Literature Review

Cancer- an overview

Cancer is a cellular disease in which the growth of a cell occurs in an uncontrolled manner (Lewin, 2004; Hanahan and Weinberg, 2000). Cancer has now become a big threat to human population and a major cause of human mortality. Among all deaths 13% occurs due to this disease in the modern world (Alberg et al., 2005). The precise number of cancer cases diagnosed each year is being increased day by day. Every year, at least 200,000 people die worldwide from cancer related to their workplace. It is estimated that approximately 20,000 cancer deaths and 40,000 new cases of cancer each year in the U.S. are attributable to occupation (http://www.who.int/mediacentre/news/notes/2007/np19/en/).

As per population census data, the rate of mortality due to cancer in India is also alarming with data showing about 8,06,000 cases by the end of the last century. Projection estimates from the WHO (2007) has shown that by the year 2030, cancer will account for about 12% of death in India. Cancer is the second most common disease in India responsible for maximum mortality with about 0.3 million deaths per year. All types of cancers including the cancers of skin, lungs, breast, rectum, stomach, prostate, liver, cervix, esophagus, bladder, blood, mouth etc. have been recognized in India. All of them posed a major threat to human lives. The causes of such high rates of cancers may be both internal, like-genetically or hormonal and external like- food habits, industrialization, over growth of population etc (Ali et al., 2011). The following bar diagram (Figure 1) gives an overview of the expected cancer prevalence in India.
Figure 1- Year wise total cancer prevalence in India (http://www.icmr.nic.in/ncrp/report_pop_2001-04/cancer_p_based.htm).

Lung cancer

Among all types of cancer, lung cancer remains to be very high in occurrence, being about 1.4 million cases per year worldwide (Jemal et al., 2012). Lung cancer includes uncontrolled proliferation and growth of abnormal cells in both the lungs, including trachea and bronchial portion. Sometimes, lung cancer cells may be metastatic. In that case abnormal cells of lung enter through lymphatic or blood circulatory system and form a secondary tumor in different other parts of the body like- breast, liver etc. According to Khuri et al. (2000) lung cancer, the single most devastating cause of cancer related deaths shows great variation in the prevalence at different geographical areas. However, lung cancer remains the leading cause of cancer-related deaths mainly in the United States, Western countries, North America and Japan. Globally since 2002, 0.85 million males and 0.33 million females have suffered from lung cancer (Ferlay et al., 2010). Earlier, Nandakumar (2001) reported that the worldwide incidence of this cancer is 14% whereas it constitutes 6.8% of all cancers in India. It is the most frequent tumor in males, and second or third most common in females In India; almost 58,000 male and 12,500 female deaths occurred since 2004 (Ferlay et al., 2010). In India, compilation of data of 24 years (1982-2005) on lung cancer has revealed that while new cases of lung cancer per one lakh male population has increased by around 160% in Chennai, 100% in Bangalore and 40% in Delhi during this period, such cases have fallen by 60% in Mumbai.
Basic causes of lung cancer can be either internal factors like inherited mutations, hormones, immune condition or environmental factors such as tobacco, diet, radiation and other infectious agents (Belpomme et al., 2007; Hammond and Horn, 1958; Roa et al., 1994; Willett, 2000) or both. Using modern molecular biology tools like microarray and rapid sequencing techniques remarkable progress into elucidation of the lung cancer pathogenesis has been achieved. The main attributes have been deciphered that help in progression of lung cancer. Some of these are-

- Growth stimulation by activating major oncogenes like protein tyrosine kinase (PTKs), epidermal growth factor (EGF), human epidermal growth factor receptor 2/neu (HER-2/neu), ras family genes (H-ras, K-ras) etc (Fong et al., 2003 and Rom et al., 2000).
- Insensitivity to anti-growth signals by down-regulating major tumor suppressor genes like p53, PTEN, TGFβ etc. (Forgacs et al., 2001).
- Evasion of apoptosis process by altering the responses of important regulators of apoptosis like BCL2 family proteins, p53 etc (Schulze-Bergkamen and Krammer, 2004).
- Limitless replicative potential by altering telomerase activity (Shay et al., 2001).
- Promoting tumor angiogenesis process (Fontanini et al., 1997), invasion (Shih et al., 2001), immune suppression (Neuner et al., 2001) and metastatic activity (Bonomi, 2002).

The main molecular abnormalities occurring during lung cancer pathogenesis has been summarized in the following figure 2:
Based on histo-pathology lung cancer is categorized into 2 major types, SCLC and NSCLC. Sometimes mixed cell lung carcinoma also occurs showing both the characteristics of SCLC and NSCLC. Several chemotherapeutic approaches are effective in combating SCLC, but therapeutic scope for NSCLC is limited and generally treated only with surgery (Fong et al., 2003; Neel et al., 2013). But, surgery sometimes develops a secondary tumor formation through metastasis. For this therapeutic limitation, development of a noble agent in combating particularly NSCLC is highly sought after.

**Non-small cell lung cancer (NSCLC) - a brief overview**

NSCLC, major types of lung cancer bears almost 85% of major lung cancer types. Only 17.3% of the people who develop NSCLC survive for 5 years. NSCLC is an aggressive neoplasm, responsible for more lung cancer deaths each year in the United States than colon, breast, pancreas, and prostate cancers combined (Ferlay et al., 2010). NSCLC is
found to occur in a great number of people in India (Noronha et al., 2012). In India, squamous cell carcinoma (44.73%) and adenocarcinoma (30.26%) are the most prevalent types of NSCLC as reported by Kenfield et al. (2008). The main causes of NSCLC are internal like- mutation, immune suppression and as well as external like- passive smoking, air pollution etc (http://ummm.edu/health/medical/reports/articles/nonsmall-cell-lung-cancer). There are three main types of NSCLC which are named for the type of cells in which the cancer develops. The major types of NSCLC are- squamous cell carcinoma (nearly 25%-30%), adenocarcinoma (nearly 40%) and large cell carcinoma (nearly 10%-15%) (Travis et al., 1995).

**Present treatment status of NSCLC**

SCLC is often being treated by drug molecules whereas NSCLC still bears a very narrow range of drug or chemosensitivity. Treatment choices for NSCLC patients depend on the stage at which the cancer is diagnosed. Patients diagnosed with stage I NSCLC usually receive surgical resection. Patients with stage IA (T1N0M0) undergo resection and are rarely treated with adjuvant chemotherapy. Patients with resected stage IB–III (any T any N M0 except T1N0M0) NSCLC show improved survival when given adjuvant chemo-therapy (El-Sherif et al., 2005). However, surgery sometimes develops a secondary tumor by triggering metastasis. According to reports, the major chemosynthetic drugs those are being used and under clinical trials for treatment of NSCLC are as listed below (Table 1) -

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<th>Drugs Approved for NSCLC</th>
<th>Drugs Approved for combinational therapy for NSCLC</th>
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<td>o Abitrexate (Methotrexate)</td>
<td>o CARBOPLATIN-TAXOL</td>
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<td>o Abraxane (Paclitaxel Albumin-stabilized Nanoparticle Formulation)</td>
<td>o GEMCITABINE-CISPLATIN</td>
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<td>o Afatinib Dimaleate</td>
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<td>o Alimta (Pemetrexed Disodium)</td>
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<td>o Avastin (Bevacizumab)</td>
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<td>o Carboplatin</td>
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<td>o Erlotinib Hydrochloride</td>
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<td>o Folex (Methotrexate)</td>
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Table 1- Major synthetic drugs used either alone or in combination for treatment of NSCLC.

(http://www.cancer.gov/cancertopics/druginfo/lungcancer)

Most of these anti-cancer drugs are aimed to trigger apoptosis process to kill NSCLC cells, as the cancer cells are otherwise immortal and can evade the process or signals of apoptosis. Apoptosis is a process of programed cell death (Elmore, 2007; Schulze-Bergkamen and Krammer, 2004; Fesik, 2005) comprising the following two major types-

Extrinsic pathway mediated- The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily. The sequences of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models (Locksley et al., 2001).

Intrinsic pathway mediated- The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-mediated events. The stimuli that initiate the intrinsic pathway produce intracellular signals (Elmore, 2007).

Beside this, there is also another pathway of apoptosis which is of perforin/granzyme mediated (Elmore, 2007). Collectively both the extrinsic and intrinsic pathways end at the
point of the execution phase which is considered as the final pathway of apoptosis. Execution pathway involves activation of execution caspase (caspase3, 9 or 7), cytoplasmic endonuclease that degrades nuclear material/ and cytoskeletal proteins (Slee et al., 2000). However, the major obstacles of using the synthetic drugs prepared to trigger apoptosis are the following:

- **Genetic mutation of NSCLC** - Development of oncogenic mutations, especially in EGFR, K-ras and ALK in NSCLC cells make them more evasive and proliferative, defying all attempts of preventive measures. Among them K-ras mutations are more frequent in non-small cell adenocarcinomas (Riely et al., 2009).

- **Lack of chemopreventive and target based efficacy of anti-NSCLC drugs** - The chemosynthetic drugs kill cancer cells and also elevate enough cytotoxicity in normal cells. In recent times, drugs like- bevacizumab (target blood vessel growth), erlotinib, cetuximab, afatinib (target EGFR), crizotinib (target the ALK gene) have been designed to modulate particular gene/receptor that would furthermore block uncontrolled cell proliferation and trigger apoptosis. But also the target based therapy has its limitation as most of these drugs bear severe common side-effects, namely, nausea, vomiting, diarrhea, constipation, swelling, fatigue, and eye problems. Some side effects can be severe, such as low white blood cell counts, lung inflammation (http://www.cancer.org/cancer/lungcancer-non-smallcell/detailedguide/non-small-cell-lung-cancer-treating-targeted-therapies).

**Complementary and alternative medicines (CAM) as anti-NSCLC agent**

To avoid the above mentioned problems of using chemosynthetic drugs, people now are more inclined to complementary and alternative medicine (CAM), which shows preferential activity to raise apoptosis process only onto their target cells sparing the normal ones. The high cost, side effects and therapeutic limitations of conventional medicines are the key factors that drive people more towards the use of CAM which includes- homeopathy, herbal extracts, acupuncture etc. People can easily obtain plant extracts or botanical supplements on an over-the-counter basis. These drugs are also effective in raising apoptotic efficacy avoiding the present mutation status of the cells which is highly sought after. Collectively, CAM includes such kind of drugs which show target specific anti-cancer efficacy leaving the normal counterparts and avoiding the present genetic mutations (Go et al., 2001; Ernst, 2001a, 2001b).
Among CAM, homeopathy plays a pivotal role. Homeopathy, a holistic method of treatment, in which either plant extract (as mother tincture) or ultra-low doses of highly diluted remedies (potentized forms) are prepared to treat several diseases (Boericke, 2004; Khuda-Bukhsh, 1997, 2006) including cancer (Boericke, 2004; Biswas et al., 2005; Banerjee et al., 2010; Preethi et al., 2012).

Plant extracts obtained as homeopathic mother tinctures are major sources of natural substances like flavonoids, terpenoids, alkaloids etc. All these components individually or collectively show profound efficacy in treating cancer. Therefore, plant extracts have been considered as potent agents for anti-cancer drug formulation. Several reports are already available to show the efficacy of certain plant extracts in treating NSCLC either alone or in combination with any chemosynthetic drugs (Park and Pezzuto, 2002).

In homeopathy, potentized forms are often made by serial dilutions with fixed number of agitations of the plant extracts at each step of dilution (Khuda-Bukhsh, 1997, 2003, 2006). Drugs thus dynamized by serial dilutions and repeated agitations, are generally used more in chronic diseases than in acute cases, although there are some cases of acute diseases, where some practitioners get benefits from the diluted preparations as well (Boericke, 2004). However, when the drug attains the potency 12C, it becomes diluted to $10^{-24}$, which is beyond the Avogadro’s limit. Such dilution beyond Avogadro’s limit raises the controversy about the lack of physical existence of any drug molecule, and therefore, its efficacy is questioned. However, several studies by Kasab (2009) and Khuda-Bukhsh (1997, 2006) have shown efficacy of such highly potentized homeopathic remedies in biological system. This potentized drug has been claimed to be effective in treating several diseases including cancer in the literature (Boericke, 2004; Gaskin, 2005). Plant extracts are rich source of several bioactive components like flavonoids, alkaloids, terpenoids, isothiocynates, phenols etc. as shown in the following table 2.
Several studies have been made to check the efficacy of those active ingredients after being isolated from their respective plant extracts. Various studies indicated the efficacy of several isolated components from plant extract against NSCLC, both in vitro and in vivo either alone or in a combination (Nobili et al., 2009; Graham et al., 2000; Saha and Khuda-Bukhsh, 2013; Park et al., 2012). Especially the isolated compounds from cruciferous vegetables (Zhao et al., 2001), grapes (Singh et al., 2011), broccolis (Spitz et al., 2000), tea (Arts, 2008) have been found to be effective in treating NSCLC. Among the all active components, flavonoids or more specifically flavonols plays a major active role in treating NSCLC (Shapiro et al., 1999; Ren et al., 2003). Earlier studies by Bai et al. (1998) and Caltagirone et al. (1997) showed several flavonoids as potent anti-lung cancer agents.

Quercetin belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources (Cody, 1986; Quercetin (Monograph), 1998; Formica and Regelson, 1995). Earlier reports by several workers evaluated quercetin as a potent anti-inflammatory (Comaladela et al., 2005) and antioxidant activities (Kahraman et al., 2002; Lamson et al., 2000; Robaszkiewicz et al., 2007) agents. Few reports suggested quercetin as a pro-apoptotic agent against breast cancer (Avila et al., 1994), gastric cancer (Yoshida et al., 1990), colorectal cancer (Ranelletti et al., 1999), colon cancer (Koishi et al., 1992) etc. Very few studies have done till date about the effect of quercetin on NSCLC.
However, few workers have reported quercetin as an effective agent in raising apoptosis in a NSCLC cell line, *in vitro*. (Kanadaswami et al., 2005; Youn et al., 2013).

In recent years, nanotechnology has been playing a major role in the development of anti-cancer drug formulation (Kawasaki et al., 2005; Kim et al., 2007; Sahoo et al., 2007). Nanotechnology is being used to improve the bio/cellular availability of drugs either by encapsulating them in bio-degradable nanoparticles or by using the drug molecules reducing some metal elements into nano form (Misra et al., 2010). Plant extracts or their bioactive components are often being delivered through a nanoencapsulated form into the cells. Nanoencapsulated drugs are released into the cell more precisely so that the same drug molecule can show its function at much lower concentrations (Ravi Kumar, 2000; Mu et al., 2003). Encapsulating the preferred drug molecules in bio-degradable nanoparticles like PLGA, PLA etc (Shive and Anderson, 1997) have earlier been reported to show better efficacy in killing cancer cells also the NSCLC as per reported by (White et al., 2006; Wang et al., 2013; Zaki, 2013).

*Thuja occidentalis*, (Figure 3) one of the major plants of family- Cupressaceae, largely produced in part of north-east of the United States, south-east of Canada (British Herbal Pharmacopoeia, 1983.) and has been known for its medicinal value (Sunila et al., 2005; Dubey et al., 2008, 2009a; Wharf, 1999).

![Figure 3- Thuja occidentalis (family- Cupressaceae)

In homeopathy, ethanolic leaf extract of this plant is used as mother tincture for treating several diseases (Boericke, 2004) including cancer (Sunila et al., 2006, 2011). Earlier, Sunila et al. (2006) reported that Thuja extract can act as major anti-metastatic agent against
lung cancer. Potentized forms of Thuja have also been reported to be effective in treating several diseases (Frenkel et al., 2010; Banerjee and Banerjee, 2012). Pharmacological studies revealed that Thuja extract is rich source of essential oils (1.4%-4%), flavonoids, coumarins, terpenoids and tannin like components. The essential oil of the fresh leaves (related to the monoterpenic fraction) contains 65% thujone, 8% isothujone, 8% fenchone, 5% sabines and 2% α-pinen as the main monoterpenes. Other monoterpenes, namely, carvotanacetone, origanol, origanes, myrcen and camphen, have been described (Tsiri et al., 2009; Naser et al., 2005; Dubey et al., 2009b; Neth et al., 1995). Recently Chang et al. (2000) has found further bioactive constituents in Thuja extract. Many of these components have been reported to have anti-cancer property individually. Thujone, one of the major components of Thuja leaf extract have been reported earlier to have anti-cancer property against skin melanoma cells in vitro (Biswas et al., 2011).

Activity of *Thuja occidentalis* extract, its potentized/diluted forms and its bioactive components against NSCLC is relatively an unexplored area of research. Very few studies have been done till date in showing the efficacy of this plant as a whole, particularly in treating NSCLC. Therefore, the present study was mainly focused on exploring the effects of *Thuja occidentalis*, its homeopathically potentized diluted forms and bioactive components in treating NSCLC. Beside this, the efficacy of highly potentized from of Thuja i.e. Thuja 30C was analyzed in ameliorating the BaP (major carcinogen that induces NSCLC)-induced normal mice lung cell cytotoxicity. Furthermore, the effect of quercetin, one of the major components of Thuja leaf extract was also evaluated against NSCLC. To increase bio/cellular availability, quercetin was also encapsulated in PLGA-nanoparticles and thereafter its efficacy was analyzed against NSCLC cell lines. The overall aim of this study, was, therefore to explore and verify the reported anti-cancer potential of Thuja extract (mother tincture), and to evaluate the anti-cancer effects of isolated flavonols-rich fraction against NSCLC. Further the diluted (dynamized/potentized) forms of Thuja; quercetin, one of the major components of Thuja leaf extract and its PLGA nano-encapsulated form; were also tested for their possible anti-cancer potential. The overall aim was to help in the development of an effective drug formulation against NSCLC from critical analysis of results obtained in the present study, if possible.