3. REVIEW OF LITERATURE

Cancer is one of the most important health problems of the current era and also a leading cause of death among populations. Cancer is known medically as a malignant neoplasm all involving unregulated cell growth. In cancer, cells divide and grow uncontrollably forming malignant tumors and invade nearby parts of the body. A tumor is a mass of abnormal cells which grow uncontrollably in the body. In its natural course, tumor mass continues to grow invading the surrounding tissues and finally tumor cells get access to the lymphatic and vascular systems spreading to distant organs which results in metastasis these tumors are referred as malignant tumors, benign tumors refers to a tumor that are localized and has not spread to other parts of the body, because they lack the invasive properties of cancer.

The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. Under normal circumstances, cells of the same type are somewhat equal in size, have a common shape and spread out evenly. The cells grow slowly a process known as mitosis, adhere to each other and spread out in a layer formation to collectively form a tissue (Kleinsmith, 2006).

The cell cycle or cell-division cycle is the series of events that take place in a cell leading to its division and duplication.

In cell cycle at least two types of cell cycle control mechanisms are recognized: one type of mechanisms are a set of check points that monitor completion of critical events and delay progression to the next stage of necessary, other type of mechanisms are a cascade of protein phosphorylations that relay a cell from one stage to the next. The control involves a highly regulated Kinase family (Morgan Do, 1995).
Figure 1: Cell-division cycle for mammalian cells.

*Courtesy: [http://www.abcam.com](http://www.abcam.com)*

In the first type of cell cycle regulation check points sense flaws in critical events such as DNA replication and chromosome segregation. When check points are activated by under replicated or DNA damage then signals are relayed to the cell cycle-progression machinery. These signals cause a delay in cycle progression, until the mutation has been averted (Elledge, 1996). Two main check points exist: the G1 / S checkpoint and the G2 / M checkpoint. p53 plays an important role in triggering the control mechanisms at both G1 / S and G2 / M checkpoints. The second type of cell cycle is regulated by kinase activation, generally requires association with a second subunit that is transiently expressed at the appropriate period of cell cycle. The periodic cyclin subunit associates with its partner “cyclin-dependant kinase”(CDK) to create an active complex with unique substrate specificity. Regulatory phosphorylation and dephosphorylation fine tune the activity of CDK-cyclin complexes. This activation helps the coordinated entry into the next phase of the cell cycle. CDKs are constitutively expressed in cells whereas cyclins
are synthesised at specific stages of the cell cycle in response to various molecular signals ensuring well-delineated transitions between cell cycle stages (Robbins et al., 2004).

Superficially, the connection between the cell cycle and cancer is obvious: cell cycle machinery controls cell proliferation, and cancer is a disease of inappropriate cell proliferation. When normal cells become old or damaged, they die and are replaced with new cells. However, sometimes this orderly process goes wrong, and then the genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division (Kleinsmith, 2006). As a consequence, the DNA repair process is constantly active as it responds to damage in the DNA structure. DNA repair is coupled with DNA damage responses that are commonly referred to as checkpoint response. These checkpoints enable cell cycle arrest, which provides time for repair and avoids further damage until the DNA damaging agent is cleared from the cell. When normal repair processes fail, and when cellular apoptosis does not occur, irreparable DNA damage may occur including double-strand breaks and DNA cross-linkages (Bjorksten et al., 1971).

When there is absence of apoptosis in normal cell at required time results in uncontrolled cell division. The extra cells forms a mass of tissue called tumor. Most cancer types result from accumulation of multiple errors in DNA that may affect primarily the regulatory pathways in the cell. A disregulation of the cell cycle components may lead to tumor formation.

**Types of cancer**
Cancer types can be grouped into broader categories. They are classified according to the tissue and cell type from which they arise. The main categories of cancer include:

- **Carcinoma**: cancers derived from epithelial cells. Cancer that begins in the skin or in tissues that line or cover internal organs. (*eg:* cervical carcinoma)
- **Sarcoma**: cancers arising from connective tissue (bone, cartilage, fat, muscle, blood vessels). Each of which develop from cells originating in mesenchymal cells outside the bone marrow.
Leukemia: cancer that starts from hematopoietic (blood forming) cells such as the bone marrow and causes large numbers of abnormal blood cells and enter the blood.

Lymphoma and myeloma: cancer that begins in the cells of the immune system.

Central nervous system cancer: Cancers that begin in the tissues of the brain and spinal cord (Varricchio; Claudette, 2004). Each of these categories has their sub-division according to specific cell type, location in the body and structure of the tumor.

There are over 200 different known cancers that afflict humans. Cancer in general and specific cancers in particular organ are morphologically distinct from normal cells histopathologically such as breast, prostrate, lungs, colon, pancreas, eye cancer, gall bladder, head and neck cancer, oral cancer, ovarian, skin, stomach, throat, urethral, vaginal, vulvar, cervical cancer, endometrial cancer, etc.

When mutation in some genes like the cell cycle inhibitors p53, pRb, etc may cause the cell to multiply uncontrollably forming a tumor. In cervical cancer caused by human papillomavirus expresses E6 and E7 genes from HPV which results in tumor development by suppressing p53, pRb tumour suppressor proteins involved in cell cycle regulation and cause cancer.

p53

p53 known as protein 53 or tumor protein 53 functions as a tumor suppressor that is involved in preventing cancer. It regulates the cell cycle. It is described as “the guardian of the genome”. p53 is a protein encoded by TP53 gene with a molecular mass 53 kDa. It has many mechanisms of anticancer function and plays a role in apoptosis, inhibition of angiogenesis and genomic stability. In its anti-cancer role several mechanism involved are - it can activate DNA repair proteins when DNA has sustained damage, It can induce growth arrest by holding the cell cycle at the G1 / S regulation point on DNA damage recognition and provides time for DNA repair proteins to fix the damage and then continue the cell cycle. p53 can initiate apoptosis, if DNA damage is irreparable (Matlashewski et al., 1984).
The retinoblastoma protein pRb is a tumor suppressor protein that is dysfunctional in several major cancers (Murphree and Benedict, 1984). Function of pRb is to prevent excessive cell growth by inhibiting cell cycle progression. It is also a recruiter of several chromatin remodelling enzymes such as methylases and acetylases. pRb belongs to the pocket protein family, have a pocket for the functional binding of other proteins like an oncogenic protein, such as those produced by cells infected by high-risk types of human papillomaviruses, bind and inactivate pRb which can lead to cancer (Munger and Howley, 2002). pRb prevents the cell from replicating damaged DNA by preventing its progression along the cell cycle through G1 into S phase. pRb binds and inhibits transcription factors of the E2F family, which are composed of dimers of an E2F protein and a DP protein (Wu et al., 1995). Phosphorylation and dephosphorylation of pRb plays important role in activation and inactivation of pRb protein.

Chemotherapy failure

When cancer cells overcome cell repair mechanism and it undergoes to irreparable cell cycle progression, irreparable mutations leads to transformation of normal cell to cancer cell. Chemotherapy is the treatment for cancer that plays a key role to overcome the transformation. In certain cancer the main obstacle in chemotherapy for cancer is drug resistance. Recently failure to chemotherapy for cervical cancer caused by human papillomavirus were found to be due to drug resistance to chemotherapeutic agents and also cross resistance to other anticancer drug which results in the development of multidrug resistance(MDR) phenotype. Although onset of cervical cancer is slow, but if infection persist for longer time leads to invasive cancer. The failure to the treatment for advanced and invasive cancer is development of multidrug resistance.

Cervical cancer

Cervical cancer is the second most common cancers among women worldwide. Cervical cancer is a malignant neoplasm arising from cells originating in the cervix. The cervix is the lower, narrow end of the uterus, it is sometimes called the uterine cervix. The cervix connects the vagina (birth canal) to the upper part of the uterus. The part of
the cervix closest to the body of the uterus is called the endocervix. The part next to the vagina is the exocervix (or) ectocervix. The place where these two parts meet is called the transformation zone. Most cervical carcinoma starts in the transformation zone.

![Female reproductive system](http://www.nccc-online.org)

**Figure 2:** Female reproductive system.

_Courtesy: http://www.nccc-online.org_

Human papillomavirus (HPV) infection appears to be a necessary factor in the development of almost all cases (90%) of cervical cancer. HPV establish productive infections only in keratinocytes of the skin or mucous membranes. Persistent infection with high-risk HPV types different from the one that causes skin warts may progress to precancerous lesions and invasive carcinoma (Walboomers _et al._, 1999).

**Cervical cancer history**

In 400 BC Hippocrates described cervical cancer is incurable. The concept of cervical cancer as a multistage disease originated at the beginning of 20th century. In 1910 Rubin described histological changes in the cervical epithelium were precursors of invasive squamous carcinoma. In 1928 Papanicolaou developed pap test, in 1943 he reported diagnosis of uterine cancer by vaginal smear (Papanicolaou and Traut, 1941). Syverton and Berry in 1935 discovered a relationship between Rabbit papillomavirus (RPV) causing skin cancer in rabbits, but HPV is species specific and cannot be transmitted to rabbits. Human papillomavirus (HPV) was discovered by electron
microscopy in 1949 (Lowry and Schiller, 2006). Since HPV were classified by specific viral subtypes using variation in DNA sequences and in 1963 HPV-DNA was identified (Durst et al., 1983).

Global burden of cervical cancer

Cervical cancer is the second highest leading cancer in terms of incidence rate after breast cancer among women worldwide. Its mortality rates are higher in low and middle income countries (LMIC). Around 80% of global cervical cancer cases are in (LMIC) (Waggoner, 2003).

![Mortality](image)

**Figure 3:** *Estimated Age-Standardised mortality Rate for cancer. Age-Standardised Rate (ASR) and proportions per 100,000*

*Source: GLOBOCAN 2008 (IARC)*
Global prevalence of HPV infection in the general female population is estimated at 11.4%, however prevalence varies greatly from country to country. In India prevalence of HPV infection is 7.9% lower than the world average. The number of cases of invasive cervical cancer is highest in the South Asia region. In India prevalence of HPV 16 / 18 in invasive cervical cancer cases is 82.5% (Bosch and de, 2003).
Table 1: Crude & age-standardized incidence rates per 100,000 population for Cervical cancer in 12 PBCRs in India

<table>
<thead>
<tr>
<th>PBCR</th>
<th>Crude incidence rate</th>
<th>Age standardized incidence rate</th>
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<tbody>
<tr>
<td>Bangalore</td>
<td>18.8</td>
<td>21.7</td>
</tr>
<tr>
<td>Barshi</td>
<td>42.7</td>
<td>22.4</td>
</tr>
<tr>
<td>Bhopal</td>
<td>22.2</td>
<td>24.5</td>
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<tr>
<td>Chennai</td>
<td>24.4</td>
<td>30.6</td>
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<tr>
<td>Delhi</td>
<td>16.3</td>
<td>22.7</td>
</tr>
<tr>
<td>Mumbai</td>
<td>14.6</td>
<td>18.0</td>
</tr>
<tr>
<td>Ahmedabad</td>
<td>16.2</td>
<td>13.4</td>
</tr>
<tr>
<td>Karunagappally</td>
<td>19.2</td>
<td>15.0</td>
</tr>
<tr>
<td>Kolkata</td>
<td>17.5</td>
<td>19.9</td>
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<tr>
<td>Nagpur</td>
<td>19.1</td>
<td>23.2</td>
</tr>
<tr>
<td>Pune</td>
<td>20.5</td>
<td>22.5</td>
</tr>
<tr>
<td>Thiruvanthapuram</td>
<td>13.1</td>
<td>10.9</td>
</tr>
</tbody>
</table>

**Source:** National Cancer Registry Programme and World Health Organisation, Atlas of Cancer in India

*Population Based Cancer Registries (PBCRs)*

Chennai shows maximum incidence rate for cervical cancer. Age standardized incidence rate in major metropolitan cities were higher in cervical cancer cases registered.
HPV infection plays important role in cervical cancer there are low and high risk HPV types causing infection. Among them high-risk HPV were more potent for infection among all ages.

![Graph showing prevalence of low-risk and high-risk HPV types among aged 14 to 59 years, NHANES 2003-2004.](image)

**Figure 5:** Prevalence of Low-risk and High-risk types among aged 14 to 59 years, NHANES 2003-2004. National Health and Nutrition Examination Survey (NHANES) Courtesy: (Bosch and de, 2003).

Cancer of the cervix can develop in women of all ages; usually at 35-55 years have a peak age for incidence varying with population. In India the peak age for cervical cancer incidence is 45-54 years and 30-40 years in UK (Zeller et al., 2007).
There are several strains of HPV infection causes cervical cancer. 80% cases by HPV 16 and 18 that plays important role in cervical cancer (Bosch and de, 2003).

**Human papillomavirus (HPV)**

Papillomaviruses have mostly been isolated from humans, and also found in domestic mammals. Papillomaviruses are species specific and do not transmit from non-primates to humans (Orth et al., 1978). Cervical cancer is primarily caused by human papillomavirus (HPV) infection, 90% of all cervical cancers is caused by human papillomavirus (HPV). HPV infects either mucosal or cutaneous keratinocytes. All HPV types are epitheliotropic viruses, so far over 200 human papillomavirus (HPV) types are identified. These HPV types can be subdivided on the basis of their ability to infect tissue. There are low-risk and high-risk HPV types which infects mucosal keratinocytes. Benign neoplasms, such as warts and condyloma acuminatum are caused by low-risk HPV types. Malignant neoplasms, such as cervical cancer are caused by high-risk HPV types (de Villiers et al., 2004; Longworth and Laimins, 2004). High-risk HPV types HPV16, HPV18, HPV31, HPV45 are associated to malignant progression. These high-risk HPV’s produce a protein that can cause cervical epithelial cells to grow.
uncontrollably. These viruses make a second protein that interferes with tumour suppressors produced by the human immune system (Ellen Thackery, 1972).

Virion structure

Human Papillomavirus are small double stranded DNA tumour virus. Virions of human papillomavirus are non-enveloped icosahedral capsid consisting of dsDNA encapsulated by a single copy of circular 8 kb dsDNA genome and replicate in the nucleus of host cells (squamous epithelial cells). The viral genomes are associated with cellular histones, forming chromatin like structures within the capsid (Larsen et al., 1987; Doorbar, 2005). Genome have 1.34- 1.36gL\(^{-1}\) density in caesium chloride and owing to the lack of lipids, are resistant to ether and other solvents (Fuchs and Pfister, 1994). Capsids are icosahedral particles approximately 50 to 60 nm in diameter (Modis et al., 2002). The viral particle composed of 72 capsomers with a star-shaped morphology and displaying a cylindrical channel along their axis 80% of total capsid protein are L1 protein of 55kDa and remaining 20% of capsid composed of L2 protein of 70 kDa (Howley et al., 1996).

Figure 7: Human Papillomavirus (magnification 520x600).

Courtesy: www.med-ars.it
L1 protein form a network of intra and inter-pentameric disulfide interactions which stabilize the capsid (Sapp et al., 1998). L2 protein may be involved in the efficient self-assembly of the viral capsid and attraction of viral genome during viral assembly (Howley et al., 1996). Doorbar and Finnen suggested that papillomavirus particles can contain up to 36 and as much as 72 L2 proteins per particle by SDS-PAGE analyses of native HPV1 virions (Doorbar and Gallimore, 1987; Finnen et al., 2003).

**Viral genome**

All HPV types have an average of 8 open reading frames (ORFs) in their viral genome, it codes for only eight proteins. A single DNA strand transcribe to form polycistronic mRNAs and these ORFs are expressed from polycistronic mRNAs (Favre et al., 1975; Zheng and Baker, 2006). Genome is divided into three regions, a long regulatory region (LCR) of 1kbp, early region about 4.5 kbp in size and a late region about 2.5 kbp. There are no open reading frames (ORF) in the LCR, but contains the viral origin of replication and control elements for transcription and replication, eight ORFs (E1 to E8) in the early region, two (ORFs) in the late region (L1 and L2) (Kashima et al., 1990).

![Figure 8: Schematic representation of HPV genome.](image)

*Courtesy: (Hoenil et al., 2005).*
HPV early protein E1 proteins are essential for viral replication, E2 ORF codes for E2 proteins which act as transcriptional activator. Function of E3 protein is not yet known, E4 proteins seems to assist the release of virus from infected cells (Chow and Broker, 1994). E5 ORF translated as small protein E5 proteins which are responsible for cellular transformation (Stoppler et al., 1994). Important oncogenic proteins are E6 and E7 proteins encode by high-risk HPV (hrHPV) types. Genes of these two proteins are expressed after integration into the host genome. E6 and E7 genes play a role in the initiation and oncogenic progression of tumours. E6 protein in association with host E6-associated protein, which has ubiquitin ligase activity act to ubiquitinate p53 leading to proteosomal degradation. E7 (in oncogenic HPVs) act as the primary transforming protein. E7 competes for retinoblastoma protein pRb binding, freeing the transcription factor E2F to transactivate its targets, thus pushing the cell cycle forward (Schiller et al., 2010). The important mechanisms of HPV-mediated cellular transformation involved are the binding and inactivation of cellular proteins expressed by p53 and pRb genes by E6 and E7 oncogenes. E8 ORF is found in some papillomavirus contributes to cell transformation by conferring anchorage-independent growth. The late L1 and L2 are structural proteins of the individual capsomere subunits in the viral capsid. L1protein functions for virus attachment to the cell surface receptor and contributes the basic structural component of the capsid. L2 proteins are infectivity enhancing capsid protein during viral assembly (Howley et al., 1996).

**HPV genotyping**

The genotyping of HPV is differentiated by the DNA sequences of L1 proteins and mainly by E6 and E7 protein. The HPV genotypes are numbered in sequence of their discovery. To define a new HPV viral type a 105 difference in DNA sequence with respect to previously established strain is sufficient. Three genera of the papillomaviridae family are responsible for human diseases. Alpha-papillomavirus includes all genital papillomaviruses, beta-papillomavirus responsible for epidermodysplasia verruciformis and gamma-papillomaviruses responsible for cutaneous lesions (Bernard, 2005).
HPV classification

There are 150-200 types of HPV known. There are several strains of HPV infection most of which have been found to increase the risk of developing cervical cancer.

There are 16 genera of papillomaviruses identified, 5 genera contains HPV type papillomaviruses

Human papillomavirus

- Group : Group I (ds DNA)
- Order : Unranked
- Family : Papillomaviridae
- Genera: Alphapapillomavirus
  - Betapapillomavirus
  - Gammapapillomavirus
  - Mupapillomavirus
  - Nupapillomavirus

Alphapapillomavirus are HPV32, 10, 61, 2, 26, 53, 18, 7, 16, 6, 34, 54, 71,
Betapapillomavirus are HPV5, 9, 49, Gammapapillomavirus are HPV4, 48, 50, 60,
Mupapillomavirus are HPV1, 63, Nupapillomavirus are HPV41 (de Villers et al., 2004).
The clinically most important genus is referred as alpha-papillomavirus, it contains all HPV types associated with mucosal and genital lesions. DNA sequences varies in each HPV viral type and they are numbered according to their discovery in sequence. Munoz et al., classified mucosal HPV into 18 high-risk HPV (hrHPV) genotypes are HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, and HPV26, 53, 66 as probable high-risk types and 12 low-risk HPV (lrHPV) are HPV6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108. HPV16 and 18 account for more than 70% of all cervical cancer cases. HPV16 seems to be predominant; HPV 18 seems to play an equally important role in adenocarcinoma (Munoz et al., 2003).
Figure 9: Phylogenetic tree containing the sequences of 118 papillomavirus types. The L1 ORF sequences were used in a modified version of the phylip version 3.572 and based on a weight version of the neighbour-joining analysis. The tree was constructed using the tree view program of the University of Glasgow. The numbers at the ends of each of the branch identify an HPV type; c-numbers refers to candidate HPV types. All other abbreviations refer to animal papillomavirus types (de Villers et al., 2004).
**Mode of infection**

Within the tissue HPV virions infects the basal epithelial cells by micro-abrasions. Basal layer cells is the epithelial stem cells that have been infected for a lesion to be maintained and cells consists mainly of stem cells and transit-amplifying cells (Kaur and Li, 2000; Egawa, 2003). The virions associate with putative receptors such as alpha integrins and laminins, leading to entry of the virions into basal epithelial cells through clathrin-mediated endocytosis or caveolin-mediated endocytosis depending on the papillomavirus (Schiller et al., 2010). It takes many hours for post adsorption to the cell surface and internalization of virions depending on the type of HPV. Though antibodies against L1 and L2 proteins may neutralize the HPV infection, while in some cases the virions still reside on the basement membrane and cell surfaces. After infection, the early viral proteins are expressed in the infected basal cells within the lower epithelial layers (Gillison and Shah, 2003). During infection virion enters the cell and they have no envelope, since they are relatively stable and remain infectious for months in a moist environment (Orth et al., 1978).

HPV infected basal cell divide during mitosis each new daughter cell contains a set of viral genomes. After mitosis basal cell layer retains one cell and other cell migrates up through the suprabasal layer (Lehman and Botchan, 1998). Due to the oncogenic properties of the E7 protein, detached cells remain mitotically active, therefore infected cells can re-enter the S phase and enhance the expression of cellular replication factor for amplification of viral genome and late gene expression. During migration of the cell up through the strata E1 to E4 genes are translated which involves inhibition of G2 to M transition and E5 proteins stimulates cell cycle progression in the suprabasal layers (Doorbar et al., 1997). L1 and L2 capsid proteins are expressed only in terminally differentiated keratinocytes, the capsid proteins assemble into icosahedral capsids using chaperone proteins (Barksdale and Baker, 1993). In the cornified envelope E1 and E4 proteins interact with cellular keratin networks and collapse to allow escape of mature virions from the cornified cells (Doorbar et al., 1986; Mach et al., 2006). Thus viral life cycle continues to expand the infection to nearby cells.
HPV life cycle

The life cycle of HPV is tightly linked to the differentiation program of the host keratinocyte. HPV infects the basal cells of a human epithelial surface and remains in infected basal cells (Doorbar, 2005). HPV infection initially occurs through micro-abrasions of the epithelial tissue to enter cells of the basal layer. The viral genomes are maintained at approximately 20-100 episomal copies per cell (Pang et al., 1993).

Upon infection HPV DNA is released into the cytoplasm, integration of HPV DNA to host genome disrupts the virus by inhibiting expression of L1 and L2 protein. E1 and E2 are first early proteins expressed and form a complex with the viral origin of replication. E2 protein recruits E1 to the origin of replication and accompanies to regulate expression of E6 and E7 proteins (Mohr et al., 1990). When the host and HPV genomes integrate, E2 function is disrupted, preventing repression of E6 / E7. The E6 and E7 proteins inactivate two tumor suppressor proteins, p53 (inactivated by E6) and pRb (inactivated by E7). The viral oncogenes E6 and E7 present in hrHPV are thought to modify the cell cycle so as to retain the differentiating host keratinocyte in a state that is favourable to the amplification of viral genome replication and consequent late gene
expression to form capsid proteins and finally mature virion formed in the infected cell is released due to expression of E4 proteins and the virion migrates to neighbouring cell for infection (Schiller et al., 2010).

Types of cervical cancer

There are two main types of cervical cancer:

![Cervical Cancer Diagram]

Approximately 90% of cervical cancers are squamous cell carcinomas. This type of cancer originates in the thin, flat, squamous cells on the surface of the ectocervix, (the part of the cervix that is next to the vagina). Squamous cells are the thin, flat cells of the surfaces of the skin and cervix and linings of various organs. Another 10% of cervical cancers are of the adenocarcinoma type. In this type cancer originates in the mucus-producing cells of the inner or endocervix, near the body of the uterus. Since abnormality develops from the glandular cells that line the cervical canal, it can be more difficult to detect with cervical screening tests. Sometimes cancer may have characteristics of both types of carcinoma and it is called adenosquamous carcinoma or mixed carcinoma.

In cervical cells the initial changes that may occur; these precancerous cells form a lesion called dysplasia or a squamous intraepithelial lesion (SIL). Dysplasia is a common condition and the abnormal cells disappear later without treatment. Cervical intraepithelial lesion (CIL) also known as cervical dysplasia a potentially premalignant transformation and abnormal growth of squamous cells on the surface of the cervix and are eliminated by the host’s immense system. A small percentage of cases progresses to
become cervical cancer, koliocytes are cellular changes associated with HPV infection are commonly seen in cervical intraepithelial neoplasia (CIN).

CIN starts in any of the three stages, and can either progress or regress. CIN is classified in grades, CIN 1- It is grade I. represents only mild dysplasia or abnormal cell growth and least risky type. (Agorastos et al., 2005). It is confined to the basal 1/3 of epithelium. When there is infection with HPV it is typically cleared by immune response in a year.

CIN 2- It is grade II. A moderate dysplasia confined to the basal 2/3 of the epithelium.

CIN 3- It is grade III. Severe dysplasia than spans more the 2/3 of the epithelium, and may involve the full thickness. They are referred as cervical carcinoma in situ.

Corresponding to cytology CIN is also called as squamous intraepithelial lesion (SIL).

There are two types of SIL based on Bethesda system:
1. Low-grade SIL are mild dysplasia or CIN 1
2. High-grade SIL are moderate and severe dysplasia otherwise called CIN2, CIN3 (Park et al., 1998).

![Figure 11: Cervical intraepithelial neoplasia (CIN).](http://medicalpictures.net)
Stages of cervical cancer

Cervical cancer is staged by the International Federation of Gynecologists and Obstetricians (FIGO) staging system, based on clinical examination. It allows only the following diagnostic tests to be used in determining the stage: palpation, inspection, colposcopy, endocervical curettage, hysteroscopy, cystoscopy, proctoscopy, intravenous urography and X-ray examination of the lungs and skeleton and cervical conisation.

FIGO nomenclature – carcinoma of the cervix uteri.

Stage 0  : Carcinoma in situ, cervical intraepithelial neoplasia grade III
Stage I  : The carcinoma is strictly confined to the cervix
Stage Ia1 : Small cancerous area that is visible only with a microscope
Stage Ia2 : Measured stromal invasion of not >3.0mm in depth and extension of not >7.0mm
Stage Ib  : Clinically visible lesions limited to the cervix uteri or pre-clinical cancers greater than Stage Ia. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0mm and a horizontal extension of not >7.0mm. Depth of invasion should not be >5.0mm taken from the base of the epithelium of the original tissue should not change the stage allotment.
Stage Ib1 : Clinically visible lesions not >4.0cm
Stage Ib2 : Clinically visible lesions >4.0cm
Stage II  : Cervical carcinoma invades beyond uterus, but not to the pelvic wall or to the lower third of vagina.
Stage IIa : No obvious parametrial involvement
Stage IIb : Obvious parametrial involvement
Stage III : The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumor and the pelvic wall. The tumor involves the lower third of vagina. All cases with hydronephrosis or non-functioning kidney are included.
Stage IIIa : Tumor involves lower third of the vagina with no extension to the pelvic wall.
Stage IIIb: Extension of the pelvic wall and hydronephrosis or non-functioning kidney

Stage IV: The cancer has spread to other parts of the body. The carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum.

Stage IVa: Spread of the growth to bladder or rectum

Stage IVb: Spread of the abnormal cells to distant organs such as lungs (Quinn et al., 2006).

Symptoms

The early stages of cervical cancer may be completely asymptomatic. Most women do not have symptoms until cancer symptoms include:

- unusual vaginal bleeding between periods or after menopause
- continuous vaginal discharge, which may be pale watery, pink, brown, bloody or foul smelling
- pelvic pain during urination.

Symptoms of advanced cervical cancer may include

- loss of appetite,
- fatigue,
- weight loss,
- leg pain,
- back pain,
- pelvic pain,
- swollen legs,
- heavy bleeding from the vagina leads to anemia,
- bone fracture,
- urinary problems because of blockage of kidney or ureter,
- leakage of urine or faces into the vagina, when an abnormal opening has developed between the vagina and the bladder (Canavan and Doshi, 2000).
**HPV detection and diagnosis methods**

One of the most effective ways of preventing and controlling cervical cancer is regular screening and early diagnosis. The diagnosis of HPV infection can normally be achieved by cytology, histology, and colposcopy. Cervical cancer is first detected with a pap test that is performed as part of a regular pelvic examination. There is smear based cytology and liquid-based cytology.

**Pap test**

Pap test is very efficient for detection of cervical abnormalities part of a regular pelvis examination. Georgious Papanikolaou was inventor of the “pap smear”. The preparation of vaginal and cervical smears, physiologic cytological changes during the menstrual cycle, the effects of various pathological conditions, and the changes are seen in the presence of cancer of cervix and the endometrium of the uterus. The vaginal spread with a metal or plastic instrument called a speculum; a swab was used to remove mucus and cells from the cervix and examined through microscope (Miller *et al.*, 1990). In traditional pap test the cells are scraped from cervix and examined on a slide but in liquid based technique cells are first suspended and then applied to a glass slide removing much of the mucus, blood and inflammatory cells. Though liquid-based methods are more expensive than conventional pap tests but they have sensitivity of 80% for the detection of CIN (masus-Awatai and Freeman-Wang, 2003).

**Colposcopy**

Following an abnormal pap test, a colposcopy is usually performed. The physician uses a magnifying scope to view the surface of the cervix. Schiller test was performed; the cervix may be coated with an iodine solution that causes normal cells to turn brown and abnormal cells to turn white or yellow. If any abnormal areas are observed a colposcopic biopsy may be performed. A loop electrosurgical excision procedure (LEEP) is a cone biopsy used to determine whether abnormal cells have invaded below the surface of the cervix (Miller *et al.*, 1990).
Further molecular tests for the clinical detection of HPV have been developed for women with abnormal cervical cytology. Serological testing of HPV was commonly performed using an ELISA test for antibodies to type-specific virus-like particles (VLPS). Most persons with HPV infections do not develop antibodies, unfortunately current serologic testing of HPV antibodies has relatively low sensitivity and serological response are non-type specific. Therefore serologic studies of HPV are considered to be only research tools for detection of HPV (Silins et al., 1999).

**Molecular test**

Detection of HPV DNA was reliable diagnostic tool available to identify specific HPV types. HPV-DNA test are currently available diagnostic tool for detecting HPV DNA to identify specific HPV types. Although methods is expensive but it has higher sensitivity than cytological screening detection of viral genome either directly by Southern blot hybridization or by Polymerase chain reaction (Howley et al., 1996).

It can detect CIN lesions at an earlier stage than cytology. The subsequent combined pap and HPV test should be done at least once in 3 years. HPV DNA testing is also recommended as an alternative to additional procedures (colposcopy and cytology) when there are abnormal pap results. Regular screening is required for abnormal cervical cells because usually it take 6-12 months for the clearance of transient infection (Wright et al., 2004).

**Vaccines**

The prophylactic vaccine approach became possible in 1991. Vaccines are given before HPV infection to prevent viral entry before onset of infection, which helps the immune system to recognize virus and clear the infection. Vaccines play main role in viral entry by generating virus neutralising antibodies directed against the L1 and L2 capsid proteins. Individual receiving the vaccine pose no infections or oncologic risk, zhou et al., demonstrated particles do not contain viral DNA and constructed with HPV-16 L1 capsid proteins self assembled into conformational VLPs that resembled native virions in a recombinant system. These particles are empty capsids that contain the
major neutralising epitopes of the native vision (Zhou et al., 1991). Vaccine development has focused on hrHPV types since HPV 16 and 18 accounts for approximately 70% of cervical cancers. Approach for L1 VLP vaccines gives type-specific protection (White et al., 1998). Two different vaccines that have been developed to prevent infection from HPV 16 and 18 and one of these offers added protection against HPV 6 and 11 (which cause genital warts). Vaccine called ‘Gardasil’ manufactured by Merck and ‘Cervarix’ developed by GlaxoSmithKline (GSK) has been licensed for use in several countries including USA. Both vaccines need to be administrated with 3 doses can be given to females aged 9-26 years, and are most effective if given before the female’s first sexual encounter (Kim et al., 2009).

Gardasil is a quadrivalent vaccine with HPV 6,11,16,18 VLPS and an adjuvant aluminium hydroxide that boosts immense response. Yeast *Saccharomyces cerevisiae* is used to produce L1 VLPS using recombinant DNA technology. The vaccine was generally well-tolerated, and there were no serious vaccine-related adverse events. It has completed phase 3 clinical trials and was licensed by the FDA on June 2006 (Villa et al., 2005).

Cervarix is a bivalent vaccine with HPV-16 and 18 VLPS to boost the immune response aluminium salt plus monophosphoryl lipid A (AS64) is used as adjuvant and produced using baculovirus-infected insect cells. Both the vaccines are administered in a three-dose regimen each at 0.5ml injected intramuscularly but the time duration of each administration differs in both vaccine types. In addition cervarix may provide cross-protection against HPV types 31 and 45 (Harper et al., 2006). Both companies probably included aluminium based adjutants to stabilize the vaccine during cold storage and reduce the dose required to induce peak antibody filter (Schiller and Lowry, 2006).

There are some drawbacks in vaccine because both vaccines protect women against only two hr HPV types, since remaining 30% of cervical cancer caused by other hr HPV types, so there still needs study for screening and development of broad spectrum vaccine for cervical cancer. There are certain questions to be addressed in vaccine
development including the duration of immunity, long-term safety the optimal age for vaccination the optimal program of screening for cervical lesions (kung-liahng wang, 2007).

**Treatment for cancer:**

Treatment varies based on the type of cancer and its stage (Moscow et al., 2007).

Treatment of cervical cancer depends on:
- The stage of the cancer
- The size and shape of the tumor
- The woman’s age and general health
- Her desire to have children in the future.

**Surgery**

If the cancer is confined to one location and has not spread, the most common treatment approach is surgery to cure the cancer. Early cervical cancer can be cured by removing or destroying the precancerous or cancerous tissue. There are various surgical ways to do this without removing the uterus or damaging the cervix, so that a woman can still have children in the future.

**Types of surgery for early cervical cancer include:**

- Loop electrosurgical excision procedure (LEEP) - that uses electricity to remove abnormal tissue
- Cryotherapy - freezes abnormal cells.
- Laser therapy - uses light to burn abnormal tissue.

If surgery cannot remove all of the cancer, the options for treatment include radiation therapy, chemotherapy or both. Some cancers require a combination of surgery, radiation and chemotherapy (Kim et al., 2009).
Radiation therapy

Radiation therapy uses high-energy radiation to shrink tumors and kill cancer cells. X-rays, gamma rays, and charged particles are types of radiation used for cancer treatment. Treatment for more advanced cervical cancer may include radiation therapy to treat cancer that has spread beyond the pelvis, or cancer that has returned. Radiation therapy is either external or internal. External radiation therapy beams radiation from a large machine onto the body where the cancer is located. Internal radiation therapy uses a device filled with radioactive material, which is placed inside the women’s vagina next to the cervical cancer. The device is removed after sometime (Kent, 2010).

Chemotherapy

The use of drugs to kill cancer cells is called chemotherapy. Chemotherapeutic drugs can be given by oral administration or injection. Chemotherapy is considered a body-wide (systemic) treatment, since the medicines travel through the bloodstream to the entire body. Some of the drugs used for cervical cancer chemotherapy include 5-fluorouracil (5FU), cisplatin, carboplatin, ifosfamide, paclitaxel and cyclophosphamide (Kim et al., 2009).

Chemotherapy is the method can be used even to control advanced cancer. The advantages are easy for administration, economic; the patient will require only occasional hospitalization.

Classification of chemotherapeutic drugs

Drugs for chemotherapy are broadly classified into several categories based on the factors such as synthetic agents. These drugs are chemically synthesized they are mainly targeted towards the DNA of the cancer cells.

Some synthetic agents are:

Alkylating agents: They chemically alter the cellular DNA by adding alkyl groups to the electronegative groups. They are cisplatin, oxaliplatin, carboplatin, chlorambucil, cyclophosphamide, mechloethamine and melphalan, ifosfamide, melphalan, busulfan.
**Anti-Metabolites:**- These anti-metabolites stop cell division, some of the important drugs include methotrexate, fludarabine and cytarabine, 5-fluorouracil, capecitabine, gemcitabine.

**Microbes derived agents:**- They are mainly obtained from the microbes. Mainly many enzymes are included in this category some are Topoisomerase inhibitors this includes irinotecan, topotecan, amasacrine, etoposide phosphate and etoposide, teniposide.

**Anti-tumor antibiotics:**- They are Dactinomycin, daunorubicin, doxorubicin and idarubicin. Epirubicin (Joensuu, 2008).

**Phytochemical agents:**- These are plant-based chemicals which block the cell division by inhibiting microtubule function. These include the plant derived toxins and their pigments like xanthanoides, Alkaloides, flavanoides etc. they act as mitotic inhibitors such as paclitaxel, docetaxel, vinblastine, vincristine (Wu, 2006). The anticancer drugs are generally used to kill cancer cells. These drugs all interfere with cell cycle, they have different target site in cell, inhibits certain signalling pathways to terminate the cancer cell growth.

Cancer develops overtime when certain normal genes starts mutating. These gene mutations occurs due to a complex mix of factors related to lifestyle, hereditary and environment, chemicals (carcinogens), diet, exercise, radiation, infection by viruses (HPV, EBV, hepatitis B and C) (Anand et al., 2008).

Various plant-derived agents like genistein, curcumin, epigallacatechin gallate(EGCG), resveratrol, indole-3-carbinol, and proanthocyanidin have been shown to be able to affect the efficacy of traditional chemotherapeutic agents (Prasad et al., 2001; Davis, 2007).
Russo et al., and steward had demonstrated phytochemicals as biological targets in mammalian cells which involved in inflammatory process, mechanism of defence against foreign agents (radiations, viruses, parasites), and oncogenic transformation like apoptosis evasion, alterations of cell cycle control angiogenesis and metastases. A number of uses of phytochemicals suggest that a daily intake of phytochemicals can reduce the incidence of several types of cancers (D’Incalci et al., 2005; and Russo et al., 2005).

**Dietary phytochemicals**

There are vast number of heterogenous class of molecules present in the diet they are generally called as phytochemicals includes vitamins like carotenoids and food polyphenols like, phytoalexins, phenolic acids indoles, flavonoids (Sporn and Suh, 2002; Suh, 2003).

More than 10,000 phytochemicals have been described and among them flavanoids include about 6000 compounds (Hairborne, 1993). These phytochemicals play major role in chemotherapy for cancer patients. They are present in the herbal constituents fruits, berries and spices. The herbal constituents like quercetin, kaempferol, naringenin, silymarin, catechins. Fruits and berries include grape fruit, orange, apricot, and strawberry. In spices are mint, rosemary, garlic, piper nigrum, curcumin, ginseng, onion (Bansal et al., 2009).

Phytochemicals shows therapeutic efficiency in mono-treatments or in association with classical chemotherapeutic drugs, can give a double positive effects like.

1. It can synergize with cytotoxic drugs by increasing their efficacy and lowering the toxic side effects on normal cells.
2. Combined treatment can delay the onset of resistance to cancer cells (Maria Russo et al., 2010).
Chemotherapy is applied to patients who already developed tumors and invasive cancers. Phytochemicals can be found in free form in the blood only if they are taken at pharmacological doses (hundreds of milligrams). Therefore, the drugs are evaluated for pharmacological doses and drug administration by mouth or injected into the blood. Drugs with high doses are primarily metabolized in the liver and low doses are metabolized in the intestine. Adequate dosage of drug is to be evaluated because drug saturate the metabolic pathways of conjugation like methylation, sulfation, and glucuronidation and are potentially bioavailable and biologically active (Scalbert and Williamson, 2000).

Phytochemicals are functionally pleiotropic and possess multiple intracellular targets by affecting different cell signalling processes in cancer cells with limited toxicity on normal cells. The association of phytochemicals with chemotherapy shows synergistic effects that target simultaneously multiple pathways and help to kill cancer cells and slowdown the onset of drug resistance (Maria Russo et al., 2010).

**Pharmacological role of phytochemicals**

Some ginsenosides from Chinese ginpeng are able to sensitize drug-resistant breast cancer cells to paclitaxel (Wang and Yuan, 2008). Polyphenol quercetin present in many foods such as onions, apples, berries, and tea is able to potentiate the cytotoxic effect of cisplatin while protecting normal renal cells from cisplatin toxicity (Lamson and Brignall, 2000). Quercetin and busulfan against human leukemia cells showed synergistic effect (Hoffman et al., 1989). Quercetin can work synergistically with doxorubicin in cultured multidrug-resistant human breast cancer cells. It increases cytotoxic effect of cyclophosphamide (CY) and decreases resistance to gencitabine, topotecan, vincristine, tamoxifen, paclitaxel, and doxorubicin (Scambia et al., 1994).

Green tea increases the concentration of chemotherapeutic agent in tumor but not in normal tissue by enhancing its antitumor activity. Catechins from green tea can increase the therapeutic effect of doxorubicin in drug-resistant tumor in animal studies (Lamson and Brignall, 2000).
Genistein from soy food is an isolflavones can potentiate the action of tamoxifen, this isolflavones can attenuate the tumoricidal activity of tamoxifen in oestrogen dependant breast cancer by inhibiting the tumor cell growth in oestrogen receptor-negative human breast cancer cells (Constantinou et al., 2005).

Several naturally occurring plant agents like flavonoids can enhance the drug bioavailability by inhibiting ATP transporters-mediated drug efflux in vitro, suggesting that such interactions could occur also in vivo. Lycopene is the most abundant carotenoid in tomato *lycopersicon esculentum*. The effect of lycopene is observed in prostate cancer, hepatocyte and breast cancer cell lines results in antiprolifretive activity by blocking the cell cycle at G0 / G1 phase (Van Breemen and Pajkovic, 2008).

Resveratrol, a phytoalexin present in the root of the weed *Polygonum cuspidetum*, well known in Asian medicine. It cause block of cell cycle progression by acting on modulating gene expression to down-regulate cyclin D1,D2 and E expression and upregulating the expression of the CDK inhibitors. It also activates pro-apoptotic factors and deactivation of anti-apoptotic compounds and inhibition of metastatic progression (Ahmad et al., 2001). Capsaicin from red chilli (*Capsicum annum*) modulates interleukin-8 production and constitutive regulation of cell proliferation and induced NFKB activation in malignant melanoma cells (Patel et al., 2002).

Curcumin from *Curcuma longa* L. can enhance the tumorocidal efficacy of cytotoxic chemotherapy (Sagar et al., 2006). Curcumin induced cell growth inhibition and apoptosis of pancreatic cells by down-regulation of notch signaling pathway (Wang et al., 2006). In in vivo studies a combination of curcumin and gemcitabine inhibited pancreatic cancer growth in nude mice better than gemcitabine alone, suggesting chemosensitizing effect of the molecule (Kunnumakkara et al., 2007).
Figure 12: *Turmeric plant with rhizome exposed.*

**Scientific classification**

- **Kingdom**: Plantae
- **Class**: Liliopsida
- **Subclass**: Commelinids
- **Order**: Zingiberales
- **Family**: Zingiberaceae
- **Genus**: *Curcuma*
- **Species**: *Curcuma longa*
Turmeric (Curcuma longa L.) is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae (Govindarajan, 1980). It can be grown in diverse tropical conditions; It is native to tropical South Asia and needs temperature between 20°C and 30°C, and a considerable amount of annual rainfall under rain fed or irrigation conditions (Peret et al., 2005). The plants reach a height of upto 1m. Leaves, alternate, obliquely erect or sub-sessile are oblong-lanceolate and dark green, surmounting leaf sheaths tapering near the leaf and broadening near the base, enveloping the succeeding shoot. Flowers which are seen occasionally on cylindrical spikes bearing numerous greenish white bracts, are narrow, yellowish white. Inflorescence is terminal on leafy spurious stems appearing between the sheaths. Most cultivated varieties are sterile triploids, but stray inconspicuous fruits are known (Govindarajan, 1980).

The underground rhizome, which is processed into the spice, consists of two distinct parts. The mother or egg-shaped primary rhizome, several long cylindrical multi-branched secondary rhizomes growing downward from the primary rhizome. The two forms are differentiated based on their shape in the western trade. Though both are from same plant the bulbs as C. rotunda and the finger-like cylindrical forms as C. longa both in bulb and finger forms, is bright yellow or dull yellow in appearance with a rough or polished surface. The measurement of bulb form is usually 4-5cm long and 3cm in diameter.

The turmeric fingers are tapering cylinders 7 to 15mm thick and 2.5 to 7.5 cm in length. When cut or fractured the rhizome, the break is clean not splintering or fibrous. Both have a number of transverse annulations, root scars, and cut surfaces, where the two growth forms and secondary branches are separated. In the transverse cut surface, the endoderm is clearly seen as a light yellow circle separating the darker yellow cortex and central cylinder, inspite of the spread of an orange-yellow color over the entire surface (Govindarajan, 1980).
**Anatomical characteristics.**

The rhizome has multi-layered, thin-walled cells in radial rows forming the cork tissue, with tangential oblong cells of the epidermis on the outside and thin-walled parenchymal cells of the cortex on the inside. The central cylinder of parenchymal cells is separated from the cortex by a thin layer of oblong cells of the endoderm. It contains an orange-yellow component are scattered throughout the entire parenchymal tissue and also contains oleoresin cells containing oil, resin, starch as dominant constituent. There are vascular bundles as in ginger but bast fibers are absent. The starch granules are 15-30µm in size being flat and disc shaped. The starch granules swell on boiling (at 73 to 82°C) extending tangentially and separating from each other, wrapping around nucleus (Sankaracharya and Natarajan, 1973).

**Cultivation of turmeric**

The turmeric cultivation requires preparation of land, tillage, irrigation and pest control. Land with preferably loamy soil is prepared by plowing 6-8 times to secure fine tilth. Flat beds are prepared in red soils or raised beds in black dry loams. For good irrigation and drainage ridges and furrows are prepared. Sowing is done during mid-June to mid-July. Both the mother rhizomes and fingers are used for sowing. The mother rhizomes are planted as such or split into two and fingers are cut into pieces each 4-5cm long. The rhizomes are pre-treated by dipping in fungistatic solutions. Before sowing farm yard were manured with nitrogen, phosphates and potash, a similar dose of nitrogen and potash were used at 2\textsuperscript{nd} and 4\textsuperscript{th} month after weeding (Anjaneyulu and Krishnamurthy, 1968).

**Harvesting and curing**

After 7-9 months turmeric crop is ready for harvesting. For harvesting, dry field was irrigated and rhizome bunches are carefully lifted and cleaned to remove by soaking in water. The mother rhizome and fingers are separated; some mother rhizomes are kept for seed and remaining are processed by curing. The green rhizomes are boiled in water, after cooking the boiled rhizomes are spread on clean floor allowed to sun dry for
10-15 days. Dried rhizomes are polished and wrapped in gunny bags for export (Desikachar et al., 1959).

Classification of turmeric germplasm collection based on:

1. Morphological characteristics of the rhizomes—such as size of mother rhizomes, number of nodes and intermodal distance.
2. Vegetative characters such as number of tillers, height of plant, number and size of leaves.
3. Yield and components as recovery of dry turmeric, curcumin content, volatile oil results in four distinct groups. Among them group III showed high yield of curcumin and volatile oil contents. With these studies superior variety was selected for successive generations.

Turmeric varieties

India is one of the leading producer and exporters of turmeric in the world. The varieties of *C. longa* under cultivation have probably evolved by unconscious selection. There are over thirty named varieties are grown in India. They vary in grass morphological characters, yield, composition and susceptibility to microbial and insect attack and may be due to agro-climatic and cultural practices, some variations may be inherent. These varieties are known by local names or the name of the region/area where they are originated (Govindarajan, 1980).

Some popular varieties are Duggirala, Tekurpeta, Sugandham, Amalapuram, Erode, Aleppey, Moovattupuzha and Lakadong. The improved varieties of turmeric are Suvarna, Suguna, Sudarsana, Prabha, Prathiba, Krishna, Sugandham, Roma, Suroma, Ranga, Rasmi, Rajendra, Sonia, Allepey, Supreme, Kedaram. Andra Pradesh, Tamil Nadu, Orissa, Karnataka, West Bengal, Gujarat, Meghalaya, Maharashtra, Assam are some of the important states cultivate turmeric.
**Turmeric International trading**

India is the global leader of turmeric exports. Internationally other major exporters are Thailand, other Southeast Asian countries, Taiwan, United Arab Emirates (UAE), United States of America (USA), Bangladesh, Japan, Srilanka, United kingdom, Malaysia, South Africa, Netherland and Saudi Arabia are the major importer of turmeric from India (Govindarajan, 1980).

**Turmeric chemical composition**

In general turmeric contains total of curcuminoids which is about 4-6%, and contains 2-4% essential oil and 2-3% of fixed oil and various volatile oils including turmerone, atlantone and zingiberone. It includes other constituents like sugars, proteins and resins. The value of the turmeric products is based on their curcuminoids content and estimated based on its absorbance at 420 nm.

Krishnamurthy *et al*, describes the processing and color content of turmeric varieties found that the maximum color content varies with varieties, harvest maturity, and falls to nearly half its value if the harvest is delayed. Generally bulbs have more color than fingers but in curing process the color is distributed resulting in a more or less uniformly colored product (Krishnamurthy *et al.*, 1975).

The curcuminoids composition of different species of *Curcuma* has also been shown to differ. The value of turmeric oleoresin entirely depends on the color value determined by the specific absorption of the curcuminoids in an extract. The selection of raw material is based on cleanliness of rhizomes and high content of color from the curcuminoids. The value of turmeric is determined by the specific absorption of the curcuminoids in an extract. In some varieties rhizomes are deep yellow to orange-yellow color that implies high curcuminoid content (Sankaracharya and Natarajan, 1973).

**Solvents for extraction of curcuminoids**

Solvents of defined purity are allowed by National and International food laws. The choice of solvents for extraction is restricted to the few solvents in the processing of food materials (WHO, 1971). Choice of solvent is dictated by the efficiency of extraction.
of characteristic components, optimization in respect of yield of oleoresin with desirable handling properties. It is important that economy of desolventizing to the permitted residual solvent level and recovery of solvent from the final product (Srinivasan, 1952). Hexane, heptane, acetone, alcohol and ethylene dichloride have generally been used in the extraction of oleoresins of spice. From consideration of solubility of the active constituents, the curcuminoids are poorly soluble in hydrocarbon solvents.

Alcohol and acetone are good extractants to be high because of extraction of non-flavor components. Alcohol introduces excise and administrative problems and so is not preferred. Ethylene dichloride are being relatively selective extraction having sufficient low boiling point, but impurities in the solvent such as traces of high boiling fractions accumulating in the product create damages to the equipment in presence of water. Acetone appears to be choice of solvent for good quality of turmeric extraction. Acetone as solvent was slightly superior to alcohol and ethylene dichloride.

Soxhlet extraction of turmeric powder with acetone gave a yield of about 5.0% containing 42% curcuminoids in 4 to 5 hours, prolong extraction upto 24 hours gave fractionally higher yield of curcuminoids. Acetone extract contains curcuminoids content on the high side. Laboratory extraction study of turmeric gave data on yield and extraction efficiency of curcuminoids in relation to cultivars, particle size and hot vs. cold extraction. The yield of oleoresin and efficiency of extraction of curcuminoids are higher with the finer grind than with the coarse grind, both by the soxhlet and cold percolation methods (Krishnamurthy et al., 1976).

Curcuminoids are polyphenols having a pronounced yellow color. A curcuminoids is a curcumin or a derivative of a curcumin with different chemical groups that have been formed to increase solubility of curcumin and make them suitable for dry formulation. They are poorly soluble in water at acidic and physiological pH and lipids. They are soluble in dimethyl sulfoxide (DMSO), acetone but they also hydrolyze rapidly in alkaline solutions (Jayaprakasha et al., 2006). They are readily decomposed when exposed to bright light, high temperature or oxidative conditions (Schieffer, 2002). Curcumin is the main coloring substance in turmeric (Curcuma longa) and two related
compounds like demethoxycurcumin and bisdemethoxycurcumin are altogether known as curcuminoids (Govindarajan, 1980).

Curcumin is Diferuloyl methane or Di-cinnamoyl methane. It is yellow crystalline, odorless powder. It is poorly soluble in water, petroleum ether, hexane and benzene and highly soluble in alcohol, acetone and ethyl ether. It has molecular weight of 368.4 and melting point of 184-186°C. In a finely powdered form curcumin can be dispersed in oil. With excess potassium hydroxide, curcumin forms a characteristic salt, as globular radiating flame colored crystals (Srinivasan, 1953). The relative Rf value was 1 using chloroform:ethanol (25:1) as eluent.

Srinivasan investigated the presence of other related components was investigated by column chromatography on silica gel with benzene as eluting solvent. The total colored components. The dominant component eluting first was to be curcumin and second to elute was demethoxycurcumin and the third was bisdemethoxycurcumin (Srinivasan, 1953). Curcumin formed two distinct diacetyl compounds, natural curcumin was reported to be accompanied by structural or stereo isomers (Roughly, and Whiting, 1973).

![Chemical structures of three curcuminoids](image)

**Figure 13:** The chemical structures of three curcuminoids.

*Courtesy: (Deepak et al., 2003)*
Demethoxycurcumin was an amorphous orange yellow product with a chemical name as 4-hydroxy cinnamoyl (feruloyl) methane. It is highly soluble in acetone and alcohol and poorly soluble in petroleum ether and hexane. It has molecular weight of 338 and melting point at 172.5 – 174.5°C. Using chloroform:ethanol (25:1) as eluent the relative Rf value was 0.66.

Bisdemethoxycurcumin was yellow plates eluted as third compound in column chromatography. The chemical name as bis-4-hydroxy cinnamoyl methane. It is poorly soluble in ether, water and hexane and highly soluble in alcohol and acetone. It gives a pink color with brilliant orange fluorescence with boric-oxalic acid reagent. It has a molecular weight of 308.1 and melting point at 224°C. The relative Rf value was 0.41 using chloroform:ethanol (25:1) as eluent (Khalique and Amin, 1967).

Analytical studies of curcuminoids

The absorption spectra of three components vary slightly with their maxima at 429nm for curcumin, 420nm for demethoxycurcumin and 419 nm for bisdemethoxycurcumin. The ultraviolet and infrared spectra are in conformity with the structure assigned by degradative reactions. Although they are not apparent at room temperature, the enolic protons can be observed in the NMR in deuterio acetone solutions at -90°C, the NMR spectra indicate that in chloroform solutions, these diketones exist entirely in the enolic form (Roughly, and Whiting, 1973).

Estimation of the total curcuminoids is done on the alcoholic extract of the powdered rhizome by measurement of optical density at their absorption maxima, 420 to 425nm. Analysis showed that curcumin has two methoxyl groups the second fraction DMC had one methoxyl group and the third fraction BDMC had none. Based on the structure assigned to curcumin earlier by Kostanecki and Lampe, the possible structure was in the enolic form (Govindarajan, 1980).

Curcumin is a polyphenol derived from the rhizome of *Curcuma longa* L. (turmeric) possess various pharmacological uses. The yellow-pigmented fraction of turmeric contains curcuminoids such as curcumin, demethoxycurcumin,
Bisdemethoxycurcumin and cyclocurcumin (Goel et al., 2008). Traditionally, turmeric and natural curcuminoids have been used in folk medicine for its therapeutic effects.

**Medicinal uses of curcuminoids and its derivatives**

Curcuminoids posses several pharmaceutical activities like antioxidant, anticancer, anti-inflammatory, anti-microbial, hepatoprotective, thrombosuppressive, hypoglycemic, antiarthritic (Aggarwal et al., 2009, Maheshwari et al., 2006).

Curcuminoids exhibit a protective effect by accelerating antioxidant defense mechanisms and attenuating mitochondrial dysfunction in the brain of diabetic rats (Rastogi et al., 2008). Curcuminoids induces apoptosis and suppress the growth of human brain GBM 8401 cells through mitochondria and caspase-3-dependent pathway (Huang et al., 2010). Curcuminoids inhibited proliferation and increased apoptosis in the colon cancer cells appeared to be associated with the cellular uptake of curcuminoids (Hsu et al., 2007).

Curcumin can inhibit Lovo cells growth and the cellular mechanism responsible for the action is to arrest the cell cycle in S, G2/M phase and to induce apoptotic cell death (Chen et al., 1999). Curcumin inhibit the growth of human ovarian cancer cells by inducing apoptosis through up-regulating caspase-3 and down-regulating gene expression of NF-κB (Zheng et al., 2006).

Mazzanti et al., demonstrated curcumin molecule suppresses proliferation and induces apoptosis through down-regulation of constitutively active NF-κB and IκB kinase in pancreatic cell lines (Mazzanti et al., 2009). It also inhibits cell growth by down-regulation of COX-2, EGFR, ERK1/2 in pancreatic cell line (Lev-Ari et al., 2006). Curcumin shows anti-proliferative mechanism against multiple myeloma cell lines. It blocks the constitutively active IKK and inhibited NF-κB activation, leading to the suppression of proliferation and induction of apoptosis (Bharti et al., 2003).
Sung et al., studied both in vitro and in xenograft model in nude mice that curcumin overcome chemo-resistance and enhance the activity of thalidomide and bortezomib used to treat patients with multiple myeloma (Sung et al., 2009).

Several studies on colorectal cell lines showed curcumin inhibits NF-κB activation, neurotensin-mediated activator protein-1, PGE-2 (prostaglandin E2) and epidermal growth factor receptor (EGF-R), Ca2+ mobilization and down-regulates CoX-1/2, IL-8 MMP2/9 gene induction and colon cancer cell migration (Chauhan, 2002; Anand et al., 2008). Curcumin act as a drug transporter-mediated MDR reversal agent and effective anticancer agent in hormone-independent MDR breast cancer (Labbozzetta et al., 2009).

Demethoxycurcumin(DMC) and bisdemethoxycurcumin (BDMC) revealed matrix metalloproteinase-3 (MMP-3) expression in invasive breast carcinoma cells (Boonrao et al., 2010). Demethoxycurcumin(DMC) induced Bc-2 mediated G2 / M arrest and apoptosis most effectively than curcumin(C) and bisdemethoxycurcumin(BDMC) in human glioma U87 cells (Luthra et al., 2009). Demethoxycurcumin has anti-proliferative activity on MCF-7 human breast tumor cells. It exerts a cytostatic effect at G2/M phase arrest in cell cycle (Simon et al., 1998).

Yodkeeree reported for the first time that DMC inhibited adhesion, migration and invasion of MDA-MB-231 human breast cancer cells by targeting NF-κB and modulation of expression of invasion-associated proteins in MDA-MB-231 cells (Yodkeeree et al., 2010). DMC and BDMC, tetrahydrocurcumin (THC) inhibit the Wnt/beta-catenin pathway by decreasing the amount of the transcriptional coactivators P300 (Ryu et al., 2008).

Yong et al., demonstrated BDMC for fumigant activities on the important phytophagous mite Tetranychus cinnabarinus at its different life stages. BDMC has stronger repellency activity against the mite (Yong et al., 2011). BDMC has been identified as a novel anti-inflammatory signaling pathway that leads to the expression of
heme oxygenase-1 (HO-1) in macrophages. Thus showing anti-inflammatory effects (Kim et al., 2010).

Chemotherapeutic effect of curcuminoids was studied in proliferation in the HCT116 human colon cancer cells. BDMC induces G1 / S arrest in cell cycle. BDMC is a direct effect of the drug and concomitant to the mitotic block. It also induces rapid DNA double strand breaks (Basile et al., 2009).

**Multidrug resistance**

The development of multidrug resistance (MDR) is a major impediment to the chemotherapeutic treatment of many forms of human cancer. Resistance of cancer cells to multiple chemotherapeutic drugs (a mechanism termed MDR) is a major obstacle to the success of cancer chemotherapy and has been closely associated with treatment failure. Multidrug resistance is a significant challenge in the treatment of cancer. This type of resistance develops against various “naturally occurring” drugs with different structure and mechanism of action such as anthracyclines, taxanes, epipodophyllotoxins, and vinca alkaloids (Ambudkar et al., 2003).

Tumor cells that are initially sensitive to a broad range of drugs can frequently develop resistance to a group of anti-cancer drugs. Employing multiple cytotoxic drugs with different mechanisms of action was not the solution to this problem. Treatment with many drugs can significantly increase the frequency of mutation that would produce resistance in cells. Cancer appeared to develop resistance simultaneously to many different anti-cancer drugs. The MDR cancer cells may subsequently develop cross-resistance to several unexplored and structurally unrelated chemotherapeutic agents (Gottesman and Ling, 2006). Multidrug resistance (MDR) result in heritable changes in cancer altered levels of specific proteins or mutant proteins which allow cancer cells to survive in the presence of may different cytotoxic agents (Gottesman and Ling, 1995).
Various mechanisms are involved in drug resistance in cancer cells. These mechanisms include decrease uptake of drug, alteration in cellular pathway and increased active efflux of drugs, increase drug metabolism and alter drug targets.

**Figure 14:** *Mechanism of MDR towards cancer chemotherapeutic drugs. Cancer cells can develop resistance to multiple drugs by various mechanisms as depicted. Mechanisms include decreases uptake of drug, reduced intracellular drug concentration by efflux pumps, altered cell cycle check points, altered drug targets, increased metabolism of drug and induced emergency response genes to impair apoptotic pathway.*

*Courtesy: (Chai et al., 2010).*

Cancer cell develops resistance to chemotherapeutic drugs by natural (intrinsic) and acquired resistance. Intrinsic drug resistance may be caused by pharmacologic obstacles and also due to inherent properties of the tumor cell. Drug administration plays important role in intrinsic drug resistance, results in poor absorption of drugs, inadequate dosage, wrong treatment schedule, drug interaction, inadequate clinical trail. Intrinsic
drug resistance is that occurs at the level of the tumor cell. This characteristic arises during the progression to malignancy (Mc Vie, 1984).

**Mechanisms involved in intrinsic drug resistance**

- Poor uptake into tumor cell
- Poor drug catabolism
- Lack of tumor-suppressor gene function (RB p53)
- Over expression of proliferative signals (E2F, CM4C)
- Increased level of anti-apoptotic protein (Bcl-2)
- Increased extra cellular survival signals (TGF-β) (Cordon-Cardo, O’Brien, 1999).

In acquired resistance a new tumor has arisen from a small number of mutant tumor cells pre-existing in the population that has escaped initial therapy and have a growth advantage to over the rest of the tumor. This resistance is induced by drugs, due to newly acquired properties of the tumor cell, tumors not only became resistant to the drugs originally used for treatment also became cross-resistant to other drugs with different mechanism of action (Goldie and Coldman, 1979).

**Mechanisms involved acquired drug resistance**

- Decreased influx of drug
- Increased efflux
- Increased inactivation of drug or activated drug
- Decreased activation of drug
- Increased target enzyme level
- Altered target (also decreased binding to target)
- Increased DNA repair (Goldie and Coldman, 1979).

The most common reason for acquisition of resistance to a broad range of anticancer drugs is expression of one or more energy-dependant transporters that detect and eject anticancer drugs from cells. One of the major reasons for acquired resistance is over expression of efflux proteins belongs to the ATP binding cassette (ABC) family of
transporters (ABC transporter). Other mechanism of resistance including insensitivity to drug induced apoptosis and induction of drug-detoxifying mechanisms plays important role in acquired anticancer drug resistance (Gottesman, 2002).

**ABC transporter**

ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carryout certain biological processes including translocation of various substrates across membranes and non-transport related processes such as translation of RNA and DNA repair (Jones and George, 2004).

Classical multidrug resistance is attributed to the elevated expression of ATP-dependant drug efflux pumps belong to the superfamily of ATP-binding cassette (ABC) transporters. Efflux mediated by ABC drug transporters leads to decreased cellular accumulation of anti-cancer drugs. There are 48 different ABC transporters have been identified in human genome coded by 48 genes are classified on the basis of identical sequence in their ATP-binding domains (ABDs) (Deeley et al., 2006).

ABC transporters divided into seven different classes (A-G) based on sequence similarities. Members of four of these classes (A,B,C, and G) have been shown drug resistance on cultured cells. ABC transporters have a consensus ATP-binding region of approximately 90-110 amino acids, which includes walker A and walker B motifs, C region lies between these walker motifs as a linker or dodecapeptide region (Higgins, 1992).

These transporter proteins harness the energy of ATP binding or hydrolysis to drive conformational changes in the transmembrane domain (TMD) and consequently transports molecules (Hollenstein et al., 2007). ABC transporters are large membrane-bound proteins consisting of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) which mediate the active transport of substrate drugs out of the cell by hydrolysis of ATP via ATPase enzyme where both the functional ATP site are essential for ATP hydrolysis (Szakacs et al., 2006). Two domains undergo
dimerization in the presence of ATP because of extensive inter-subunit contacts to the nucleotides located in the interface of the two subunits. Similar nucleotide-dependant dimerization of ABC, is a common feature shared by all members of this super family (Hoffmeyer et al., 2000).

In this model the substrate binding site alternates between outward and inward-facing conformations. Two principal conformations of the NBDs formation of a closed dimmer upon binding two ATP molecules and dissociation to an open dimmer facilitated by ATP hydrolysis and release of inorganic phosphate (pi) and adenosine diphosphate (ADP). Switching between the open and closed dimer conformations induces conformational changes in the TMD resulting in substrate translocation. The conformational changes in the nucleotide-binding domain (NBD) as a result of ATP binding and hydrolysis is the ATP-switch model (Higgins and Linton, 2004).

![Diagram](image)

**Figure 15:** Proposed drug efflux mechanism for ABC transporters. Substrate and ATP bind to ATP transporters. After ATP hydrolysis, the substrate is effluxed out of the cell. Phosphate group is released and the substrate is then excreted to extracellular matrix.

*Courtesy: (Chai et al., 2010).*

Mainly three members of the ABC transporter family, ABCB1 (MDR1, p-glycoprotein), ABCC1 (MRP1) and ABCG2 (BCRP, MXR, ABCP) appears to play important roles in the development of MDR in cancer cells. Over expression of these three major ABC transporter were frequently observed in cancer cell lines selected with chemotherapeutic drugs (Szakacs et al., 2006).
ABCB1 is the major factor in MDR to chemotherapeutic regimen and has a wide substrate profile. All of the substrates are hydrophobic compounds with molecular weights ranging from 0.3-2 kDa (Sauna and Ambudkar., 2001). MDR 1 gene in human was of the first members of a large family of ATP-dependant transporters among ABC family (Higgins, 1992).

**MDR-1 gene**

The expression of MDR gene plays important role in multidrug resistant phenotype and also confers to cross resistance to a large group of lipophilic cytotoxic compounds. Multidrug resistance in human and rodent cell lines results in decreased intracellular drug accumulation and correlates with the increased expression of MDR genes due to the presence of efflux pump (Gottesman and Pastan, 1988). Different patterns of amplification of MDR-related genomic sequences observed among multidrug resistant cell lines have suggested that the mammalian MDR genes constitute a small multi-gene family, indicating two MDR genes in human genome and three genes in mouse and hamster genomes (Van der Bliek et al., 1986).

The human mdr genes were found on human chromosome 7q21-31 (Fojo et al., 1986). The mdr1 gene consists of over 100 kb and is encoded by 28 exons and 27 introns, although only 26 introns interrupt the coding sequence of the gene (Chen et al., 1990). The Methionine start codon is coded for at the beginning of exon 2, while exon 1 contains both an upstream and downstream promoter. Two alternate polyadenylation sites exist., including a poly (dA) tail and an ATTAAA polyadenylation sequence 15 nucleotides upstream (Gros et al., 1986).
Increased p-glycoprotein expression in many multidrug resistant cell lines results from amplification of the MDR1 gene. (Scotto et al., 1986). The human multidrug-resistance (MDR1) gene encodes a membrane transporter p-glycoprotein (p-gp) a member of ATP binding cassette family (Borst et al., 2000).

**p-glycoprotein (p-gp)**

p-glycoprotein is permeability glycoprotein and also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) encoded by MDR1 gene (or ABCB1 gene). p-gp is a well characterized ABC transporter of MDR / TAP subfamily which transports a wide variety of substrates across extra and intra cellular membranes (Ueda et al., 1987).
p-gp is a 170 kDa plasma membrane glycoprotein or phosphoglycoprotein. P-gp are single chain protein composed of two homologous halves each containing six transmembrane (TM) domains and an ATP-binding utilization domain separated by a flexible linker region (Hrycyna et al., 1998). p-glycoprotein is encoded by 1280 amino acids. A total of 12 segments consist mostly of water-insoluble amino acids to cross the plasma membrane of the cell (Gottesman and Pastan, 1988). Each homologous halves contains a hydrophobic membrane associated domain with 250 amino acid residues followed by hydrophilic nucleotide binding fold with 300 amino acid residues (Gros et al., 1986). The N- and C- terminus and two nucleotide binding folds are located intracellularly and the first extracellular loop is glycosylated (Kartner et al., 1985).

The two ATP sites are capable of hydrolyzing ATP, the stoichiometry of ATP hydrolysis is 1 mol ATP/mol of p-gp and that ATP hydrolysis and drug transport are obligatorily linked (Ambudkar et al., 1997). In the first extracellular loop the p-gp is glycosylated at three sites (N91,N94 and N99). The glycosylation required for the proper trafficking of the transporter to the surface. p-gp can detect and bind a large variety of hydrophobic natural-product drugs to enter the plasma membrane (Schinkel et al., 1993).

Figure 17: structure of P-glycoprotein efflux pump.

Courtesy: (Schinkel et al., 1995).
Function of p-gp

ATP binding and hydrolysis is essential for the proper functioning of p-gp for drug transport. Binding of drugs results in activation of one of the ATP-binding domains and hydrolysis of ATP cause major change in the shape of p-gp which allows the release of drug into the extracellular space (Ramachandra et al., 1998). The second molecule of ATP is hydrolyzed to restore the p-gp to its original state to repeat the cycle of binding and release of drugs (Sauna and Ambudkar, 2000). p-gp drug substrates appears to be that all are hydrophobic with a molecular mass of 300-2000 Da. Anionic compounds are not transported by p-gp (Ford and Hait, 1990).

Selected Substrates of P-glycoprotein

Anticancer drugs: Vinca alkaloids (vincristine, vinblastine), Anthracyclines (doxorubicin, daunorubicin, epirubicine), Epipodophyllotoxins (Etoposide, Teniposide), Paclitaxel (taxol), Actinomycin D, Topotecan, Mithramycin, Mitomycin C.

Other cytotoxic agents: Colchicine, Emetine, Ethidium bromide, Puromycin.

Cyclic and linear peptides: Gramicidin D, Valinomycin, N-Acetyl-leucyl-leucyl-norleucine, Yeast a-factor pheromone.

HIV protease inhibitors: Ritonavir, Indinavir, Saquinavir,

Other compounds: Hoechst 33342, Rhodamine 123, Calcein-AM.

Drug Transport Mechanism

Reduced drug accumulation appears to be a very common mechanism of MDR in vitro and in vivo studies in cancer.

In the classical transporter mechanism substrates are encountered in the cytoplasm and pumped directly out of the cell, called “hydrophobic vacuum cleaner” model. P-gp substrate entering the cell from the extracellular medium are intercepted at the plasma membrane and extruded to the exterior without entering the cytosol (Gottesman and Pastan, 1993).
In the Flippase model Pgp operate as a drug flippase, moving hydrophobic drug molecules from the inner to the outer leaflet of the plasma membrane (Higgins and Gottesman, 1992; Higgins, 1994).

Drugs appear to gain access to the binding site after moving to the cytoplasmic leaflet by spontaneous flip-flop from the outer leaflet, it can be slow process for many compounds.

(a) The ‘pump’ model for drug transport. The three-dimensional structure of P-gp consists of a single drug-pore (shown in red). Chemotherapeutic drugs (green) diffuse through the lipid membrane and are transported out of the cell by P-gp in an ATP-dependant manner.

(b) The ‘flippase’ model for drug transport. A drug interacts with lipids of the membrane before it interacts with P-gp. The drug can then interact with P-gp and is transported from the inner leaflet directly into the extracellular medium. Alternatively, drug intercalated into the inner leaflet of the lipid bilayer is ‘flipped’ into the outer leaflet and released into the extracellular space. Movement of drug from the inner to the outer leaflet is a relatively quick process, whereas drug movement from the inner leaflet to the cytosol is relatively slow (Johnstone et al., 2000).
Figure 18: Two mechanisms of action for drug efflux by P-glycoprotein (P-gp).

Courtesy: (Johnstone et al., 2000).

P-glycoprotein reduces intracellular drug accumulation by change in intracellular pH. P-glycoprotein regulates the plasma membrane pH gradient ($\Delta p$H) and electrical potential ($\Delta \Psi$). Many of the known p-gp substrates are weak bases having positive charge at neutral pH. An increased intracellular pH or reduced intracellular accumulation of these compounds by passive diffusion across the plasma membrane (Roepe et al., 1993).
**MDR Modulators**

Modulators are that reverses drug resistance. Several generations of p-gp modulators are developed in reversing p-gp mediated multidrug resistance (MDR). The basic strategy to circumvent MDR is to co-administration of an anticancer drug with a chemosensitizer that impairs p-gp function resulting in enhanced intracellular anticancer drug accumulation (Leonard *et al.*, 2002). A large number of non cytotoxic compounds known as chemosensitizers or MDR modulators sensitize resistant cells for the action of cytotoxic drugs.

Chemosensitizers include calcium channel blockers, camodulin antagonists, steroids, cyclic peptides and drug analogs. Most reversing agent blocks the drug transport by acting as competitive or non competitive inhibitors by binding either to drug interaction sites or to other modulator binding sites leading to allosteric changes (Dey *et al.*, 1997).

**Selected modulators of p-glycoproteins**

- **Calcium channel blockers**: Verapamil, Dihydropyridines
- **Antiarrhythmics**: Quinine
- **Antihypertensive**: Reserpine
- **Antibiotics**: Cephalosporins
- **Immunosuppressant**: Cyclosporine A
- **Steroid hormones**: Progesterone
- **HIV protease inhibitors**: Saquinavir, Disulfiran (Kioka *et al.*, 1989).

**MDR cell lines**

Since cancer appears to develop resistance simultaneously to many different anti-cancer drugs, There are several studies focused on mechanisms that account for multidrug resistance (MDR) has been studied using various cultured cell models (Gottesman and Ling, 2006).
Cultured mouse and hamster cells which are intrinsically more than two fold more resistant to chemotherapeutic agents served as model system for the genetic and biochemical analysis of the multiple drug-resistance phenotype. Ling and his coworkers isolated multiply drug-resistance. Chinese hamster ovary (CHO) cells and found that increased expression of a 170,000 Dalton surface p-glycoprotein is correlated with multiple drug resistance (Ling and Baker, 1978).

The basis of drug resistance have been studied in laboratory using drug resistance cell lines that have been isolated by exposing to increasing amounts of any one of the chemotherapeutic agents such as adriamycin, daunomycin, doxorubicin, actinomycin-D, taxol, vinca alkaloids etc, to various cancer cells in order to develop resistance to that particular drug. These isolated resistant cells are frequently resistant to particular drug but also shows cross resistant to other drugs.

**Model cell line for drug resistance**

Protocols for isolating resistant cell line variants with freshly cloned cell populations using single step selection were developed in order to maximize the probability of obtaining variants resulting from single mutations (Ling and Thompson, 1974).

Akiyama *et al.*, screened human cultured cancer cells for two properties: high efficiency of cloning and sensitivity to common MDR drugs such as colchicine, vinblastine and doxorubicin. Among a half dozen or so cell lines we tested, one stood out- a line called KB which was reported to be a human nasopharyngeal carcinoma (Akiyama *et al.*, 1985). After working with this line they discovered that it was actually a subclone of HeLa, a cervical adenocarcinoma cell line that had contaminated and overgrown quite a few putative human cancer cell lines and had contaminated and distributed as KB (Nelson-Rees and Flandermeyer., 1976). Human cervical cancer cell line (HeLa) contains HPV18 DNA, comparison of HeLa cell DNA as well as of DNA from the HeLa-derived cell line KB reveals the presence of HPV 18 specific sequences without major structural reorganization. HeLa (KB), growth properties and drug
sensitivity has proved to be a very useful human cancer cell line for studying development of drug resistance (Boshart et al., 1984).

Gene amplification is a common mechanism of drug resistance in cultured cells, and that amplifying a gene would facilitate its eventual cloning. The KB cell line series (KB-8, KB-8-5, KB-8-5-11 and subsequent, more resistant populations such as KB-C1) have subsequently been shown to overexpress only MDR1 among all known ABC transporters, supporting original strategy for selection KB-8-5 was subsequently found to have a level of MDR1 expression similar to many human cancers (Ling and Thompson, 1974; Akiyama et al., 1985).

**Colchicine resistant cell line**

Isolating variant cell lines resistant to colchicine was to obtain mutants with altered colchicines binding protein (tubulin) as an initial approach towards creating a class of cell division mutants. Higher levels of colchicine resistance were selected from clonal populations of lower resistance. Multiply drug-resistant sublines have been isolated from human KB cells by several sequential single-step selections in medium containing colchicine. It has not been possible to acquire high levels of resistance of KB cells to colchicine in a single step protocol. This finding suggests that more than one genetic alteration is required to achieve high levels of colchicine resistance. Resistance cell lines were isolated by stepwise selection of KBChR lines. KBChR8-5 cell line has been isolated in second step with 10ng/ml colchicine. To enhance the mutation rate in cell lines, the cells were treated with ethyl methane sulfonate (EMS) for 24 hours ant the cells were then incubated in EMS-free medium for 5 days to prevent expression of the resistant phenotype. Then colchicine was added to the medium at varying concentration for stepwise selection.
Stepwise selection of KB cell line for isolation of KBchR 8-5 cell line.

**KB-3-1**

Step 1………………….↓ EMS, 5ng/ml colchicine (14 days)

**KBChR-8-5**

Step 2………………….↓ EMS, 10ng/ml colchicine (14 days)

Finally cells were isolated and grown in medium without colchicine prior to testing of drug resistance (Ling and Thompson., 1974; Akiyama et al., 1985).

The relative resistances of colchicine-resistant sublines to colchicine were 3-8 times in second step for isolating KBChR8-5 cell line. Further KBChR8-5 cell line is four times more resistant to colchicine than the parental cell line and showed cross-resistant to Adriamycin, vincristine, vinblastine, actinomycin and puromycin and showed multiple drug-resistance phenotype (Akiyama et al., 1985).

Collateral sensitivity of MDR cells

In in vitro studies, a phenomenon of drug-resistant cells is that the development of resistance to one agent can center greater sensitivity to an alternate agent than seen in the original parental cell line szybalski et al., described this phenomenon as collateral sensitivity (Szybalski and Bryson, 1952).

Such an approach is to exploit resistance by identifying drugs that targets MDR cells over the non-resistant parental cells from which they were selected (Szybalski and Bryson, 1952; Hall et al., 2009). It has been demonstrated using compounds like verapamil which selectively kills p-gp expressing cells and reduce p-gp expression at sub-toxic concentrations. This strategy holds promise for overcoming multidrug resistance (MDR) (Muller et al., 1994). Collateral sensitivity is abbreviated as (RR) for resistance ratio. Drug resistance phenotypes are usually assessed in terms of relative resistance (RR) values for a panel of cytotoxic anti-tumor drugs (RR patterns). The extent
of amplification or overexpression of the MDR1 gene correlates positively with the level of RR (Bradley et al., 1989).

Collateral sensitivity is assessed in vitro by determining the cytotoxicity (IC$_{50}$) of a compound against a parental line relative to its MDR subline. It is calculated as the ratio of compounds IC$_{50}$ for parental cell divided by its IC$_{50}$ for MDR cells. A RR value $>1$ indicates that the compound kills MDR cells more effectively than parental cells (Bradley et al., 1989; Hall et al., 2009).

Expression patterns of the MDR genes

Steady-state levels of MDR1, mRNA correlate with the levels of drug resistance in multidrug resistant cell lines (Shen et al., 1986). The primer sequences were chosen for their divergence from the homologous sequences of the MDR1 gene. A pair of MDR1-specific primers derived from different exons in the homologous region of the MDR1 gene was selected for amplification of a comparably sized fragment of MDR1 cDNA and β2 – microglobulin cDNA was used as an internal standard to normalize the amounts of cDNA used in different PCR reactions. The gene expression was studied based on enzymatic amplification of RNA sequences by PCR (Saiki et al., 1986).

In comparison of parental drug sensitive and multidrug-resistant cell lines the parental cell lines did not showed detectable expression of MDR1 but resistant cell lines showed over expression of MDR1-specific PCR product. Among colchicine-selected KB cell lines increasing levels of multidrug resistance and MDR1 mRNA expression was observed (Roninson et al., 1986). Et-743 is a marine alkaloid isolated from Ecteinascidia turbinata the expression level of MDR1 decreased after treatment with Et-743 for 72 hours in KB-C-2 cells. This result enhances the accumulation of VCR/DOX by down-regulating MDR1 expression (Kanzaki et al., 2002). Salvicine is a novel diterpenoid quinine compound isolated from a Chinese medicinal plant. Salvicine on MDR-1 mRNA expression is MDR K562 / A02 cell using RT-PCR were observed, when treatment with salvicine 1µm for 24 hours lead to a dose-dependant down regulation of mdr-1 mRNA expression in MDR cells (Miao et al., 2003).
Effect of drug combination

Co-incubation of phytochemicals with anticancer drug was analysed by MTT assay it reveals that phytochemicals can increase the cytotoxicity of anticancer drugs in MDR cells. Chearwae et al., demonstrated curcuminoids on cytotoxicity of vinblastine in KB-V1 and KB-3-1 cells revealed that curcumin had increased the sensitivity to vinblastine by decreased IC\textsubscript{50} in KB-V1 than other two curcuminoids (Chearwae et al., 2004).

Cell cycle analysis Flow cytometry (FCM)

The main markers used in Flow cytometry (FCM) are involved in the assessment of cell cycle parameter, single labeling by propidium iodide (PI) gives cell percentages in the different cell cycle phases. The markers of cell cycle progression such as Proliferating cell nuclear antigen (PCNA) and their cell expression can be analyzed using FCM (Lacombe and Belloc, 1996).

Salvicine induced apoptosis in DOX-selected MDR subline K562/A02 cell line which is resistant to a variety of conventional anticancer drugs. Treatment with salvicine for 24 hours induced apoptosis to the same degree in both parental and resistance cell lines in a dose-dependant manner by Flow cytometry (Miao et al., 2003).

In summary, the resistance of tumor to multiple chemotherapeutic drugs (MDR) is a serious barrier to the treatment of human cancer. Phytochemicals can be used as effective chemosensitizer to be used in combination with conventional chemotherapy which helps cellular accumulation of drugs controlled at the molecular level in presence of chemosensitizers and reverse resistant property of MDR by reducing the expression of efflux protein that exploit the drug efflux and allow to retain the anticancer drug in tumor and enhance susceptibility of tumor cells to apoptosis by anticancer drugs. Combination of drugs maximizes the cytotoxicity of MDR cells seems to be one of the promising approach to improve resistance cancer chemotherapy.