2. Review of Literature

2.1. Cervical Cancer

Cancer of the cervix is the commonest genital tract malignancy in the female and it has been ranked second-most type next only to breast cancer. About half a million new cases are seen worldwide each year, most occurring in developing countries (Ertem, 2009; Awodele et al., 2011). In 2008, approximately 5,30,000 women were diagnosed with invasive cervical cancer worldwide and 2,75,000 women died from it. Cervical cancer is the top cancer type which occurred in women of most East African and South Asian countries both in terms of incidence and mortality (Ferlay et al., 2010; Arbyn et al., 2011). India accounted for a quarter of the world’s estimated cervical cancer burden of 5,29,000 cases with the occurrence of 2,75,000 deaths in 2008 (Ferlay et al., 2010). Cervix region is the most frequent primary site of cancer among Indian women with an estimated incidence and mortality rates of 27 and 15 per 100,000 women respectively in 2008 (Ferlay et al., 2010). Furthermore, India shows the highest rates of cervical cancer worldwide, especially among rural populations (Rajkumar et al., 2000; Swaminathan et al., 2009a). A risk factor is something that increases chances of developing a disease or condition. Epidemiological studies have identified a number of risk factors such as infection with certain oncogenic types of human papilloma viruses (HPV), sexual intercourse at an early age, multiple sexual partners, multiparity, long-term oral contraceptive use, tobacco smoking, low socio economic status, infection with Chlamydia trachamatis, micronutrient deficiency and a diet deficient in vegetables and fruits, that contribute to the development of cervical cancer (IARC Working Group, 1995; Walboomers et al., 1999; Ferenczy & France, 2002). However, the primary underlying cause for cervical cancer is reported to be that of human papilloma virus (HPV) (Marrazzo et al., 2001).

2.2. Human Papilloma Virus

Papillomavirus infections in humans are known to cause a variety of benign proliferations; these include warts, intraepithelial neoplasias, anogenital papillomas, oral laryngeal and pharyngeal papillomas (Zur Hausen, 1996). Molecular and epidemiological evidences have now established that HPV types associated with
anogenital neoplasms, including condylomata, cervical dysplasia & cervical carcinoma and these are always sexually transmitted (Lowy et al., 1994). The involvement of HPV in cancers of the vulva, anal canal, vagina and penis is currently being identified and also the possible infectivity of HPV in cutaneous cancer, oral cancers and other cancers of the upper aero digestive tract is being investigated (Bosch & Munoz, 2002). In humans, specific papillomavirus types have been associated with over 99% of cervical cancer biopsies (Walboomers et al., 1999). They are considered to be the “high-risk” types and include, in order of prevalence; HPV types 16, 18, 31 and 45 (Bosch, 1995). Although there are several strains of HPV infection, the two strains; HPV 16 and 18, account for more than 70% of all cervical cancer cases; five other strains; HPV 31, 33, 35, 52 and 58 account for an additional 20% of cases (Bosch & Desanjose, 2003). However, there is considerable regional and country-wise variation in this association, with HPV 16/18 prevalence in invasive cervical cancer cases ranging from 65% in South/ Central America to 76% in North America (Smith et al., 2007). In India, prevalence of HPV 16/18 in invasive cervical cancer cases is 82.5% (National Cancer Registry Programme and World Health Organization, 2002).

2.2.1 Structure and genome organization

Papillomavirus in humans has been studied extensively and more than 120 HPV types were reported to date (De Villiers et al., 2004). Human Papillomaviruses are epitheliotropic, non-enveloped, double-stranded DNA viruses that infect mucosal and cutaneous epithelia in a wide variety of higher vertebrates. The HPV virion has a diameter of ~55 nm and the genome of virus is approximately 8 kilobases (kb) size (Seedorf et al., 1985). The HPV genome is divided into three regions - long control region (LCR), early region coding for six proteins (E1, E2, E4, E5, E6 and E7) and late region encoding two late proteins (L1 and L2). The early genes are non-structural genes coding proteins for viral DNA replication, transcription and cellular transformation whereas the late genes are structural genes responsible for the formation of virus capsid (Hebner & Laimins, 2006).
2.2.2 Human Papillomavirus early proteins

2.2.2.1 E1 Protein

The E1 protein is highly conserved among HPV types, the 73-kDa E1 protein mediates the replication of virus; it binds to a specific DNA sequence (E1 binding site; E1BS) in the viral origin of replication and forms the hexameric complexes with the help of a second viral protein, E2 (Frattini & Laimins, 1994). The resultant complex has helicase activity which initiates synthesis of progeny DNA (Wilson et al., 2002). The papillomavirus E1 protein functional regions have been identified. The C terminal has helicase and ATPase activities. Amino acids change in the ATPase domain (P479 → S479) inactivates the ATP binding site and disrupts the function of E1 protein (Hughes & Romanos, 1993). The ATPase domain of E1 protein interacts with E2 protein and α subunit p70 of the DNA polymerase (Masterson et al., 1998). A segment of approximately 160 amino acids immediately upstream of the ATPase/helicase domain is referred as the DNA-binding domain (DBD; White et al., 2001). A segment of about 50 amino acids located within the terminus of E1 functions as a localization regulatory region (LRR), which contains a nuclear export sequence (NES) and a nuclear localization signal (NLS) (Deng et al., 2004).

2.2.2.2 E2 protein

The E2 protein has three distinct regions. First the carboxy terminal, a dimerization domain that initiates the homodimers formation, which recognizes and binds to the 12 bp palindromic DNA sequences (ACCGNNNNCGGGT) within the LCR. This region is called E2-Binding Sites (Desaintes & Demeret, 1996). Second, the middle region of E2 - the hinge region, is crucial for regulation of E2 proteins stability and identifying their nuclear localization in others (Zou et al., 2000). The amino terminal domain, which is important for transcription regulation and replication of viral DNA (Desaintes & Demeret, 1996) is the third region. At higher concentration of E2 protein acts as a transcriptional repressor and at low levels it activates transcription from the viral LCR. Some of the investigators have demonstrated that in HeLa cells E2 protein expression induced the apoptosis by restoring the functions of p53 as a result repression of E6 expression (Desaintes et al., 1997). Interaction of E2 with the minor capsid protein L2 leads to the inhibition of the
transactivation of E2 for both BPV and HPV proteins (Okoye et al., 2005; Heino et al., 2000).

In the early history of papilloma virus research, there was an ORF designated E3, which was identified later as anomaly. No attempt was made on E3 which was later renamed as HPV-ORF. Hence there is no protein designated as E3 in papillomaviruses.

2.2.2.3. E4 Protein

The HPV E4 gene is located in the early genomic region yet it is generally expressed as a late gene with a role in productive infection (Howley, 1995). The E4 protein localizes and aggregates into cytoplasmic and nuclear inclusion granules within the differentiating layer of the infected epithelium and in the basal layer, indicating both an early and late function (Roberts et al., 1993). The E4 facilitates and supports viral genome amplification and also regulates expression of late genes. The E4 protein plays a major role in virus maturation and virus release.

2.2.2.4. E5 Protein

The E5 protein enhances the oncogenic nature of the E6 and E7 (Stoppler et al., 1996). The BPV E5 protein acts as the strong transforming protein. Whereas, in HPV infections; E5 has only weak transforming activity (Schiller & Lowy, 1996). The E5 plays a role in early stage of HPV infection but is dispensable for maintenance of malignant transformation (Zur Hausen, 1996). In cultured cells expression of HPV 16 E5 enhanced the activity of epidermal growth factor receptor (EGFR) (Crusius et al., 1998; Pim et al., 1992) co-immuno precipitation studies have demonstrated that HPV 16 E5 can also form a complex with EGFR 24 when both proteins are over expressed (Hwang et al., 1995). Through activation of EGFR, E5 can interfere with several signal transduction pathways, including the mitogen-activated protein (MAP) kinase pathway (Crusius et al., 1997). HPV 16 E5 also inhibits the process of apoptosis induction by Fas-ligand, tumour necrosis factor related apoptosis inducing ligand (TRAIL) (Kabsch & Alonso, 2002) and by UV- light (Zhang et al., 2002).

2.2.2.5. E6 Protein

The E6 gene of papilloma virus is located at the 5’ end of the viral early region. The papilloma virus E6 gene is the one of the first genes expressed during PV
infection. The E6 protein is required for the productive life cycle of the papilloma viruses which has been implicated in supporting the stable maintenance of viral DNA in cultured keratinocytes (Park & Androphy, 2002; Wu et al., 1994).

Hudson et al. (1990) and Munger et al. (1989a) demonstrated the major transforming activity of the high-risk HPV E6 protein in cultured cells and its ability to increase immortalization of human keratinocytes in association with the E7 protein. The HPV E6 binds to the cellular ubiquitin-ligase, called E6 associated protein (E6-AP), which in turn binds to p53. This interaction leads to the p53 degradation and cell cycle disruption (Fehrmann & Laimins 2003). In addition, E6 binds to various other cellular proteins and these are broadly divided into four classes: proteins involved in cell polarity and motility, transcriptional co-activators, tumor suppressors & apoptosis inducers, and DNA replication and repair factors.

2.7.3.6. E7 Protein

The E7 gene is situated at the immediate downstream of the E6 gene. The E7 protein is made up of approximately 100 amino acid residues. The HPV16 E7 protein has been studied most extensively as it was the first high-risk HPV oncogene product to be discovered (Kanda et al., 1988; Yutsudo et al., 1988). HPV16 E7 induces elongated S-phase and abrogates the cell-cycle checkpoints (Martin et al., 1998; Peacock et al., 1995). In healthy human cells high-risk HPV E7 protein leads to the chromosomal instability (White et al., 1994). HPV E7 plays a major role in the virus life cycle and is necessary for the stable maintenance of HPV episomes in epithelial cells (Flores et al., 2000; Thomas et al., 1999). The E7 protein is responsible for cellular transformation during the life cycle of high-risk and low-risk HPVs (Oh et al., 2004b). The differentiation state of the host keratinocyte influences the replicative life cycle of HPVs. The host cellular DNA replication machinery is necessary for HPV genome synthesis, but these differentiated cells do not intrinsically support DNA replication (Stubenrauch & Laimins, 1999). HPV E7 retains 26 differentiating keratinocytes in a DNA replication competent state (Cheng et al., 1995). The ability of E7 proteins to induce unscheduled DNA replication is important for cellular transformation and it is a necessary factor of the HPV replication strategy (Figs. 5 & 6).
The host cell integrated genes E6 and E7 are expressed and their protein products are responsible for the oncogenesis. E7 interferes with transcriptional activity of p53, inactivates the cdk inhibitor p21\textsuperscript{CIP1} and blocks pRb binding to E2F. The pRb and p53 inactivation interferes with the integrity of important cell checkpoints and the cellular apoptosis mechanism. The E6 protein disrupts apoptosis by binding to p53 and targeting this tumor suppressor for proteolysis via the ubiquitin pathway. A schematic description of the E6 and E7 function that obstructs normal cell cycle progression is given in Figs. 5 & 6.

Figure 5. Viral genes replication, assembly and shedding
Munger et al. (2004) and Munoz et al. (2006) have studied the expression and HPV positive cells differentiation, the late promoter activation. These induction led the cell to expression of late genes and new virions. In the uppermost layers of the epithelium, DNA is packaged into newly formed virus capsids and shed into the environment as a cargo within epithelial squamae.

Dunne et al. (2006) and Singh (2005) have observed that the HPV was largely asymptomatic, making it difficult to recognize and detect among the general population, which will limit any behaviour modification. Vaccinations may thus provide a solution for prevention. 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. Gardasil, a prophylactic vaccine shows maximum efficacy if used by girls pre-exposure to HPV. Prophylactic HPV vaccines are associated with VLPs and are made up of HPV L1 proteins at present. As VLPs, has no viral DNA therefore these are not associated with the development of infection even though their structure is similar to actual virus morphologically. By intra-muscular (I/M) injection,
high levels of systemic anti HPV L1, immunoglobulin G antibodies are formed due to 
VLPs.

Munoz et al. (1996) and Stanley (2006) have found that investigated the 
current prophylactic HPV vaccines are effective in purified virus-like particles 
(VLPs), i.e. the viral capsid, without the viral DNA, composed of the main envelope 
protein L1 of the oncogenic HPV types. Risk of infection with HPV is directly 
proportional to age. Prophylactic HPV vaccines are found to be effective in treating 
HPV infection and this has been proved in a number of randomized clinical trials.

The FDA reports that HPV vaccines, gardasil (approved in 2006) and cervarix 
(approved in 2009), are safe for females ages 9 to 26 years. As of 2009, gardasil is 
also licensed, and considered safe for males ages 9 through 26 years. Boys and young 
men may choose to get this vaccine to prevent anal cancer and genital warts. Both 
vaccines were tested in thousands of people around the world before they were 
approved. These studies showed no serious side effects and no deaths have been 
linked to either vaccine. Common, mild side effects include pain where the shot was 
given, fever, dizziness, and nausea. People may faint after getting any vaccine, 
including HPV vaccines. Fainting after getting a shot is more common in teens than in 
young children or adults. To keep people from getting hurt from fainting, a 15-minute 
waiting period for people of all ages is recommended after any vaccination. Both 
HPV vaccines are being monitored for side effects, especially rare ones not seen in 
the study trials. CDC & FDA doctors and scientists still review all reports of serious 
side effects reported to the Vaccine Adverse Event Reporting System (VAERS) to 
watch for potential new vaccine safety concerns that may need further study (The 
VAERS is a national reporting system that looks at reports of side effects after 
vaccinations.) The American Cancer Society will watch those reviews and report any 
concerns about the safety of the vaccines (Saslow & Lawson, 2012).

Miller et al. (1990) reported that the cervical cancer is preventable and curable 
if detected and treated at an early stage. Since early detection predicts better 
prognosis, one of the most effective ways of preventing and controlling cervical 
cancer is regular screening and early diagnosis. The most effective method of 
screening employed in the developed world has been cytology based using pap
smears, which has contributed considerably to reducing incidence of, and mortality from, cervical cancer. However this method of screening requires excessive resources in terms of laboratories, equipment, trained personnel and transport of specimens (Miller et al., 2000). Lack of adequate financial and human resources in developing country settings has prevented the quick uptake of such cytology based screening programmes at the population level.

Dabash et al. (2005) reported that there are socio-cultural barriers to cervical cancer screening in India. Lack of privacy and confidentiality during screening, cultural norms encouraging modesty among women and insufficient importance given to women’s health issues to be of significant barriers to cervical cancer screening. It is advocated only for women, at least to begin with, and the target age group is adolescent girls. The parents may not give consent and the young adult women may not be willing to go for such a vaccine. The health care providers may also be reluctant to recommend the vaccine to general population due to their personal beliefs and anxieties about the parental reactions (Basu & Chowdhury, 2009).

Epidemiological studies have significantly contributed to our understanding of cancer associated risk factors and thus prevention of cancer. The above information clearly created the awareness among women about screening procedures (Pap test) and vaccination programmes against cervical cancer. Apart from major risk factors, this survey also helped to find out some of the minor risk factors of cervical cancer.

Identification and development of natural products for their use in cancer prevention have attracted a lot of attention globally. Herbal extracts with their proven potential and less side effects in therapeutics have replaced the synthetically derived drugs in modern allopathic medication system (Sakthivel & Guruvayoorappan, 2012). Evidence has been provided that dietary phytochemicals may play important role as chemopreventive or chemotherapeutic agents in the prevention of many diseases, possesses antimutagenic effects and indeed modulating and stimulating the immune system (Raskin et al., 2002; Rates, 2001).
2.3 Anthocyanidins and their applications

Several studies have shown that the anthocyanidins and their cytotoxic effects on number of diseases. Guarini et al. (1992) examined the anticancer effects of caffeic acid phenyl ester suppressed proliferation of HO-1 (Human Melanoma cells) and GBM – 18 (Human glioblastoma). Chemopreventive mechanisms of anthocyanins include scavenging free radicals, reducing cell proliferation, up-regulating/inducing apoptosis and modulating mitogen activated protein kinase (MAPK).

Karlis et al. (2001) finding suggested that dietary polyphenol cyanidin, is a potent inhibitor of mitogen activated metabolic activity, which increases the free intracellular Ca$^{2+}$ and cellular growth of cultured colon carcinoma cells. Pan & Ho (2008) found chemoprevention, a potential approach to prevent cancer make use of natural dietary compounds or synthetic molecules to arrest, inhibit, reverse or delay the carcinogenesis process. Dreiseitel et al. (2008) reported inhibition of CYP3A4 activity by ACN and their aglycons, proanthocyanidins in a concentration dependent manner and also suggested that number of sugar moieties predicts a decline in anthocyanidins effects on CYP3A4.

Antimutagenic activity of anthocyanidins was reported by Gasiorowski et al. (1997) which indicated that ACN inhibited the mutagenic activity of benzo (a) pyrene and 2- amino fluorine. Haber Meyer et al. (2005) reported that delphinidin and cyanidin owing vicinal hydroxyl groups at the b-ring strongly inhibited the catalytic activity of human topoisomerase I and II in a dose dependent manner.

Esselen et al. (2009) reported that delphindin at the concentration of 10µM, decreased the etoposide – induced DNA strand breaks. Syed et al. (2008) reported that delphidin inhibited HGF induced phosphorylation and activation of c-Met in the MCF – 10A breast cell line.

Meiers et al. (2001) have reported that cyaniding and delphhindin carrying free hydroxyl group in the 3, 3’ and 4’ position were potent inhibitors of EGFR protein tyrosine kinase activity while malvidin carring a sugar residue in the 3rd position and methoxy group at the B-ring did not affect enzyme activity. Similarly, Afaq et al. (2005) reported the capability of delphinidin to inhibit EGFR activation and downstream signaling pathways.
Fridrich et al. (2007) have found the suppression of EGFR phosphorylation by delphinidin in human colon cancer cell and vulva carcinoma. Potent inhibition of EGFR activity following shut down the MAPK pathway by cyanidin and delphinidin but not malvidin was also determined by Marko et al. (2004).

Afaq et al. (2008) demonstrated that topical application of anthocyanin and hydrolysable tamin-rich pomegranate fruit extracts prior that of 12-o-tetradecanoylphorbol-13-acetate (TPA) resulted in a significant inhibition of TPA mediated induction of epidermal ODC activity and ODC protein expression. Similarly, Thole et al. (2006) provided evidence that fractions from the American elderberry Sambucus canadensis containing ACN inhibited in vitro ODC activity. Bomser et al. (1996) reported that ACN from fruit extracts four Vaccinium species (blueberry, bilberry and lingonberry) were relatively weak inhibitors of TPA- induced ODC activity on mouse epidermal cells.

Marko et al. (2004) proved that malvidin, bearing methoxy substituent in the 3’-and 5- position, inhibited cAMP hydrolysis (IC₅₀ value of 23µM) in human colon cancer cells and that the PDE-inhibitory properties were impaired by the lack of replacement with hydroxyl substituents of methoxy groups. Fritz et al. (2006) further corroborating the inhibitory activity of malvidin on cAMP – PDE, indicated that the compound at a concentration of 5 µM significantly decreased the phosphorylation of ERK1 and ERK2, thus hampering their downstream signaling cascade activation.

Kuo et al. (2004) reported that prodelphinidin B-23, 3’-di-O-gallate inhibited proliferation of squamous lung carcinoma cells by arresting cancer cell cycle progression at the G0-G1 phase through are the induction of p21^{WAF1/Cip 1}. Similarly, Wu et al., (2007) found that the 14-fold increase in the expression of p21^{WAF1} was associated with inhibition of colon cancer cell proliferation.

Malik et al. (2003) found that the exposure of colon cancer cells to an anthocyanidin-rich extract resulted in cell cycle arrest at the G1/G0 and G2/M phases, increased the expression of the p27Kip1 and p21WAF 1/Clip 1 genes and finally in 60% cancer cell growth inhibition.

Lamy et al. (2006) demonstrated that delphinidin is potent angiogenic inhibitor. Martin et al. (2003) reported that delphinidin inhibited serum and vascular
endothelium growth factor induced endothelial cell proliferation. Oak et al. (2006) showed that delphinidin inhibited angiogenesis in vivo by influencing endothelial cell migration and proliferation. Oak et al demonstrated that delphinidin and cyaniding markedly reduced the PDGF_{AB} induced phosphorylation of p38 MAPK and JNK without affecting that of ERK1/2 and prevented vascular expression of VEGF.

Favot et al. (2003) as well as Martin et al. (2003) have reported that delphinidin inhibition of human umbilical endothelial cells proliferation was correlated with the blockage of cell in the G_0/G_1 phase and that delphinidin counteracted the proliferative effect of vascular endothelial growth factors through the restored induction of cyclin kinase inhibitor p27^{kip1} and p21^{WAF/Cip1}.


Hafeez et al. (2008) demonstrated that delphinidin inhibited the growth and induced apoptosis of prostate cancer cells through the decreasing phosphor- NF-kB/p50 at ser^{259} and phosphor – NF-kb/p50 at ser^{536} in the nuclear fraction which decreased the NF-kB DNA binding activity. Tsoyi et al. (2008) reported that ACN from black soyabean inhibited UV B mediated PGE_2 production in human keratinocyte cell line and decrease in COX – 2 expression and transcription activity levels also measured.

Kwon et al. (2007) analyzed the molecular events underlying the inhibitory activity of peonidin on 12-o-Tetradecanoyl – phorbol – 13 acetate (TPA) induced mouse dermal cell transformation. Hou et al. (2007) established that prodelphinidin (PDG) inhibited LPS- induced COX-2 expression and PGE_2 production in RAW264 cells through downregulation of the m- RNA levels of COX – 2 achieved by blocking the binding complex of NF-kB DNA on its promoter. Lipoxygenase (LOX), the other arachidonic acid processing enzyme, was inhibited by spectrum of anthocyanidins, as
demonstrated by Knaup et al. (2008) and the LOX inhibitory activity of ACN decreased from Delphinidin to cyanidin to peonidin and finally to malvidin derivatives.

**Reports on methylation and cancer progression**

Laird & Jaenisch (2003) opined that tumor suppressor genes can be inactivated not only through structural changes (mutation /deletion) but also by lack of expression due to promoter hypermethylation. First suppressor gene known to be hyper methylated and silenced was RB (Greger et al., 1989) and followed by p16, MHL1, VHL and E- cadherin (Santini et al., 2001).

Szyf & Targeti (2003) have shown that the DNA methylation is common and casual by the ability of diverse pharmacologic compounds and molecular technique to reactivate gene expression upon inhibiting the DNA methyltransferases.

List of hypermethylated genes:

1. Apoptosis related genes: DcR1 & DcR2 – receptor for TNFR1, Fas etc. Van Nooesel et al., (2002).
3. WNT pathway genes – APC, β-catennin, AXIN2, TCF4 WISP3, E- Cadherins and PTEN (Thorstensen et al., 2005)
4. DNA repair genes: MGMT or BRCA1 (Narayanan et al., 2003).
5. Miscellaneous: RASSFI A (Cohen et al., 2003) & DAPK (Narayanan et al., 2003) etc.

Goelz et al. (1985) suggested that tumor cells can have high hypomethylation as 60% which is less than their normal level and it occurs mainly in coding regions as well as in pericentromeric regions of the chromosomes. Eden et al. (2003) reported that ICF’s syndrome (Immuunodeficiency, chromosomal instability and facial anomalies) caused by mutations at DNMT3b.
METHYL TRANSFERASE INHIBITORS

There are two types of inhibitors namely 1. Nucleoside & 2. Non – nucleoside analog

<table>
<thead>
<tr>
<th>Nucleoside Analog</th>
<th>Non - nucleoside analog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycytidine analog</td>
<td>Mostly natural compounds</td>
</tr>
<tr>
<td>1. 5- Azacytidine *</td>
<td>1. Zebularine</td>
</tr>
<tr>
<td>2. 5- Aza -2 – Deoxycytidine*</td>
<td>MG98</td>
</tr>
<tr>
<td>3. 1-β-D arabino furanosil</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>4. 5- aza cytosine</td>
<td>1. (-) Epigallocatechin 3 – Gallate</td>
</tr>
<tr>
<td>5. Dihydro 5- aza cytidine</td>
<td>2. Procaine</td>
</tr>
<tr>
<td></td>
<td>3. Procainamide</td>
</tr>
<tr>
<td></td>
<td>4. Hydralazine</td>
</tr>
</tbody>
</table>


Table 1. Methyl Transferase Inhibitors

From the foregoing account, it is clear that more research towards drug discovery with reference to viral inhibitory compounds form plant sources is needed.