CHAPTER 2

2. REVIEW OF LITERATURE

Worldwide, head and neck squamous cell carcinoma (HNSCC) is ranked the sixth common cancer and it often generates from critical organs including the larynx, pharynx, oral cavity, and tongue (Masuda et al. 2011). Head and neck cancers has been accounted as one of the most common cancers in South and Southeast Asian countries. However, they account for only 1–4% of all cancers in the Western world (Joshi et al. 2014b). Among HNSCC, oral cancer is the predominant cancer in India, Pakistan, and other Southeast Asian countries where as oropharyngeal and tongue cancers are common in the Western world (Bhurgri et al. 2006). The prevalent habits in the respective regions might be the reason for the differences in site of disease (Sankaranarayanan et al. 1998). In India, HNSCC is the most frequent cancer and constitutes about one-third of all cancers (Trivedi et al. 2012, Takiar, Nadayil and Nandakumar 2010, Sanghvi, Rao and Joshi 1989). According to the Indian Council of Medical Research (ICMR) every year, approximately 0.2 to 0.25 million new head and neck cancer patients are diagnosed (Takiar et al. 2010, Trivedi et al. 2012). In India, it has been accounted as the most frequent cancer of males and the fifth most common among females (Organization).

2.1. Risk Factors

In the Western world, the main risk factors of HNSCC are cigarette-smoking and alcohol consumption whereas in Southeast Asia the usage of smokeless tobacco and areca nut are the prime causes (Graham et al. 1977, Dayal, Mani and Bhargava 1978). The various forms in which smokeless tobacco in use in developing countries are paan (betel quid), mava, zarda, snuff,
mashiri etc (Sridharan 2014). In India in 2010, about 930,000 deaths were due to usage of tobacco (Murray and Lopez 1997).

Human Papillomavirus (HPV) prevalence: Another risk factor in HNSCC is the prevalence of HPV which is around 50% (Cruz et al. 1996). The highest occurrence of HPV is reported in cancers of the base of tongue and tonsils (Gillison 2004). HPV-16 is the most frequent type, that is present in 30.9% of oropharyngeal carcinomas, 16% of oral cancers, and 16.6% of laryngeal cancers. Prevalence of HPV in oral cancers is similar in Europe (16%) and North America (16.1%), but greater in Asia (33%) (Kreimer et al. 2005). The prevalence of HPV in India is different in various region ranging from 33.6% in the Eastern region to 67% in South India and 15% in Western India (Balaram et al. 1995, D'Costa et al. 1998)

2.2. Molecular biology of head and neck cancers

There are manifold pathways that are de-regulated in HNSCC (Wheeler et al. 2012). Pathways that are most commonly altered include P53, epidermal growth factor receptor, signal transducer and activator of transcription, VEGF, and mTOR (Freudlsperger et al. 2011). Hence they have also been identified as probable therapeutic targets of HNSCC (Howard, Lu and Chung 2012). The TP53 and retinoblastoma (Rb) pathways are most frequently de regulated or disrupted in HNSCCs, indicating the significance of these pathways in head and neck carcinogenesis. In 60–80% cases of HNSCC somatic mutations of TP53 are seen (van Houten et al. 2002, Balz et al. 2003, Poeta et al. 2007). The TP53 pathway is mainly involved in controlling cell proliferation by regulating progression of cell-cycle and apoptosis. The loss of chromosome 9p21,17q13 loci inactivates tumor suppressor genes namely p16 and p53, respectively (Park et al. 1998). Apart from this, overexpression of cyclin D1 and p53 which are dominant negative, and increased activity of telomerase (≥ 80%), leads to de-regulation of cell cycle and induces resistance to
stimulators of DNA damage in HNSCC (Choi and Myers 2008). High-risk HPV strains (16 and 18) associated with oropharyngeal squamous cell carcinoma (as well as cervical cancer) influence cellular pathways within affected cells to activate cell growth and suppress apoptosis. The p53 tumor suppressor gene is inactivated by E6, while the retinoblastoma tumor suppressor protein (Rb) by a second HPV protein, E7. Both the HPV E6 and E7 proteins, which are encoded in the HPV-16 genome, functionally disrupt regulation of cell-cycle and DNA-repair mechanisms that leads to genetic or epigenetic changes during molecular progression of HNSCC (Ishiji 2000). E6 associates with cellular ubiquitin-protein ligase E6-AP that leads to the ubiquitination and degradation of p53, which causes deregulation of cell cycle. E7 binds to p21 and Rb which inhibits the interaction of Rb with E2F and leads to uncontrolled cell proliferation (Fouret et al. 1997).

Another oncogene that is widely overexpressed in HNSCC is epidermal growth factor receptor (80-100%) (Wheeler et al. 2012). It has been known to be linked with more aggressive phenotype with decreased survival rates, increased resistance to treatment modalities, including targeted therapies (Mydlarz, Hennessey and Califano 2010, Van Damme et al. 2010). EGFR VIII is the most common mutant variant of EGFR receptor present in 42% of HNSCC cases and is involved in enhanced proliferation (Sharafinski et al. 2010). There are many growth factors that are identified to play an important role in the development of HNSCC like basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), transforming growth factor-alpha/beta (TGF-α/β), metalloproteinases(MMPs), interleukin 8 (IL-8), signal transducers and activators of transcription 3 (STAT3), vascular endothelial growth factor (VFGF) etc. Another growth factor, the Insulin-Like Growth Factor-1 Receptor (IGF-1R) and its ligands factor Insulin-like Growth Factor-1(IGF-1) and Insulin-like Growth Factor-2 (IGF-2), are associated with the development and progression of HNSCC (Pollak, Schernhammer and Hankinson 2004, Larsson, Girmita and
The main downstream signaling pathways activated by ErbB/tyrosine kinase receptor family is the PI3-K/Akt pathway. When ligand binds to the cytoplasmic domain of EGFR receptor it undergoes tyrosine phosphorylation and subsequently activates PI3-K (Bussink, van der Kogel and Kaanders 2008). Activation of Akt leads to increased expression of many proliferative and anti-apoptotic proteins, including Bcl-xL, Bcl-2 and NF-kB. In 50–80% of HNSCCs the said pathway has been known to be activated (Lothaire et al. 2006). Several studies have shown that there is strong and independent correlation between expression of activated Akt (pAkt) and outcome in laryngeal and oropharyngeal HNSCC (Massarelli et al. 2005, Lim et al. 2005). And it has also been associated with more advanced tumor stage and progression in a number of different cancers (Kim et al. 2005). One of the downstream cell-growth regulators associated with tyrosine kinase receptors (EGFR, IGF-1R), and the PI3K/Akt signaling pathway is a serine/threonine kinase named mammalian target of rapamycin (mTOR), which has been found to be activated in 90-100% of head and neck cancers. mTOR modulates the cell cycle and ribosomal function by regulating phosphorylation of p70S6K and 4EBP1, thus triggering protein synthesis to subsequently engage in both cell growth and apoptosis (Elser et al. 2007). It has been reported that there are many components of the mTOR signaling pathways that are deregulated in HNSCC (Nguyen et al. 2012). mTOR protein is found to be high in recurrent tumors, indicating an aggressive phenotype (Clark et al. 2010). There are subtle differences in the distribution of molecular biology of HNSCC apart from the age, subsite and habit. For instance, in Europe and USA the prevalence of the p53 mutation is the most common, around 81% where as its rare in India. Multiple genetic abnormalities are common in head and cancers in India and Southeast Asia. These include a prevalence of Ha-ras mutations (35%), loss of hetero-zygositiy of Ha-ras (30%), amplification of N-ras (28%) and of N-myc (29%). These mutations in ras oncogenes are not so common in the Western world (Joshi et al. 2014a).
2.3. Clinical management of HNSCC

Over the past few decades there has been no much progress in the survival rates of HNSCC. Although the overall survival rates have remained relatively stagnant, numerous advances in treatment have occurred. Surgical resection, for decades has remained the primary treatment for advanced HNSCC, and today it has become an integrative management strategy combining surgery, chemo- radiotherapy and more recently biological therapy.

Approximately one-third of patients who are diagnosed with early stage, locally contained disease (Fung and Grandis 2010) are commonly treated with either surgery or radiotherapy both of which can yield excellent results and cure rates of better than 90% for stage I and 70% for stage II patients (Jones et al. 2004, Network 2008). The selection of either of this conventional treatment modality has been based upon a array of factors including accessibility of the lesion, organ preservation, and chance of second recurrence. Locally advanced HNSCC (stage III and stage IV) are more complicated and treatment options are less and choice is dependent on a multitude of factors including the site of the lesion and its accessibility for excision (Network 2008). Broadly, tumors of the oral cavity are generally treated with primary surgical excision and tumors of the pharynx and larynx are treated with chemo-radiation as the primary treatment (Network 2008).

2.3.1. Chemotherapy

Chemotherapeutic agents are used either as single modality or in combination with surgery or radiation. And in cases with locally advanced disease where surgical resection is impossible, concurrent chemoradiotherapy (CRT) is standard treatment option worldwide (Adelstein et al. 2003). The most widely used therapeutic agents for HNSCC include, cisplatin, methotrexate,
bleomycin, and 5-fluorouracil (5-FU) (Vermorken and Specenier 2010). Recently, promising results with 30 – 40% response rates have been achieved from studies of taxane compounds (paclitaxel and docetaxel) for recurrent and metastatic disease however these rates depend upon individual and tumor characteristics (Vermorken and Specenier 2010). However, in recent years, there has been several large studies that have compared single chemotherapeutic agents with two agent combination therapy and have found that combination treatment have synergistic results and is useful as palliative therapy but does not improve overall survival (Jacobs et al. 1992, Forastiere et al. 1992) and are also associated with toxicity or side effects.

2.3.2. Targeted Molecular therapy in HNSCC

The conventional treatment modalities like surgery, radiation and chemotherapy are not selective and cause side effects or damage the normal cells. For instance, chemo- and radiotherapy are associated with systemic toxicities that often reduce compliance and leads to the failure of completion of therapy on time (Tsao et al. 2006). So currently, the focus is on the molecular biology of HNSCC and to target selected pathways involved in the carcinogenesis in HNSCC. Targeted molecular therapy includes therapy with monoclonal antibodies, gene therapy, and other therapies. It has negligible side effects on normal cells.

Overexpression of EGFR and its ligands is recognized in more than 80% of squamous cell cancers and is associated with poor prognosis. There are many selective compounds which have been developed that target either the extracellular ligand-binding region of the EGFR like many of the monoclonal antibodies (cetuximab, panituximab), ligand-binding cytotoxic agents and immunotoxins or intracellular tyrosine kinase region like Gefitinib, Erlotinib etc (Harding and Burtness 2005). Cetuximab C225 has been the most extensively studied for targeted therapy of head and neck, lung, and colorectal cancers. Cetuximab in combination with chemotherapy and
Radiotherapy, or both have been completed for a range of indications, including non-small cell lung carcinoma (NSCLC), colorectal cancer and head and neck squamous cell carcinoma (HNSCC) (Baselga et al. 2002) and it is the first anti-EGFR monoclonal antibody that has been approved by Food and Drug Administration (FDA) for clinical use. Cetuximab has also shown an improved ability with 10-30% response rate, and 46-55% disease control in patients when used in combination with platinum-based chemotherapy. However, there are some most commonly reported cetuximab-related adverse effects like, fever, and nausea, as well as elevated transaminases and allergic reactions. Likewise, several small-molecule tyrosine kinase inhibitors of the EGFR tyrosine kinase are currently being studied. Of these, the FDA has already approved the oral quinazolines, erlotinib (OSI-774, Tarceva; Genentech, South San Francisco, CA) and gefitinib (ZD 1839, Iressa; AstraZeneca, Wilmington, DE) for clinical use. p53 gene is most commonly found to be mutated in approximately 50% of HNSCC cases (Rhee, Khuri and Shin 2004).

Gene therapy is being used to restore the function of TP53 that has been lost in HNSCC. Gene therapy resulted in tumor reduction with increase in apoptosis of cancer cells in patients with head and neck cancers. The adenovirus ONYX-015 targeted mutant p53 attacks null cells by deleting the E1B gene. It can only replicate in TP53-null cells which leads to the lysis and selective killing of cancer cells. The premalignant lesions decreased in one third of the patients, indicating its effectiveness (Rudin et al. 2003).

### 2.3.3. VEGF inhibitors

VEGF has been reported to be overexpressed in approximately 40% of head and neck squamous cell carcinomas (HNSCCs), and is associated with EGFR resistance, tumor progression, and poor prognosis. Recent anti-angiogenic therapies consist of monoclonal antibodies mainly
bevacizumab, ranibizumab. Bevacizumab when used in combination with chemotherapy showed increase in life expectancy in patients with metastatic colon cancer.

2.3.4. COX-2 inhibitors

Cyclooxygenase-2 (COX-2) is found to be overexpressed in HNSCC compared to normal mucosa. Increase in the levels of COX-2 may contribute to intiation of cancer by modulating the metabolism of xenobiotics, apoptosis, angiogenesis etc. There are studies which demonstrate the chemopreventive effect of celecoxib in oral pre-cancers and tumors in animal models (Rudin et al. 2003). COX-2 inhibition with celecoxib with radiotherapy or chemotherapy with cisplatin, paclitaxel, has shown benefit in 30% of patients (Lin et al. 2002).

2.3.5. HPV vaccines.

Several studies have shown there are significantly increased risks of developing HNSCC, particularly of the orpharynx, in HPV positive patients (Nishioka et al. 2009). HPV E6 and E7 proteins, which have increased activity in high risk HPV types (McLaughlin-Drubin and Münger 2009), modulate the tumor suppressor proteins p53 and Rb, respectively. HPV status is known to be an important prognostic factor and the molecular targeted therapies have been known to have better outcome in HPV+ HNSCC patients (Hainsworth et al. 2009). The HPV (+ve) patients are found to have significantly better survival compared to HPV (-ve) patients (Argiris et al. 2009). On an average the survival of HPV (+ve) patients treated with synchronized therapy is 6 to 8 months, which is far better than when compared to supportive care alone. Recently, the frequency of oropharyngeal cancer (HPV positive) incidence has been increased and the FDA has approved two HPV vaccines Gardasil (HPV quadrivalent), Adervari (human HPV bivalent) for use in HNSCC patients. Apart from prevention, HPV vaccines may find a therapeutic role in
HNSCC. Currently, there are a large number of approaches including live viral or bacterial, peptide and nucleic acid vaccines for inducing cell-mediated immunity against HPV (+ve) cancer cells, expressing E6 and E7 proteins.

2.4. Chemoprevention of HNSCC

According to Sporn et al, cancer chemoprevention is defined as the use of both natural and/or synthetic agents to reverse, suppress, and/or prevent initiation, promotion and progression of tumorigenesis (Sporn et al. 1976). In HNSCC, it is based upon the two concepts namely multifocal field carcinogenesis and multistep carcinogenesis. The field cancerization describes that the persistent exposure of surface epithelium to chemical, viral and/or environmental carcinogens results in genetic variation at multifocal areas throughout the oral and orophyngaeal mucosa and subsequent clonal proliferation of mutated cells (Barnes et al. 2005). Throughout the field there may be genetic changes and it increases the probability of developing multiple premalignant and malignant lesions within that field. This concept of field cancerization has described the increased risk of secondary primary tumors and recurrence of primary tumors in HNSCC patients.

The pathological analysis of field carcinogenesis has described the concept of multistep carcinogenesis in many cancers including HNSCCS as stepwise accumulation of somatic mutations over time resulting in progression from normal hyperplasia to dysplasia to carcinoma in situ to invasive carcinoma instead of different stages of tumor initiation, promotion and progression as illustrated in Fig 1.1. Blocking one or several of the steps may inhibit or delay the development of cancer. Chemoprevention is categorized into primary secondary and tertiary chemoprevention. Primary prevention focuses to prevent malignancies in healthy individuals who may belong to high risk category, such as smokers or people predisposed to cancer
development as a result of certain genetic mutations. Secondary prevention strategies seek to prevent or delay progression of the premalignant lesions into cancers in patients who are already known to have premalignant lesions (for instance oral leukoplakia). Tertiary prevention is mainly to prevent the formation of secondary primary tumor in patients cured of their initial cancer or premalignant lesions. The basis of chemoprevention trials is that blocking of the biological processes involved in carcinogenesis will halt the cancer progression or development and, in turn, reduce cancer incidence (Lippman and Hong 2002).

2.5. Chemoprevention trials in HNSCC

HNSCC has been one of the most widely researched tumor types in chemoprevention. In HNSCC patients treated with early stage or locally advanced cancers, 10% to 40% develop secondary primary tumors or recurrence. Several chemoprevention trials have been conducted and still going on which focuses on two main aspects: reversal of pre-malignancy and prevention of secondary primary tumors.

The first chemoprevention trial in HNSCC for oral leukoplakia was reported by Hong et al using high-dose 13-cis retinoic acid (13cRA) for a period of three months and reported a major decrease in size of oral leukoplakia in patients receiving the retinoid versus patients receiving placebo (Hong et al. 1986a). However, it had some side effects including the common toxicities such as cheilitis, skin dryness, hypertriglyceridemia etc. Another trial in oral leukoplakia use disotretinoin in combination with beta carotene (Khuri et al. 2006). This study was conducted in two phases. In the first phase high-dose of isotretinoin (1.5 mg/kg/day) was administrated for a period of three months and then, in the next phase patients were randomized to a dose of 30 mg/day beta carotene or a low-dose of isotretinoin (0.5 mg/kg/day) for a period of nine months. The second phase was conducted only after the patients having a positive response or attaining a
stable state. This study reached the conclusion that maintaining a low-dose isotretinoin was more effective against leukoplakia than treating with beta carotene (92% versus 45% response or stable disease) in patients who benefited earlier from high-dose of isotretinoin. However, the beneficial effects could not be sustained over time. Moreover, the trial also reported dose related toxicities to isotretinoin like dry skin, cheilitis, conjunctivitis, and hypertriglyceridemia.

In another clinical study 100,000 IU of vitamin was given twice a week along with placebo in 65 patients suffering from oral leukoplakia who tobacco or betel nut users (Stich et al. 1990). The results revealed that patients who took vitamin A had complete remissions (57% versus 3%) and there was repression of lesions versus placebo (0% versus 21%). The study also reported that combination of beta carotene and retinol had significant effects than beta carotene alone. A placebo nonrandomized Phase II trial using alpha tocopherol in patients with oral leukoplakia resulted in twenty patients (47%) responding successfully and nine (21%) showing histologic effect (Benner et al. 1993). Many more trials have been conducted with retinoids in single or combination with other agents. European Study on chemoprevention Vitamin A and N-acetyl cysteine (EUROSCAN) included 2,592 patients who had been treated for primary tumors previously (60% head and neck cancer, 40% lung cancer). They were given retinyl palmitate, N-acetylcysteine, agents, or placebo for a period of two years. This trial failed to show any benefit in terms of survival or decrease in secondary primary tumors with these agents even after 49 months of follow up study. In a similar trial which included 316 patients who had history of early-stage squamous cell cancers affecting the oral cavity or oropharynx and reported there was no difference in incidence, five year survival rate and incidence of secondary tumor rates between placebo and treatment groups (Bolla et al. 1994). From these studies it was seen that the pre malignant lesions had high risk to attain resistance and transform to much aggressive cancers. Therefore, biochemoprevention, which used a combination of retinoids with interferon
(IFN) and alpha tocopherol, was formulated to treat this group. In this clinical trial 36 patients were treated for a period of one year with IFN-alpha, alpha-tocopherol, and 13cRA who had developed advanced premalignant lesions. There was no positive outcome with oral cavity lesions but it prevented the development of laryngeal lesions. It was also found that patients with high expression of p53 had lesser response rates and much higher disease progression rates than patients compared with low expression of p53.

In another biochemoprevention trial, combination of biological agents such as Interferon- α (IFN- α) and 13-c-RA patients were evaluated in patients previously treated for locally advanced HNSCC. Combination of 13-c-RA (50 mg/twice daily), IFN- α (3X106 U/m2, 3 times/week), and α-tocopherol (1,200 IU/day) for a period of 12 months at a median of 2 years, 86% of patients were cured (Hong et al. 1986a). Overall survival reported at one year was 98% and at two year was 91%. Some toxicity like fatigue, head ache, weight loss, peripheral neuropathy etc were reported yet no patients needed growth factor support or suffered major problems hence biochemoprevention was recognized as feasible adjuvant therapy and many trials are underway to confirm the same.

According to Johnson et al. 1994 there are many studies that explored the anti-carcinogenic potential of a range of secondary plant metabolites found commonly in human diets (General 2007). There are epidemiological evidences that diets rich in fruits and vegetables confer protection against a variety of cancers (Riboli and Norat 2003). Many dietary components such as proanthocyanidins have been used for its chemopreventive efficacies in oral cancer and are based on the evidence that dietary intake of fruits and vegetables is closely related with a decrease in risk of oral cancer (Pavia et al. 2006). There results of these trials are in consistent with the guidelines of the American Cancer Society and the American Institute of Cancer Research
(AICR) advocating the significance of increased intake of fruits and vegetables for prevention of cancer (Glade 1999b). Undoubtedly, the epidemiological and clinical based efficacy studies point towards the beneficial effect of dietary components, yet there are no definitive markers to evaluate the anti-cancer efficacy of these compounds as well as to identify the risk population. However, non-toxicity, chemical diversity, effect on multiple targets, affordability and availability of phytochemicals make chemoprevention an exciting area of research against wide range of malignancies including head and neck cancer.

Phytochemicals are plant derived chemicals and can be categorized into carotenoids, alkaloids phenolics, organosulfur compounds and nitrogen containing compounds. They are known to have synergistic and overlapping mechanisms of action. They have antioxidant activity, regulate proto-oncogenes and tumour suppressor genes, induce cell cycle arrest and apoptosis, stimulate immune system and regulate epigenetic mechanisms. Recently, in a meta-analysis it was found that consumption of green leafy vegetables and carrots rich in carotenoids associated with a reduced risk of breast cancer and it has also been linked with lower risk of ovarian and pancreatic cancers (Thomas et al.). High consumption of cruciferous vegetables such as cabbage, cauliflower, and broccoli etc and isoflavones rich pulses and soy products, lycopene rich fruits and tomatoes have all been linked with reduced prostate cancer risk (Park et al. 2008, Giovannucci et al. 2002). Consumption of foods rich in flavanoids like quercetin found in onions, have been shown to lower the incidence of many cancers especially that of lung, among smokers (Knekt et al. 1997). Yet another, compound anthoxanthins, found in dark chocolate, have been identified to reduce the incidence of colon cancer similarly high intake of green tea is found to lower the risk of breast, ovarian breast and oesophageal cancer, mostly in smokers and alcoholics (Rodríguez-Ramiro et al. 2011, Sun et al. 2007). Coffee intake has been shown to reduce the risk of skin cancers both, non-melanomatous and melanoma irrespective of BMI, age,
and other physical parameters and habits including smoking and alcohol use (Loftfield et al. 2015). Not only the phytochemicals were proven for cancer prevention but were found to have clinical benefit after cancer detection particularly in combination with other healthy lifestyles. For instance, breast cancer survivors were found to lower the risk of recurrence of breast cancer by one third if they consumed fruits and vegetables more than the government recommended portion together with routine physical activity (Pierce et al. 2007). Women who had been diagnosed with breast cancer who had high levels of lignin in their serum as a result of high intake of soya, cereals, cruciferous vegetables, etc were identified to have the lowest risk of fatality in another study (Buck et al. 2011). Similarly, a low colorectal cancer recurrence rate was observed in patients consuming a lignan and polyphenol rich diet (Zhu et al. 2013). The Shanghai Breast Cancer Survival Study reported that women whose diet is rich in phytoestrogenic compounds, had a very low recurrence breast cancer rate of about 29% (Boyapati et al. 2005). Green tea consumption also was found to have similar effects in breast and colorectal cancer patients. Green tea lowered the abnormal WBC count in 30% of patients with chronic leukemia, decreased the levels of several markers of cell proliferation and also serum Prostate Specific Antigen (PSA) in men with prostate cancer (Shanafelt et al. 2009). Delay in PSA occurrence has been reported in a phase II study with pomegranate juice and other dietary studies (Ornish et al. 2005). A study of group of individuals who have already been treated for basal cell carcinoma or squamous cell carcinoma, with a high chance of recurrence due to their susceptibility to UV rays were found to have lowest rate of recurrence when they ate diet rich in lutein and zeaxanthin, such as leafy green vegetables (Heinen et al. 2007). Numerous studies evaluating the impact of phytochemicals are ongoing. They act through a wide-range and complex mechanisms and considerable advances have been made in evaluating their mode of action, however, the exact mechanisms by which these phytochemicals exert their anti-cancer
effects are not known well and are still being explored. One of the most identified cancer prevention mechanisms that most phytochemicals exhibit is by way of their antioxidant activity, elicited either by the induction of antioxidant enzymes namely superoxide dismutase (SOD), catalase and glutathione or through direct free radical absorption or by activation of Nrf2, which leads to the expression of genes responsible for production of antioxidant as well as detoxification enzymes (Reuland et al. 2013). Phytochemicals, belonging to the thiol group like sulforaphane, have been reported to inhibit the formation of electrophilic, DNA damaging chemicals (Johnson 2007).

Many studies have emphasized the antioxidant activities of phytochemicals. In one such study healthy cells were exposed to trichlocarban found in detergents which are known to mutate them into pre-malignant cells but the carcinogenesis was considerably reduced by the treatment with curcumin (Sood, Choudhary and Wang 2013). In another study a diet rich in kaempferol was given to volunteers and serum and urine were analysed, to check SOD activity and it was found to be higher with the increase in concentration of these polyphenols (Kim, Kim and Sung 2003). Indole-3-carbinol, a phytochemical isolated from cruciferous vegetables were fed to rats post exposing them to cigarette smoke and they were found to have lower lung cancer rate than placebo (Morse et al. 1990). Onions contain a compound quercetin, which when taken by the patients decreased their level of 8 hydroxydeoxy guanosine (8-OHdG), an oxidative metabolite which is a well-known marker of DNA damage and repair (Boyle et al. 2000). Quercetin have also been known to reduce the dysfunction of mitochondria induced by nitropropionic acid (Sandhir and Mehrotra 2013). Another important mechanism by which phytochemicals exerts their anticancer activity is by reducing inflammation. It has been known that prolonged inflammation is linked with oxidative stress and expression of transcription factors belonging to
NF-kappa B family which are involved in the initiation and progression of cancer. These factors regulate many genes involved in cell survival and oncogenesis.

Large number of phytochemicals like polyphenol present in green tea namely epigallocatechin-3-gallate (EGCG), curcumin, caffeic acid and caffeic acid phenethyl ester, quercetin and the phytochemicals within bilberries have been reported to inhibit NF-kappa B signaling (Carlsen et al. 2010). Apart from the above discussed mechanisms many laboratory studies have shown that phytochemicals can modulate many cellular and signalling pathway implicated in proliferation, invasion and metastasis. Ellagic acid, the main phytochemical found in pomegranate, inhibits cell growth and induces apoptosis in human prostate cancer cells (Thomas et al.). It has also been shown to increase the cell adhesion and migration markers resulting in inhibition of metastasis in breast cancer cell lines that are estrogen sensitive and resistant with no effect on normal cell lines (Malik et al. 2005). Ellagic acid was also found to inhibit a chemokine that is responsible for bone metastasis in breast cancer cells (Rocha et al. 2012). Curcumin, the main phytochemical extracted from turmeric exerts its effect by inhibiting cell proliferation, blocking the progression of cell cycle, increasing apoptosis and decreasing the invasion and migration of cells (ZHANG et al. 2007). Moreover, it has been reported to inhibit proliferation of breast cancer stem cells without any effect on normal cells. In colorectal cancer, curcumin has been found to increase the expression of tumour suppressor gene and modulate miRNA expression in breast cancer cells which in turn reduces the expression of Bcl-250. Epigallocatechin gallate (EGCG), found in green tea has demonstrated to inhibit DNA synthesis and angiogenesis etc resulting in inhibition of cancer cell proliferation. EGCG has been shown to inhibit the enzyme, ornithine decarboxylase that directs cells to divide more rapidly and evade apoptosis (Shanafelt et al. 2009). Resveratrol regulates gene expression by epigenetic modulation. It deacetylates FOXO transcription factor that leads to decrease in cell proliferation, survival and promotes apoptosis
in prostate cancer (Park and Pezzuto 2015). Caffeic acid and phenethyl ester inhibits NF-κB signaling and cell migration in vitro and inhibits metastasis of tumour models \textit{in vivo}. Luteolin, inhibits epithelial mesenchymal transition, invasion and migration (Thomas et al.). Yet other phytochemicals mainly phytoestrogenic compounds, mostly isoflavones and lignans seen abundantly in soy products and some cruciferous vegetables, exerts their anticancer activity by modulating hormone metabolism. These compound bind to the oestrogen receptor lightly and block the binding site of more harmful oestrogens, including those produced endogenously without causing the proliferation, lowering the mortality in women taking diet rich in phytoestrogens (Hecht et al. 2004). Similarly in men, these compounds modulate 5 alpha-reductase and thus reducing the endogenous levels of testosterone lowering the risk of prostate cancer.

Many natural compounds like curcumin, β-carotene, resveratrol, etc have shown promising effects in pre-clinical studies and are being investigated as prospective chemopreventive agents alone or in combination in head and neck cancer. There is increasingly convincing studies and evidences to prove that plant derived phytochemicals, provide benefits for high risk individuals as well as cancer patients. However, there are certain obstacles in the current chemoprevention trials like unavailability of exact models that mimic all the molecular alternations seen in HNSCC as well as that which consider all the risk factors involved leading to initiation and progression of cancer. However, ongoing research might overcome these limitations.

\textbf{2.6. Andrographolide (AG)}

\textbf{Source, Chemical Structures and Bioavailability studies}

\textit{Andrographis paniculata} (Acanthecea), also known as "humpedu bumi" in Malaysia, is well known for the treatment of many ailments like diabetes, rheumatism, hypertension, tonsillitis,
dysentery and diarrhea (Varma, Padh and Shrivastava 2011). The bitter taste is due to AG, a furano diterpene, that exerts most of the biological properties. Other active components include homo-andrographolide, andrographan, 14-deoxy-11,12-didehydro-andrograholide, andrographon, andrographosterin and stigmasterol. The active phytochemicals are usually extracted from the leaves and stems of the plant and the most important bioactive component of the plant is AG. X-Ray crystallographic studies have been used to elucidate the structure of the compound and the molecular stereochemistry, bond angles and bond distances were all analyzed. The compound is designated as (3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8- adimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro- 4-hydroxy-2(3H)-furanone), AG (Fig III) and it exhibits wide range of biological activities (Maiti et al. 2006).

![Fig III: Structure of AG](image)

Recently, several studies have reported its anti-cancer, anti-HIV and cardio-protective properties (Jarukamjorn and Nemoto 2008). The great benefits of phytochemical compounds reported by epidemiological and laboratory studies will prove beneficial only if the bioavailability of these compounds is optimum. Hence, there are many studies underway to determine the bioavailability of these compounds on clinical and preclinical models. In the pharmaco kinetic studies, many validated methods like HPLC, GC-MS etc were used to determine the concentration of AG in the blood plasma of humans and rats after the administration of plant extract and fixed combination
kang jang tablets. AG was found to be totally absorbed in the blood after the oral administration of extract at 20mg/kg in rats. But when the dose was increased 10 times the bioavailability was decreased almost four fold (Panossian et al. 2000). In this case 55% of the compounds were bound to plasma proteins and only remaining could enter the cells. Renal absorption is not the major route of eliminating the compound and its considered to be dose dependently metabolized. A single therapeutic dose of kang jang tablet equivalent to 20 mg of AG when administrated orally to humans a plasma concentration of 393ng/ml was reached after 1.5 to 2 hrs. The steady state of plasma concentration for multiple doses of Kang jang was 660ng/ml enough to show any anti PAF effect.

2.6.1. Clinical studies, safety and doses

Calabrese et.al., conducted a dose escalating study in 13 HIV positive and 5 non infected persons (Chao and Lin 2010). The objective of the study was to access the safety and tolerability, effects on plasma virion HIV1 RNA levels and CD4+ lymphocyte levels. It was found that AG inhibited the cell cycle deregulation induced by HIV and also raised the levels of CD4 + cells. In another study, Kalmegh tablets 500 mg was orally administrated along with other neutraceuticals to patients with advanced cancer of different parts. The concentration of total mercaptans and glutathione in plasma was measured and compared to that after 6 months. Blood parameters like CBC and biochemistry panels were checked routinely. After 6 months 16 patients out of the 20 patients studied survived and their NK function and TNF levels were found to be high. Total mercaptan levels and TNF alpha receptors were significantly reduced (See, Mason and Roshan 2002). AG also exhibited moderate to excellent anticancer activity against MCF-7 and HCT-116 cell lines in a study conducted at NCI on 9 different human cancer cell types (Desai et al. 2008). There were many studies conducted to check the safety and doses of the compound. In one such
study conducted by Guo et al., mice were fed for a period of 10 days on 500mg/kg of Kalmegh and found that there was no change in weight, appetite or production of stool. The blood counts were normal and animals were healthy. When rabbits were given intravenous administration of AG 10mg/kg, no abnormal cardio vascular responses or any abnormalities were seen in any of the organs analyzed and liver tests were also normal. Mice that were administrated a dose of 10g/kg once a day for a week was found to be safe and healthy without any complications. In other toxicity studies rats or rabbits were given 1g/kg of body weight and no adverse effect was found in animals or their kidney, liver or other organs. Singha et al., reported that pretreatment with AG at concentration of 500 mg/kg and arabinogalactan proteins at 125mg/kg of body weight would significantly reduce the toxicity in comparison to ethanol treated groups as confirmed by the liver and kidney test assays and these results were consistent with that reported for sylimarin (Singha, Roy and Dey 2007). This proved that these compounds offers protection against ethanol induced toxicity. AG was also tested against chemical induced liver toxicity in mice. The chemicals destroyed the cell membranes and cells surrounding liver. When the mice were pretreated with AG for 3 days before inducing toxicity with chemicals they provided a protective effect to the liver which was due to the antioxidant ability of the compound comparable to that reported for syilimarin, a very potent antioxidant from milk thistle.

2.6.2. Anti-cancer potential of AG.

The anti-cancer agents in current use work by preventing the growth of cancer cells, causing apoptosis or necrosis, inducing cell-cycle arrest or cell differentiation. Yet others act by modulating immune activity, by activating body’s own immune system to fight against cancer cells. The compounds that act on multiple targets are of greater demand as they are more likely to inhibit a variety of cancers in different conditions. In this regard, AG can be considered as a
strong anticancer pharmacophore candidate as it has the potential to act both directly and indirectly on the cancer cells (Varma et al. 2011).

**Fig IV: Summary of the anticancer potential of AG on various cancer cell lines and the mechanism of action**
2.6.3. Current and Future Prospects

There are numerous ongoing studies exploring different aspects of anti-cancer potential of AG worldwide. But most of them are concentrating on the cellular toxicity aspects and no much data are available on the mechanistic studies. There are no clinical data available on the anti-cancer activity of the compound. Bioavailability of a drug is the significant parameter that decides its therapeutic efficacy. Hence, more studies are needed on the pharmacokinetic characteristics of the compound to enhance the availability at the target. Recently Zhou et al., has reported an inclusion technique in his studies that claims to increase the bioavailability of the compound as well as prevent its hydrolysis in neutral and alkaline pH of gastrointestinal tract (Zhou et al. 2009). However, it has already been reported that AG gets easily absorbed into the blood plasma reaching peak concentration within 1-2 h of oral administration and P-glycoprotein is known to play important role in the absorption of AG in the intestine (Panossian et al. 2000). The metabolic fate of the compound is reported to involve a sulphonate at C-12 and the metabolic by products after oral administration of AG are sulphate compounds and sulphonic acid adducts. To establish AG as an anti-cancer agent there is the need of more mechanistic studies based upon the information provided by the preliminary studies in various cancer cell lines. Out of the different cell lines studied one study found that AG was most effective against colon cancer cell lines (Varma et al. 2011). There are some recent findings on the effect of AG on mechanism of cell death in colon cancer cell lines by Baneerje et al., However, no studies are yet available on head and neck cancer cell line with the drug. So it would be worthy to conduct studies on head and neck cancer using AG in HNSCC cell lines and elucidate the definite mechanism of action of this compound, which will lead to the development of cost effective less toxic therapeutic drug.