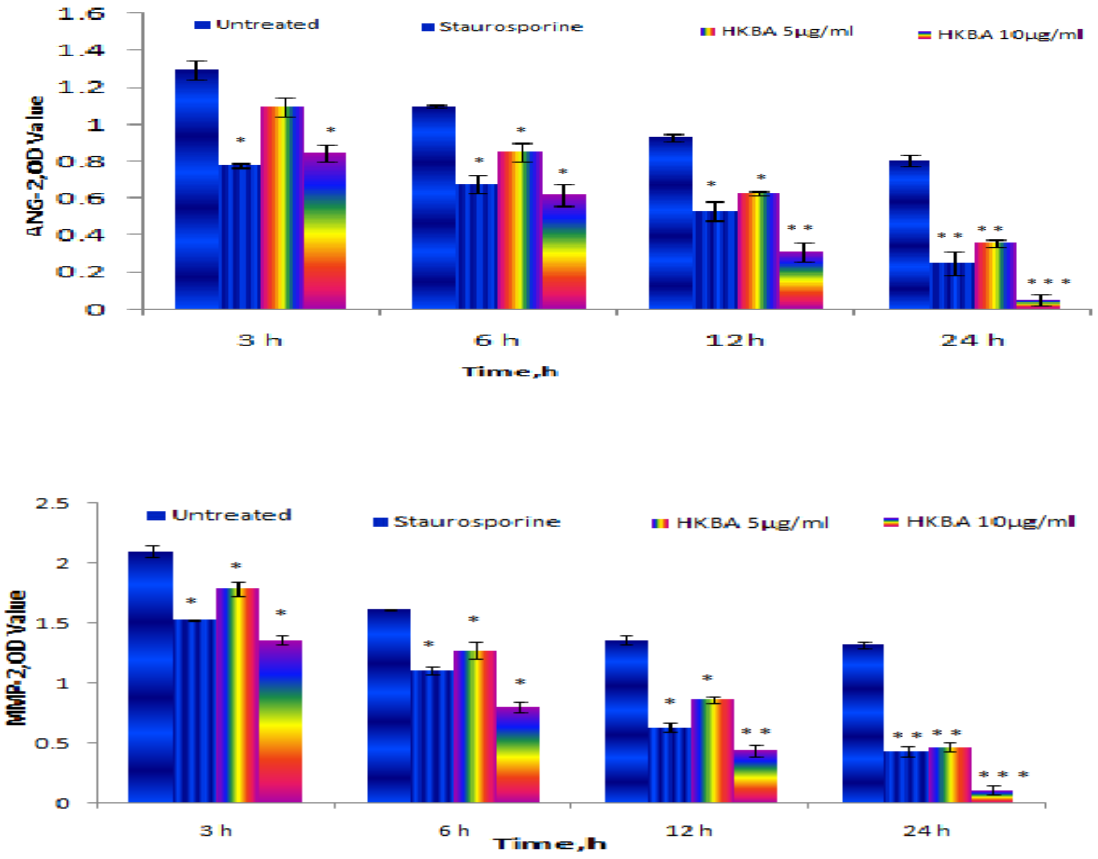


Fig. 4.33. *In vitro* inhibition of pro-angiogenic factors: Ang-2 and MMP-2. HL-60 cells (2×10^6/ 2ml/6-well plates) in culture were exposed to 5 and 10 µg/ml of HKBA for 3,6,12 & 24 h. ANG-2 (A) and MMP-2 (B) activities were determined colorimetrically by using Ang-2 and MMP-9 kits from R &D system as described in methodology. All assays were performed according to the instructions provided by the supplier. Data are Mean ±S.D. from three similar experiments. p values: *<0.05, **< 0.01 ,***0.001

4.12. Pharmacokinetic Studies

4.12.1. Determination of plasma & brain pharmacokinetics

Pharmacokinetic studies (PK) of HKBA were carried out in Swiss albino mice. Both blood plasma and homogenate of brain were evaluated for the ADME profile of HKBA. The PK studies in blood plasma showed a maximum concentration 'C_{max}' of 2.347 µg/ml and the time to reach the maximum concentration 'T_{max}' was found to be 2 hr. The half-life of HKBA in the plasma '$T_{½}$' was found to be 2.314 hrs and the area under the curve AUC was found to be 14 µg/hr/ml. Further, the volume of distribution 'Vd' and clearance 'Cl' from the plasma was found to be 23.844 L/kg and 7.141 L/hr/kg respectively (Fig 4.34).

The Brain PK studies in brain homogenate showed that HKBA was not able to cross the blood brain barrier as no drug was obtained from the brain samples

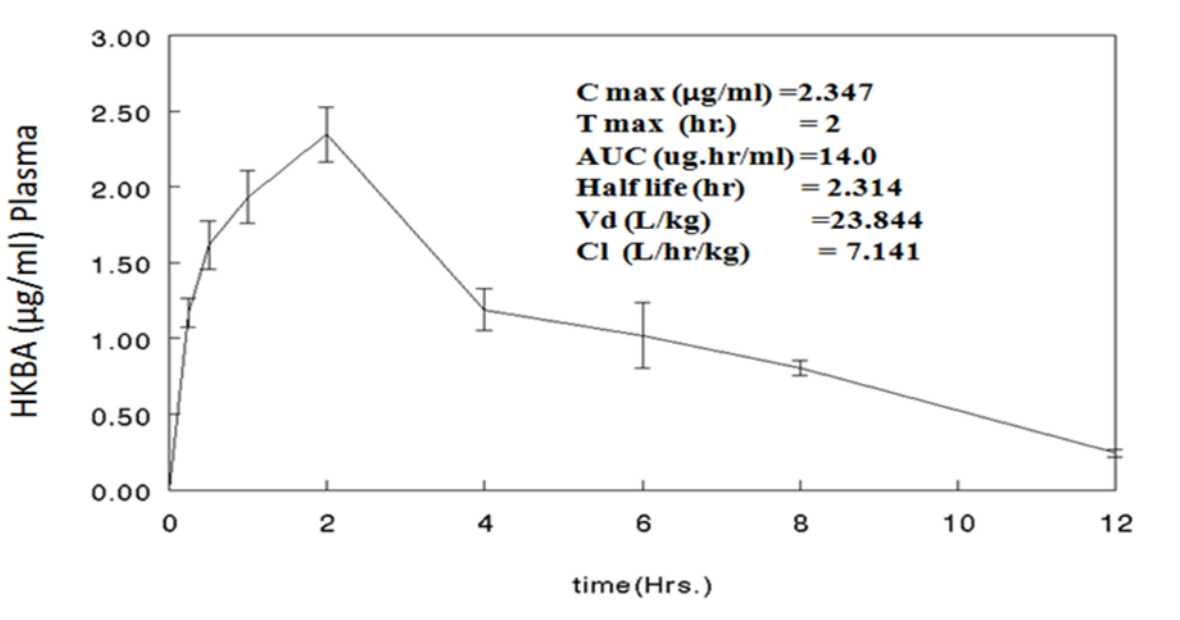


Fig. 4.34. Plasma PK studies of HKBA in Swiss albino mice.

4.13. Nanoparticle based drug delivery system of HKBA

4.13.1. Determination of Particle size, zeta potential, entrapment efficiency and yield of HKBA Nanoparticles (HKBA NPs)

Transfferin appended HKBA loaded PLGA-PEG NPs were formulated by oil-in-water single emulsion-solvent evaporation technique. Particle size and size distribution (polydispersity index) of the formulation was determined, based on dynamic light scattering (DLS), using a Zetasizer (Nano ZS, ZEN3600, Malvern Instrument) with a wavelength of 532 nm at 25^{o}C with an angle detection of 90^{o}. The encapsulation efficiency of the particles was determined by the reverse phase isocratic mode of high performance liquid chromatography (RPHPLC) method. These nanoparticles showed an average particle size of 283 ± 7.2 nm (Fig 4.35) and a zeta potential of -11.9 ± 1.4 mv. The nanoparticles gave a yield of 85 % with indirect entrapment efficiency of 97 % and direct entrapment efficiency of 66.72 % (Table 4.23).

4.13.2. Determination of *In vitro* release kinetics of drug from nanoparticles

The *in vitro* release kinetics of nanoparticles was performed by dispersing the drug loaded nanoparticles in PBS. The results revealed that more than 90 % of the drug gets released within 48 h (Fig. 4.36).

4.13.3. Determination of surface morphology by Transmission (TEM) & scanning electron microscopy

The surface morphology and shape of the formulated NPs was visualized by TEM & SEM. Most of the particles were spherical in shape although some particles were found to be elliptical (Fig. 4.37& 4.38).

4.13.4. Determination of *In vitro* cytotoxicity

HKBA NPs showed more potent cytotoxic potential against all the twelve human cancer cell lines as compared to HKBA alone. A growth inhibition of 90-100 % was observed against most of the cell lines at 100 µg/ml, at lower concentration i.e. 70, 50 30 10, 5 and 1 µg/ml activities the same was 78-98, 75-94, 73-94, 47-94, 38-69 and 18-45% respectively. 5-Flurouracil, Adriamycin, Mitomycin C and Paclitaxel were used as reference and various degree of growth inhibition was observed. Thus, HKBA showed concentration dependent cytotoxicity depending on cell lines (Table 4.24, 4.25, 4.26).

4.13.4.1. Determination of IC_{50} values

The IC_{50} values were found to be in the range of 1 to 15 µg/ml with PC-3 showing the lowest value (1 µg/ml) and Hep-2 showing the highest value (15 µg/ml). The IC_{50} values for adherent cell lines, IMR-32, SF-295, PC-3, Colo-205, HCT-15, MCF-7, OVCAR-5, Hep-2, A549 and THP-1 was calculated by SRB (Table 4.27) method and that of suspension cell lines HL-60 (Fig. 4.39) and MOLT-4 (Fig. 4.40) was calculated *via* MTT assay. The IC_{50} value in HL-60 cell line (4 µg/ml) was less than that of MOLT-4 (5 µg/ml).

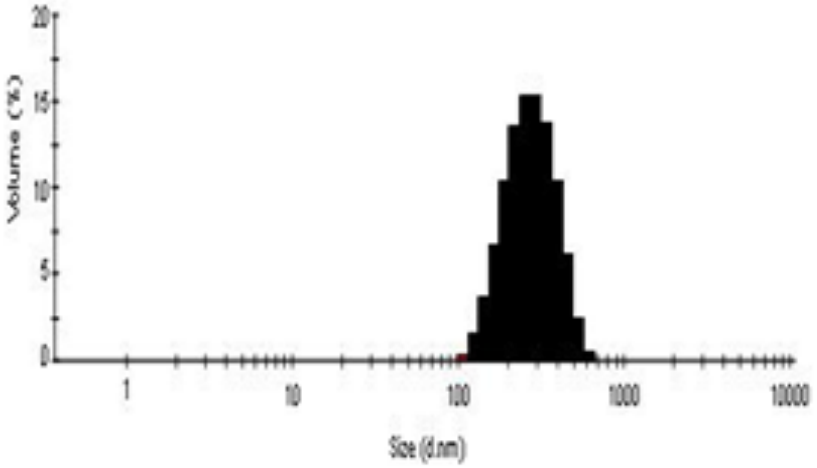


Fig. 4.35. Particle size distribution of HKBA NPs

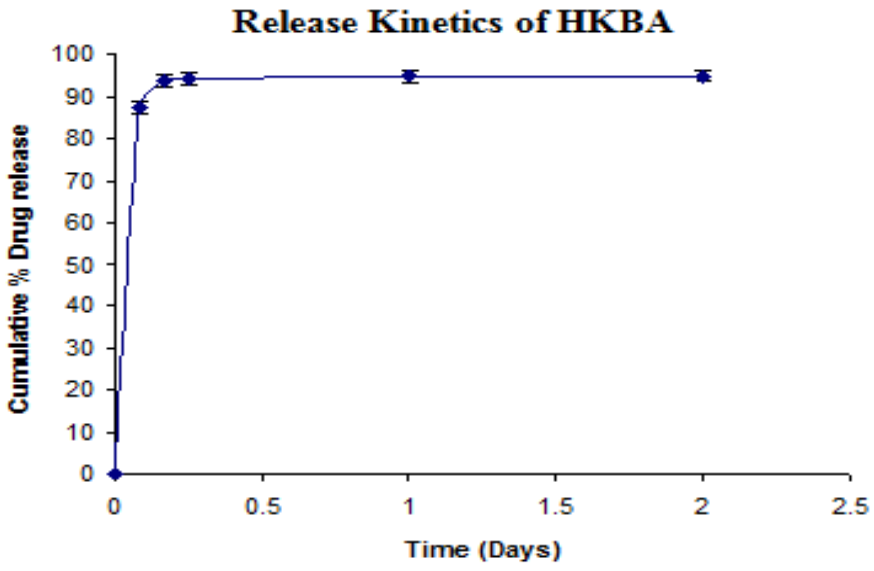


Fig. 4.36. *In vitro* release kinetics of HKBA NP's

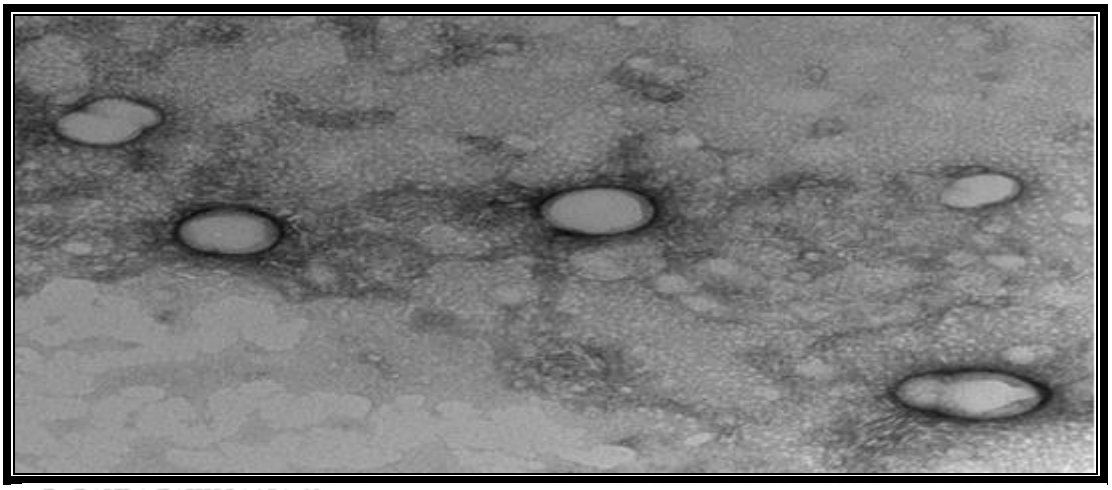


Fig .4.37. Transmission Electron Microscopy of HKBA NPs

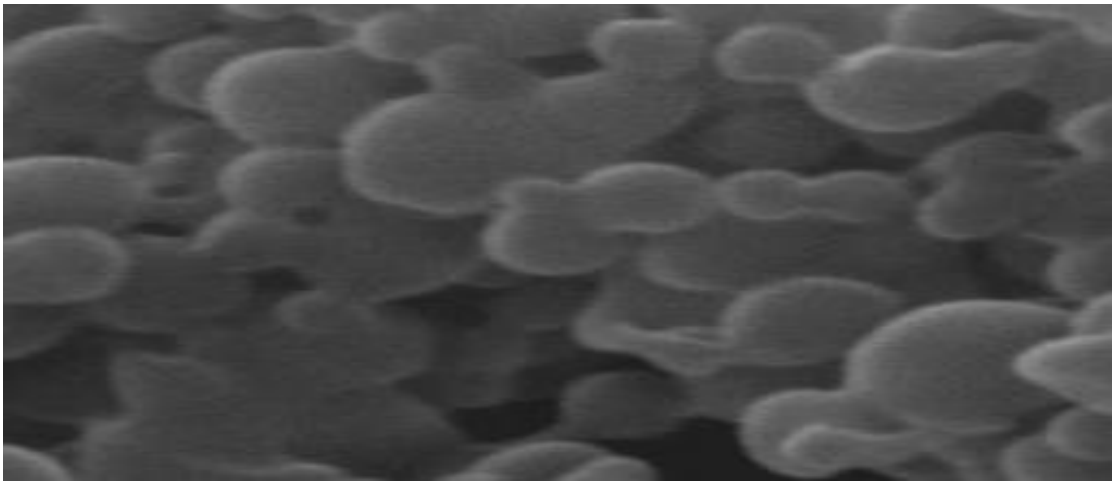


Fig. 4.38. Scanning Electron Microscopy of HKBA NPs

Sample	Loading (%)	Yield	Indirect Entrapment Efficiency (%)	Direct Entrapment Efficiency (%)	Size (nm)	Zeta Potential	PDI
HKBA NPs	10	85	97	66.72	283 ± 7.2	-11.9± 1.4	0.11± 0.009

Table 4.23. Physicochemical characterisation of drug loaded HKBA NPs

Tissue		Neuroblastoma		Prostate	Colon		Breast	Ovary	Liver	Lung	Leukemia
Cell Line		IMR-32	SF-295	PC-3	Colo-205	HCT-15	MCF-7	OVCAR-5	Hep-2	A549	THP-1
Code	Conc. µg/ml	% Growth Inhibition									
HKBA NPs	1	36	38	45	40	18	42	41	19	28	32
	5	55	56	69	59	50	63	69	38	41	59
	10	71	70	82	79	87	69	80	47	55	94
	30	86	78	89	86	93	73	83	74	81	94
	50	89	83	91	89	96	79	90	75	81	94
	70	95	90	98	93	97	88	94	78	87	95
	100	99	99	100	100	99	99	100	85	88	97
Adriamycin	1µM	65	67	-	-	-	68	-	--	-	-
Paclitaxel	1 µM	-	-	-	-	-	-	58	-	-	-
Mitomycin	1 µM	-	-	50	-	-	-	-	58	-	51
5-Fluorouracil	20 µM	-	-	-	50	62	-	-		52	70

Table 4.24. *In vitro* cytotoxicity (percent growth inhibition) of HKBA NPs. Cells were grown in 96 well plates and treated with various concentrations 1,5 10, 30,50, 70 and 100 µg/ml of HKBA NPs for 48 h. The SRB assay was performed as described in material & method section.

Cell Line	HL-60 (Leukemia)			
Conc. µg/ml	**Average O.D**	**SD**	**%Viability**	**% Inhibition**
0	0.992	0.046523	100	0
10	0.17525	0.032901	17	83
30	0.16105	0.513314	16	84
50	0.1235	0.043703	12	88
70	0.1125	0.637242	11	88

Table 4.25. *In vitro* proliferation and percent growth inhibition of HKBA NPs in HL-60 cells. Cells were treated with HKBA NPs for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Cell Line	MOLT-4 (Leukemia)			
Conc. µg/ml	**Average O.D**	**SD**	**%Viability**	**% Inhibition**
0	0.981	0.005312	100	0
10	0.20825	0.042152	21	79
30	0.1713	0.063735	17	83
50	0.15975	0.029103	16	84
70	0.107	0.053121	11	89
100	0.0375	0.021735	4	96

Table 4.26. *In vitro* proliferation and percent growth inhibition of HKBA NPs in Molt-4 cells. Cells were treated with HKBA NPs for 48 h and % growth inhibition was calculated by MTT assay as described in materials and method section

Tissue	Cell line	HKBA NPs
		Conc. µg/ml
Neuroblastoma	IMR-32	3.4
	SF-295	3.0
Prostate	PC-3	1
Breast	MCF-7	2
Ovarian	OVCAR-5	1.5
Liver	Hep-2	15
Lung	A549	9
Colon	Colo-205	2.1
	HCT-15	7.4
Leukemia	THP-1	2.9
	HL-60	3
	Molt-4	4

Table 4.27. IC_{50} values of HKBA NPs against twelve human cancer cell lines

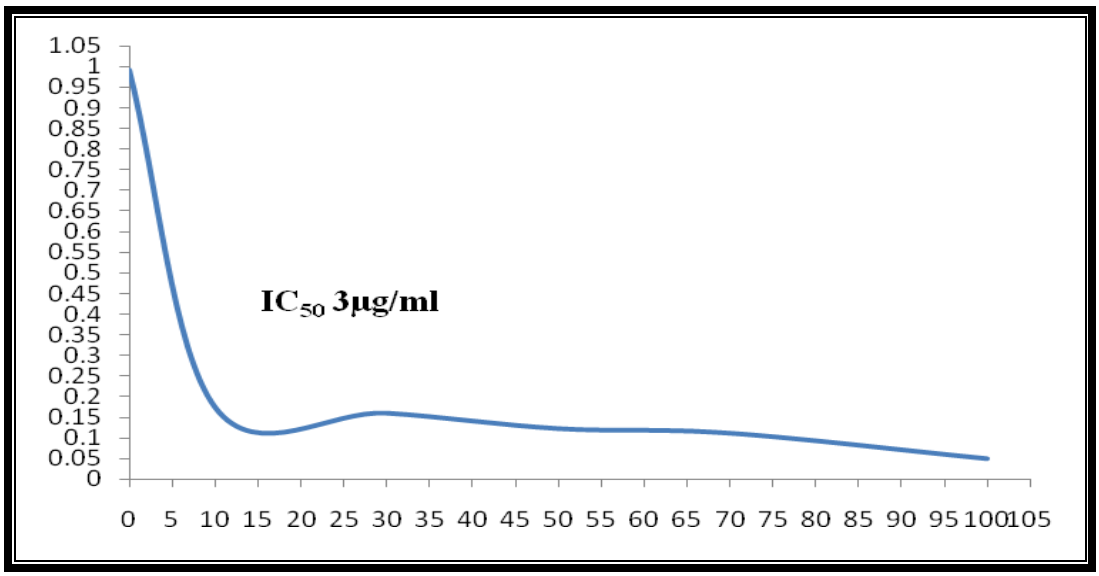


Fig 4.39. IC_{50} value of HKBA NPs against HL-60 cell line by MTT assay

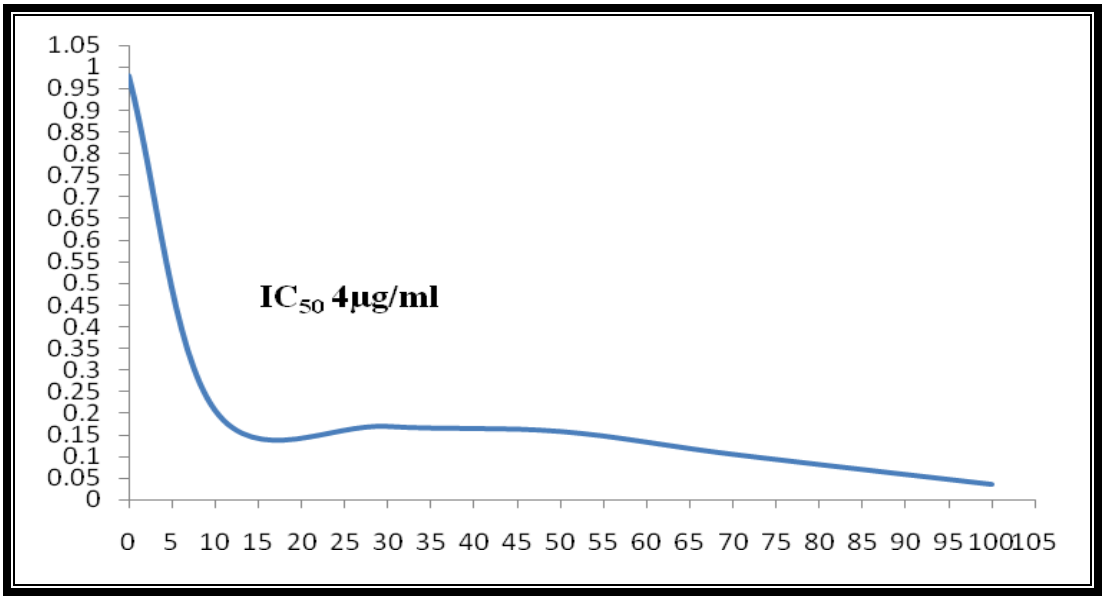


Fig 4.40. IC_{50} value of HKBA NPs against Molt-4 cell line by MTT assay

4.13.5. Determination of plasma and brain pharmacokinetics

The Pharmacokinetic studies of HKBA NPs were carried out in Swiss albino mice after administration of HKBA NPs (100 mg/kg b.wt.) orally for 2 h. PK studies in plasma showed that there was an increase in the C_{max} (5.57 µg/ml) by HKBA NPs compared to HKBA alone. Further the brain PK studies showed that very little drug (0.206 µg/100 mg) was recovered from the brain samples, indicating that HKBA NPs could not readily penetrate the blood brain barrier.

	2 h	
Animal No.	**HKBA NPs (µg/ml)Plasma**	**HKBA NPs (µg/100 mg)Brain**
1	5.043	0.190
2	4.622	0.216
3	4.548	0.2016
4	4.205	0.2069
5	3.901	0.216
Mean	**5.57**	**0.206**
SD	0.433	0.0109

Table 4.28. PK studies of HKBA NPs in Plasma and Brain of Swiss albino mice

4.14. Discussion

In Part-2 of the present study we had evaluated the anticancer potential of a hexanoyloxy derivative of 11-keto-β-boswellic acid (HKBA). The studies done with propionyloxy derivative of 11-keto-β-boswellic acid (PKBA) and the results obtained build our interest to evaluate the anticancer potential of boswellic acid with further increase in chain length. Based on the *in vitro* cytotoxicity screening, a semisynthetic derivative of KBA with hexanoyloxy substitution on the 3-hydroxyl functionality was selected for further comparative studies and to decipher the role of increasing alkoxy chain length on the anticancer potential of KBA. The therapeutic potential of HKBA was evaluated *via* both *in vitro* and *in vivo* studies. The *in vitro* cytotoxicity of HKBA was evaluated against twelve human cancer cell lines and was found to be more cytotoxic than PKBA against most of them. The IC_{50} values were also found to be lower than that of PKBA. These results thus depict a better cytotoxicity profile of HKBA. The *in vivo* anticancer activity of HKBA was evaluated in three murine tumor models including EAC, ET and S-180 and was found to inhibit the tumor growth at much lower doses than PKBA. The pro-apoptotic studies were conducted in HL-60 cell line. HKBA exhibited the ultra structural and biochemical features that characterize formation of apoptotic bodies, chromatin condensation, shrinkage of cells & bleb formation at lower concentrations of 5 & 10 µg/ml. The apoptotic ratio in HL-60 cells was found to be 90 % at 5 µg/ml which decreased to 70 % at 10 µg/ml with increase in necrotic population, indicating the more cytotoxic potential of HKBA. HKBA exhibited greater sub G_1 accumulation within 24 h of treatment. Further, it showed a significant influence on the mitochondria and resulted in the loss of mitochondrial membrane potential at lower doses than PKBA. The key element in the mitochondrial pathway is the efflux of cytochrome c, from the mitochondria to the cytosol. Cells undergoing apoptosis show increase of cytochome c in the cytosol, with a corresponding decrease in mitochondrial potential. Loss of mitochondrial membrane potential ($\Delta\psi mt$) by HKBA and leakage of cytochrome c in turn causes the activation of intracellular caspases degradation of poly (ADP-ribose) polymerase and thus resulting into DNA fragmentation. Likewise PKBA, HKBA inhibited the enzymatic activity of both topoisomerases I&II, however, the inhibition was found at

lower doses than PKBA. Several reports have revealed that chemotherapeutic agents that target topoisomerases I & II set in motion a series of biochemical changes that culminate in cell death (Bielawski et al., 2006). These antitumor drugs interact with both topoisomerases I & II simultaneously. This mechanism of action appears to be advantageous, because selective inhibition of topoisomerase I has been reported to increase topoisomerse II enzyme activity and vice-versa, which may be important for the development of drug resistance. In this regard, a single compound able to inhibit both topoisomerase I and II may present the advantage of improving anti topoisomerase activity, with reduced toxic side effects, with respect to the combination of two inhibitors (Salerno et al., 2010). Although, dual inhibitors theoretically overcome these problems, however, some of the inhibitors like Intoplicin and XR11576 have been found to be associated with liver toxicity, diarrhoea, nausea, vomiting & alopecia (Salerno et al., 2010; de Jonge et al., 2004). Besides this, the chemopreventive effects of well known topoisomerase targeting drugs such as camptothecin and etoposide have also been found to be associated with multiple and severe side effects such as myelosuppression, thrombocytopenia, anaemia, bone marrow and gastrointestinal toxicity (Hartman and Lipp., 2006). Boswellic acids in contrast to this are known to have good safety profile showing no severe side effects (Gupta et al., 1997; Mantle et al., 2001; Safayhi et al., 2000). In addition, our studies with PKBA showed that it was non-toxic to the normal cells, the present investigation also showed that in spite of the increased anticancer potency at lower doses, HKBA seems to be selective for tumor cells, since no apoptosis was observed in normal cells. The above studies lead us to conclude that HKBA is a more potent cytotoxic agent than PKBA and induces apoptosis through intrinsic or mitochondrial dependent apoptotic pathway in HL-60 cells at lower doses.

Also, HKBA showed *in vitro* inhibition of proangiogenic factors ANG-2 and MMP-2 in a dose and concentration dependent manner, which further added to its anticancer potential.

The pharmacokinetic studies of HKBA were performed in Swiss albino mice and the studies revealed a C_{max} 2.347 µg/ml with a plasma half life of 2.314 h. The other parameters being evaluated also revealed its good PK profile; however, the brain

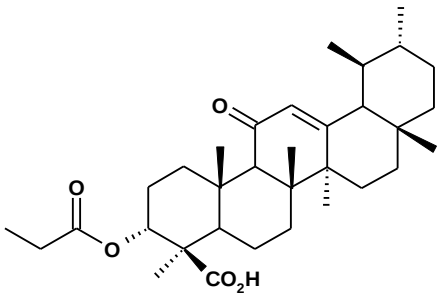
pharmacokinetic studies revealed that, HKBA could not penetrate the blood brain barrier.

In order to improve the therapeutic efficacy of HKBA and to utilize it for brain targeting, a nanoparticle based drug delivery system of HKBA was developed. Biodegradable co-polymer, PEG-PLGA was used for the preparation of nanoparticles. Further the particles were appended with transferrin proteins to make them better for brain targeting. Nanoparticles of HKBA (HKBA NPs) when subjected to *in vitro* cytotoxicity studies showed more cytotoxic effect with much lower IC_{50} values than HKBA alone. The comparative pharmacokinetic studies of HKBA NPs after 2 h revealed a C_{max} of 5.57 µg/ml in plasma and C_{max} of 0.206 µg/100 mg brain.

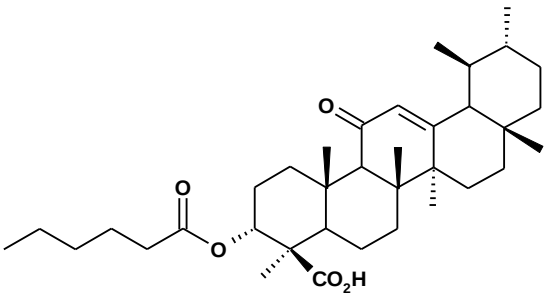
Together, these studies suggest that HKBA has lower dose *in vitro* cytotoxic and *in vivo* anticancer potential. The *in vitro* apoptosis studies revealed that HKBA induces apoptosis in HL-60 cells *via* mitochondrial dependent pathway at lower concentration than PKBA. Further, the pharmacokinetic studies revealed that HKBA has oral bioavailability. These studies nevertheless provide a substantial support for the use of HKBA in cancer chemotherapy.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Cancer is the general name for over 100 medical conditions involving uncontrolled and dangerous cell growth. It is the second largest health problem of the world after cardiovascular diseases in both developed and developing countries. The most common treatments involve surgical removal of the tumor, radiation therapy, chemotherapy, immunotherapy and combinations thereof. Instead of all these therapies available it is still difficult to get rid of cancer because cancer cells can return, grow and spread again so large that it may not be curable. In order to counteract this problem, major attention has been shifted to the discovery of new anti-cancer agents with no or minimum side effects. About 60 % of the anticancer drugs are derived from plant sources including paclitaxel, camptothecin, vincristine, vinblastine, vinleurosine, vinrosidine and podophyllotoxin. The development of anticancer drugs from plant resources depend on multiple target oriented approaches that will result in herbal medicine or an isolated active compound. However apart from this consideration the selection of a suitable plant for its pharmacological study is very important and decisive step. Additionally, semi synthesis processes of new compounds, obtained by molecular modification of the functional groups of lead compounds are able to generate structural analogues with greater pharmacological activity and with fewer side effects. Based on this strategy we have choosen two semi synthetically modified analogues of 11-keto-β-boswellic acid (KBA) namely, propionyloxy derivative of KBA (PKBA) and hexonyloxy derivative of KBA (HKBA) for the current study.



PKBA



HKBA

Further, a nanoparticle based drug delivery system of theses analogues was designed for utilizing them for brain targeting. The brief objectives of the present work were as follows:

- To evaluate the *in vitro* cytotoxic potential of PKBA and HKBA against a panel of human cancer cell lines
- To determine their *in vivo* anticancer potential against murine tumour models.
- To study the mechanism of cell death induced by these analogues.
- To determine their pharmacokinetic profile.
- To design a nanoparticle based drug delivery system of these analogues.

In vitro cytotoxic potential of PKBA and HKBA was evaluated against twelve different human cancer cell lines namely IMR-32, SF-295 (both neuroblastoma), PC-3(Prostate), Colo-205 & HCT-15 (both Colon), MCF-7(Breast), OVCAR-5(Ovarian), Hep-2(Liver), A549 (Lung), THP-1, HL-60 & MOLT-4 (all three Leukemia). PKBA and HKBA were found to induce cytotoxicity in all the twelve cancer cell lines being evaluated. PKBA was found to show lower IC_{50} values (5.95 to 18 µg/ml) than the previously reported boswellic acids including acetyl-11-keto-β-boswellic acid (AKBA). HKBA on the other hand being more cytotoxic, showing lower IC_{50} values than PKBA (2.4 to 13.9 µg/ml). The cytotoxic efficiency of these test compounds was compared with known anticancer drugs including adriamycin, paclitaxel, mitomicin-C and 5-fluorouracil.

The *in vivo* anticancer potential of PKBA and HKBA was evaluated in murine tumor models. The studies revealed PKBA to induce 100 % growth inhibition in Ehrlich ascites carcinoma (EAC) model at a dose of 100 mg/kg in Swiss albino mice. In solid tumor models the inhibition was found to be dose dependant upto the tune of 33.78 and 66.74% in Ehrlich Tumor (EAT) and 34.25 and 68.02% in S-180 solid tumor models at 100 and 150 mg/kg, respectively. In case of HKBA, at intraperitoneal doses of 50 & 60 mg/kg b.wt. the growth of Ehrlich Ascitic Carcinoma (EAC) was inhibited by 47.54 % and 67.29 % respectively. In case of solid tumors, HKBA showed a growth inhibition of 35.65 % and 50.64 % at 50 and 60 mg/kg b.wt. respectively in EAT model and 38.35 % and 54.36 % at the respective doses in S-180 model.

The mode of action of cell death mediated by PKBA and HKBA was studied using different multiparametric apoptosis assays. All the mechanistic studies were carried out using human leukemia HL-60 cell line and the observations were made after 24 hours unless otherwise specified. PKBA (5 & 10 µg/ml) and HKBA (1& 5 µg/ml)

exhibited cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation based on fluorescence and scanning electron microscopic studies. Both PKBA (100 µg/ml) and HKBA (50 µg/ml) were found to be non-toxic to normal monkey kidney CV-1 cell line. The induction of DNA damage was determined by comet assay, which revealed that PKBA at 20 µg/ml was able to induce comet formation in HL-60 cells, where in the tail DNA which determines the actual DNA damage was found to be 56.79 %. HKBA, on the other hand showed comet formation at 10 µg/ml, with a tail DNA of 68.77 %. FITC labelled Annexin V binding studies of PKBA showed a dose dependant increase in apoptotic population, which was found to be 70.74 and 96.74 % respectively at 10 and 20 µg/ml after 24 h of incubation. The apoptotic cell population of HKBA after 24 h was found to be 80.78 % & 73.44 % respectively, the necrotic population at the respective concentrations was found to be 19.22 % and 26.56 %. The cell cycle analysis of PKBA (1, 5, 10, 30 and 50 µg/ml) and HKBA (1, 5 & 10 µg/ml) showed concentration dependent increase in hypo diploid sub-G_1 cells (2.91, 3.93, 57.13, 78.16, 80.10 & 95.81% respectively for PKBA and 11.70, 74.33 & 88.13 % respectively for HKBA) and arrest of the G_2/M phase of cell cycle. Further, PKBA (5, 10 & 20 µg/ml) and HKBA (5, 10 & 20 µg/ml) were found to alter mitochondrial membrane potential and also induced release of cytochrome c from mitochondria. The released cytochrome c lead to the activation of intracellular caspases in a time and concentration dependent manner. These were also found to cleave a DNA repair enzyme PARP and finally resulted into DNA fragmentation in the cells. The dose and time dependent response was observed in all the parameters where different concentrations and time periods were used. The induction of apoptosis by these semisynthetic derivatives was due to the inhibition of enzymatic activity of topoisomerases I & II. PKBA was found to inhibit the enzymatic activity of both topoisomerases I & II at 20 µg/ml, however HKBA was found to inhibit the same at 10 µg/ml. These studies nevertheless confirm the proapoptotic nature of both PKBA & HKBA. HKBA comparatively showed the same effects at lower concentrations. The induction of apoptosis was mediated *via* mitochondrial dependent pathway in both the cases.

The anticancer potential of PKBA (10 & 20 µg/ml) and HKBA (5 & 10 µg/ml) was further supported by their effect on pro-angiogenic factors Ang-2 & MMP-2. The inhibition was found to be in both concentration and time dependent manner.

The pharmacokinetic studies of these analogues were carried out in Swiss albino mice. The PK studies revealed very good PK parameters including C_{max} and half life in plasma. The PK studies of PKBA in plasma revealed a C_{max} of 10 µg/ml and the time taken to reach C_{max} was 1 h (T_{max}) with a half life of 4.43 h. PK studies of HKBA revealed a C_{max} of 2.347 µg/ml with a plasma half life of 2.314 h. The other parameters being evaluated also revealed the good plasma PK profile of PKBA & HKBA. However, the PK studies in brain showed that neither of these test compounds could penetrate the blood brain barrier.

For brain targeting, a nanoparticle based drug delivery system of these semisynthetic analogues was developed. Nanoparticles were prepared by solvent evaporation method using PEG-PLGA co-polymer and conjugated them with transferrin ligands. The nanoparticles of these analogues showed more cytotoxic potential, greater plasma uptake and were also able to penetrate the blood brain barrier.

Conclusions

- Both *in vitro* & *in vivo* studies revealed that, PKBA was a better anticancer agent than the previously known boswellic acids including AKBA, thus revealing the importance of acyl group at 3-α-hydroxy position on the anticancer potential of KBA.
- In order to determine the effect of increasing acyl chain length at 3-α-hydroxy position of KBA, HKBA was synthesized and subjected to anticancer studies. The results obtained revealed that, HKBA was a more potent anticancer agent than PKBA, showing *in vitro* antiproliferative and apoptotic potential at low doses. The *in vivo* studies also revealed the low dose antitumor potential of HKBA.
- The pharmacokinetic studies revealed that both PKBA & HKBA were orally bioavailable, showing a good half life in the plasma. However, the brain PK studies revealed that none of these analogues could penetrate the blood brain barrier.

- For brain targeting, a nanoparticle based drug delivery system was designed and it was found that nanoparticles of these analogues showed better cytotoxic potential, greater plasma uptake and were also able to penetrate the blood brain barrier.

The data presented in this thesis provides an enhanced anticancer potential of two newly synthesized boswellic acids for the first time. These investigations seem to provide a substantial support for the use of PKBA and HKBA in cancer chemotherapy.

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List of Publications

1. **Gousia Chashoo**, Shashank K. Singh, Paraduman R. Sharma, Dilip M. Mondhe, Abid H. Dar, Arpita Saxena, Samar S. Andotra, Bhahwal A. Shah, Naveed A. Qazi, Subhash C. Taneja, Ajit K. Saxena. A Propionyloxy derivative of 11-keto-β-boswellic acid induces apoptosis in HL-60 cells mediated through topoisomerase I &II inhibition. *Chemico-Biological Interactions* **(2011): 189: 60–71.**

2. **Gousia Chashoo**, Shashank K. Singh, Dilip M. Mondhe, Parduman R. Sharma, Samar S. Andotra, Bhahwal. A. Shah, Subhash C. Taneja, Ajit K. Saxena. Potentiation of the antitumor effect of 11-keto-β-boswellic acid by its 3-α-hexanoyloxy derivative (*Article in Press. The European journal of pharmacology*)

3. Aviral Jain, **Gousia Chashoo**, Shashank K. Singh, Ajit K. Saxena and Sanjay K. Jain. Transferrin-appended PEGylated nanoparticles for temozolomide delivery to brain: in vitro characterisation. *Journal of Microencapsulation* **(2011): 28/1: 21-28.**

4. Doma M. Reddy, Jada Srinivas, **Gousia Chashoo**, Ajit K. Saxena, H. M. Sampath Kumar. 4β-[(4-Alkyl)-1,2,3-triazol-1-yl] podophyllotoxins as anticancer compounds: Design, synthesis and biological evaluation. *European Journal of Medicinal Chemistry* **(2011): 46 (6): 1983-1991**

5. D.M. Reddy, N.A. Qazi, S.D. Sawant, A.H. Bandey, J. Srinivas, M. Shankar, S.K. Singh, M. Verma, **C. Gousia**, A. Saxena, D. Mondhe, A.K. Saxena, V.K. Sethi, S.C. Taneja, G.N. Qazi, S. Kumar. Design and synthesis of spiro-derivatives of parthenin as novel anti-cancer agents, *European Journal of Medicinal Chemistry* **(2011), doi: 10.1016/j.ejmech.2011.04.030**

6. Ahmed Kamal , Ashwini Kumar , Paidakula Suresh , Satyam Kumar Agrawal, **Gousia Chashoo**, Shashank K. Singh , A. K. Saxena. Synthesis of 4b-N-polyaromatic substituted podophyllotoxins: DNA topoisomerase inhibition, anticancer and apoptosis-inducing activities. **Bioorganic and Medicinal Chemistry (2010): 18/24: 8493-8500.**

7. Asama Mukherjee, Sushanata Dutta, **Gousia Chashoo**, Ajit Kumar Saxena and Utpal Sanyal. Anti-tumoral and Toxicological Studies with 2-[4-{3-(2-Chloroethyl)-3-nitrosoureido}butyl]-napthalimide, a Novel Nitrosourea **Molecule. Journal of Cancer Molecules (2010): 5/2: 41-47.**

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11. Uppuluri V. Mallavadhani, Satyabrata Mohapatra, Anita Mahapatra, **Gousia Chashoo**, Ajit K. Saxena. A Novel Anticancer Secosteroid from an Indian Traditional plant, Morinda tinctoria Roxb (**Under Review in the journal of Planta Medica**)

12. Doma Mahendhar Reddy, Parvinder P. Singh, Sanghapal D. Sawant, Jada Srinivas, Tabasum Sidiq, **Gousia Chashoo**, Anamika Khajuria, Ajit K. Saxena, Halmuthur M. Sampath Kumar, Ghulam. N. Qazi Identification of novel anticancer and immunomodulatory peptides in Oryza sativa based fermented food by MALDI analysis (**Under Review in the Journal of Food Chemistry**).

Patents:

1. Kamal, Y.V.V. Srikanth, M.N.A. Khan, M. Ashraf, I.Sehar, **G. Chashoo**, P.R. Sharma, A.H. Dar, S.Bhushan, S.K.Singh, D.M.Mondhe and A.K. Saxena. 2-Anilino nicotinyl linked 2-amino benzothiazole conjugates as potential anticancer agents, apoptosis inducers and process for preparation thereof. **Indian and PCT Patent filed**

Abstracts presented in conferences/symposia:

1. Gousia Chashoo, Shashank K. Singh, Sachin B, Harish C. Pal, S. Singh, D. M. Mondhe, Ajit K. Saxena, S. C. Taneja, R. K. Johri and G. N. Qazi . Pharmacodynamic & Pharmacokinetic Evaluation of a Boswellic Acid Analog: A potent Topoisomerase-I Inhibitor. International Symposium on Genomic Instability and Cancer, Srinagar (Kashmir) 2007.

2. Gousia Chashoo, Shashank K. Singh, Sachin B., Harish C. Pal, S. Singh, D. M. Mondhe, Ajit K. Saxena, S. C. Taneja, R. K. Johri and G. N. Qazi. Boswellic acids analogues: worthy "MAGIC BULLET(S)" as Cancer Therapeutics. International Conference on Cancer Nanotechnology, New Delhi 2009.

3. Gousia Chashoo, Shashank K Singh, Paraduman R Sharma, Dilip M Mondhe, Abid H Dar, Arpita Saxena, Samar S Andotra, Bhahwal A Shah, Naveed A Qazi, Subhash C Taneja, Ajit K Saxena. A Propionyloxy derivative of 11-keto-β-boswellic acid induces apoptosis in HL-60 cells mediated through topoisomerase I & II inhibition. International Conference on Mitochondrial Research and Medicine (ICMRM), Shri Mata Vaishno Devi University, Katra 2010.

Honors/awards

1. Senior Research Fellowship (SRF) award from Council of Scientific and Industrial Research (CSIR), New Delhi, India.

2. Achieved best paper award (in oral presentation) for the paper entitled "A Propionyloxy derivative of 11-keto-β-boswellic acid induces apoptosis in HL-60 cells mediated through topoisomerase I &II inhibition" in International Conference on Mitochondrial Research And Medicine (ICMRM) on 12-14, November 2010 held at Shri Mata Vaishno Devi University, Katra.