Introduction

Cancer is a cellular disease in which cells display uncontrolled growth, invasion and sometimes metastasis (Lewin, 2004). It is the most fatal disease worldwide which accounts for 7.6 million deaths (around 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030 (Ferlay et al., 2010).

Lung cancer is one of the most common and leading causes of cancer death with estimated 1.3 million cases per year worldwide (Jemal et al., 2012). It is the most frequent cancer among men and fourth most frequent cancer in women. Overall, the chance that a man will develop lung cancer in his lifetime is about 1 in 13 and for a woman, the risk is about 1 in 16. Over 85% of patients harboring lung cancer eventually die during the first year (Ferlay et al., 2010). This extreme mortality has not changed significantly during the last three decades.

Lung cancer is the uncontrolled growth of abnormal cells in one or both lungs including trachea and bronchus. Besides interfering with the lung functions, cancer cells can spread from the tumor into the bloodstream or lymphatic system from where they can spread to other organs. The main causes of lung cancer include carcinogens (such as those in tobacco smoke, industrial waste), ionizing radiation and viral infection etc. Common symptoms of lung cancer are shortness of breath, coughing (including coughing up blood) and weight loss (http://www.medicinenet.com/lung_cancer/article.htm).

Based on the histo-pathological classification, lung cancer is mainly differentiated into two categories such as, small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Sometimes lung cancer may bear characteristics of both the types; this is known as mixed small cell/large cell carcinoma. SCLC, also called “oat cell cancer”, accounts for 14% of all cases of lung cancer. This type of lung cancer often starts in the bronchi near the centre of the chest, grows more quickly and is more likely to spread to other organs of the body through lymph nodes. SCLC is mainly attributable to smoking. Only 6.2% of the people who develop SCLC survive for 5 years. Whereas, only 17.3% of the people develop NSCLC survive for 5 years. NSCLC accounts for 85% of all lung cancer cases (Ferlay et al., 2010).
There are three main types of NSCLC, which are named for the type of cells in which the cancer develops:

- Squamous cell carcinoma- About 25%-30% of all lung cancers are of this type. Generally this type of cancer is linked to smoking and tends to be found in the middle of the lung.
- Adenocarcinoma- This type accounts for about 40% of lung cancers which is found in the outer part of the lung.
- Large cell carcinoma- About 10%-15% of lung cancers are of this type, which can start growing in any part of the lung and spread quickly.

Although several chemotherapeutic approaches are fruitful against SCLC, but therapeutic scope for NSCLC is limited as it bears a very narrow range of chemo- or drug-sensitivity. SCLC usually responds better to chemotherapy and radiation while NSCLC is generally treated with surgery (Fong et al., 2003; Neel et al., 2013). But, surgery sometimes develops a secondary tumor formation through triggering metastasis. For this, target based therapy has been developed to combat NSCLC. Synthetic drugs like- gefitinib, erlotinib, cisplatin etc. have been used against NSCLC (Eberhard et al., 2005; Tracy et al., 2004; Sikdar and Khuda-Bukhsh, 2013a).

Most anti-cancer drugs are aimed to inhibit cancer cell progression and survivability by triggering apoptosis signaling. Since cancer cells are known to attain immortalization, induction of apoptosis in them by any drug is considered to be highly solicited in the field of anti-cancer drug formulation research. Apoptosis, a form of programmed cell death is primarily of two types- a) extrinsic pathway mediated and b) intrinsic pathway mediated (Elmore, 2007). However, the major obstacles of using the synthetic drugs which are prepared to trigger apoptosis are-

- Chemosynthetic drugs are not very much target specific and thus raise normal cell cytotoxicity.
- Development of oncogenic mutations, especially in epidermal growth factor receptor (EGFR), Kirstein rat sarcoma (K-ras) viral oncogene homologue, anaplastic lymphoma kinase (ALK) etc in NSCLC cells make them more adaptive and fit to grow in all adverse conditions, defying all attempts of preventive measures. Among them K-ras mutations are more frequent in non-small cell adenocarcinomas (Riely et al., 2009).
Due to subsequent mutation, NSCLC cells are tend to be fit against those synthetic drugs which otherwise target one particular receptor/molecule and not the major ones, and also raise normal cell cytotoxicity.

Therefore, people are more inclined to develop such kind of drugs which exert preferential motion to activate apoptosis process to kill cancer cells sparing the normal ones, and also can work effectively despite being opposed by the specific mutation that prevents anti-cancer drugs to act. Complementary and alternative medicines (CAM) are such kind of drugs which exhibit target specific anti-cancer efficacy sparing the normal cells and thus is highly sought after (Ernst, 2001a, 2001b). Among CAM, homeopathy is a holistic method of treatment in which either plant extract (as mother tincture) or ultra-low doses of ultra-high diluted remedies (potentized forms) are prepared to ameliorate and cure several diseases (Boericke, 2004; Khuda-Bukhsh, 1997, 2003, 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. Therefore bioactive ingredients isolated from such extracts are often used as complementary and alternative medicines (Nobili et al., 2009; Graham et al., 2000; Saha and Khuda-Bukhsh, 2013). In recent years, nanotechnology has been playing a marked role in the development of anti-cancer drug formulation (Kawasaki et al., 2005; Kim et al., 2007; Sahoo et al., 2007). To increase the bio/cellular availability, plant extracts or their bioactive components are often being delivered through a nanoencapsulated form into the target cells. Nanoencapsulated drugs are released into the cell in a more precise way so that the same drug molecule can serve its function at much lower concentrations (Ravi Kumar, 2000; Mu and Feng, 2003). Few plant extracts, their bioactive components and homeopathically potentized forms are recently being reported to have anti-NSCLC effect (Ancuceanu, 2004; Feng et al., 2010; Elkady, 2013; Lu et al., 2013; Singh et al., 2011; Sikdar and Khuda-Bukhsh, 2013b; Sikdar et al., 2013c). But, so far as the author is aware, details of their mechanistic study on any noble plant extract as an anti-NSCLC agent has not been reported so far.

We have tried in this study to fill up this lacuna and evaluate the potentials of a plant leaf extract, *Thuja occidentalis* (obtained in the form of homeopathic mother tincture from Boiron Laboratory, France), its homeopathically ultra-high diluted forms and active components in combating NSCLC. Furthermore to increase the bio-availability we have
also nanoencapsulated one of the major components of that plant extract and evaluated its anti-NSCLC property, if any. *Thuja occidentalis*, one of the major plants largely produced in part of north-east of the United States and south-east of Canada, has been known for its medicinal value (Sunila et al., 2005; Dubey et al., 2008, 2009; Wharf, 1999; Naser et al., 2005; Biswas et al., 2011). In homeopathy, ethanolic leaf extract of this plant is used as a mother tincture for treating several diseases including cancer (Sunila et al., 2006, 2011; Boericke, 2004). But activity of *Thuja occidentalis* extract, its potentized/diluted forms and its bioactive components against NSCLC is a relatively unexplored area of research. Therefore, this study mainly focuses to explore the effects of *Thuja occidentalis*, its homeopathically potentized diluted forms and bioactive components against NSCLC. Furthermore to improve cellular availability and cell specific sustained release, nano-encapsulation of one of the major active components of Thuja extract has been attempted and its anti-NSCLC efficacy was explored so that the scientifically validated information may help in the development of an effective drug formulation against NSCLC.

In the course of the present work, effects of ethanolic leaf extract of *Thuja occidentalis* was tested against a K-ras mutated NSCLC cell line A549. Thereafter, cytotoxic efficacy of homeopathically potentized forms of Thuja such as Thuja 5C, 9C and 30C were tested against A549 cell line, *in vitro*. To evaluate the efficacy of ultra-high diluted forms of Thuja i.e. Thuja 30C, it was tested for its possible efficacy in amelioration of Benzo(a)pyrene (BaP) toxicity in normal mice perfused lung cells. Later a fraction, predominantly rich in flavonols, has been isolated from *Thuja occidentalis* leaf extract and its efficacy to induce apoptosis in NSCLC cell line (A549) *in vitro* was examined. Furthermore, the effect of this isolated fraction was also evaluated in inhibiting BaP induced mice lung carcinogenesis, *in vivo*. Quercetin was one of the major flavonols present in this isolated fraction of Thuja, but it was difficult to isolate pure quercetin molecule from the flavonols fraction mixture containing both quercetin and kaempherol, because of the molecular and structural similarity of both these ingredients. For this reason, to test if quercetin has any apoptotic efficacy, we had to procure commercially available pure quercetin from Himedia, India. We then examined its possible anti-cancer efficacy on NSCLC cell line, *in vitro*. However, quercetin, a hydrophobic flavonoid is poorly soluble in water; therefore, it needed a suitable approach to make its bio-availability increased. With a view to increase its bioavailability, we encapsulated quercetin by PLGA nanoparticles. The PLGA loaded quercetin was then
tested against two NSCLC cell lines- A549 and H460 to see if it could increase the apoptosis process at a very lower concentration.

With this background scenario, the main objectives of this study are:

- **Evaluation of anti-cancer potential of ethanolic leaf extract of *Thuja occidentalis* and its potentized forms against NSCLC *in vitro*.
- **Isolation of flavonols-rich fraction from ethanolic leaf extract of *Thuja occidentalis* and evaluation of its anti-cancer potential against NSCLC both by *in vitro* and *in vivo* studies.
- **Evaluation of anti-cancer potential of quercetin, a major component of flavonols-rich fraction of *Thuja occidentalis* and its PLGA nano-encapsulated form against NSCLC *in vitro*.

The total work has been divided into 3 chapters. All these chapters have again been subdivided into several parts to deal with different aspects of the study in a systematic way.

**Chapter 1. Evaluation of anti-cancer potential of ethanolic leaf extract of *Thuja occidentalis* and its potentized forms against NSCLC *in vitro***

A. Evaluation of anti-cancer and anti-proliferative potentials of ethanolic leaf extract of *Thuja occidentalis* (TO) against NSCLC cell line, A549.

B. Cytotoxic efficacy of potentized forms of *Thuja occidentalis* against A549 cells.

C. Amelioration of Benzo(a)pyrene (BaP) induced normal mice lung cell toxicity by ultra-high dilution of Thuja (Thuja 30C).

**Chapter 2. Isolation of flavonols-rich fraction from ethanolic leaf extract of *Thuja occidentalis* and evaluation of its anti-cancer potential against NSCLC both by *in vitro* and *in vivo* studies***

A. Apoptotic potential of flavonols-rich fraction (FRF), isolated from ethanolic leaf extract of *Thuja occidentalis* against NSCLC cell line, A549.

B. Apoptotic and anti-proliferative potentials of flavonols-rich fraction (FRF), isolated from ethanolic leaf extract of *Thuja occidentalis* against Benzo(a)pyrene induced mice lung carcinogenesis.
Chapter 3. Evaluation of anti-cancer potential of quercetin, a major component of flavonols-rich fraction of *Thuja occidentalis* and its PLGA nano-encapsulated form against NSCLC *in vitro*

A. Apoptotic potential of quercetin against NSCLC cell line, A549.

B. Apoptotic and anti-proliferative potentials of nano-PLGA-encapsulated quercetin against NSCLC cells, A549 and H460.