CHAPTER 1

INTRODUCTION
In present time, cancer is a leading cause of death in human beings that continually rises throughout the world. Cancer mainly arises from normal cells that acquired aberrantly proliferation capacity and ultimately turn into malignant. Cancerous cells then grow clonally through the process of mitosis into tumors and have the potential to metastasize. Normal cells transformed into cancerous cells through various process of mutation in genomic DNA such as replication errors, central chemical instability of certain DNA bases, or from attack by free radicals generated during metabolism, carcinogens and various radiations like ionizing and U.V. (Cooper, 2000). Continuously expanding the growth pattern of numbers of cancers in both developing and developed countries is the global threat that challenges prevention and treatment aspect to reduce cancer. It is reported that the survival rate of cancer patients in India is quite low in comparison with other countries like, in case of stomach cancer, the survival rate is just 19% in comparison to Korea that have much higher rate i.e. 58% and 25-30% in other developed countries. Liver cancer condition in Indian peoples is more serious with very low survival rate, i.e. 4% in comparison to others (10-20%) (Verma, 2014).

Various chemical agents or xenobiotics play an important role to induce cancers in which nitrosamines, a group of potent human carcinogens. Nitrosamines are broadly grouped into volatile and tobacco specific compounds like nicotine, nornicotine, anabasine, anatabine derived nitrosamines (Godden, 1983). Formation of nitrosamines occurred in both exogenously through various nitrosating agents and endogenously through either pyrrolidine or nicotine (Tricker et al., 1994; Hecht et al., 1999). Most of the intermediates that are formed during nitrosamine metabolism have capability to either directly tie up with DNA or react with others to form various alkylating agents that cause mutation or cancer (Hecht, 1998). N-Nitroso compounds (NNCs) like N- nitrosamines are found in drinking water, food stuffs, rubber products and tobacco products (Cárdenes et al., 2002). Nitrosamines occurrence are also a foremost concern in the emerging field of industries. Occupational safety and Health administration (OSHA) reported the occurrence of various amines i.e. nitrosating agents, in rubber, cork, linoleum and plastics products that take participate in the formation of numbers of toxic and carcinogenic nitrosamines. These amines become nitrosated with NO (nitric oxide) that are present in air via mixing, milling, extrusion, molding and curing processes. The level of NDMA, NDEA and other nitrosamines in various rubber and tire industries is more or less than 50 μg nitrosamine/day like in rocket fuel production, NDMA uptake is >50 μg/day whereas
in soap and surfactants industries, it is <5 µg/day (N-Nitrosamines, MAK value documentation, 2012). Due to the mixing of industrial waste products into rivers, presently ground water also get crippled with nitrosamines contamination which is a somber problem that needs attention of people to reduce the risk of nitrosamine toxicity and cancer.

Hepatocellular carcinoma (HCC) or liver cancer is the third most frequent death causing cancer in male and seventh in women (El-Serag, 2011), although, it is more prevalent in Asia and Africa. The pervasiveness of liver cancer is continuously increasing in India due to disturbed life style, increasing rate of hepatitis B and C, large scale consumption of alcohol and smoked products like biddi, hukka, cigarette, tobacco products like pan-masala, gutka, anabolic steroids, arsenic, type 2 diabetes etc. (www.cancer.org). This is because of metabolizing role of liver. HCC, a major threat worldwide, is attributed to immune responses that induce inflammation and apoptosis, lead the accumulation of various genetic and epigenetic obliterations. High mortality rate due to HCC i.e. 7 lakhs deaths every year makes it a threat full disease (Eortc, 2012). In India, incident rate for HCC is 0.7 to 7.5 whereas less for women i.e. 0.2 to 2.2/1 lakh population/year (Acharya, 2014).

*N- Nitrosopyrrolidine (NPYR)* is a potent carcinogenic nitrosamine that induces predominantly liver tumors and also lung tumors (Wong *et al.*, 2003; Langenbach, 2013). It is mainly found in sidestream of cigarette smoke, fried bacon and other cured meats (IARC, 2004; Kerry and Kerry, 2011) and also in drinking water (Charrois *et al.*, 2004). Exogenous exposure range of NPYR to human is 0.01-0.15 µg/day. Mean dietary exposure of NPYR and other nitrosamines is 0.20-0.31 µg/day whereas secondary amines or nitrosating agents exposure range from 7.4 mg/day (Tricker *et al.*, 1994) which are found endogenously in blood, saliva, gastric juice, urine and feces. NPYR exerts its carcinogenic activity through metabolic activation by CYP450 enzymes in liver (Morales *et al.*, 2010) and form various intermediate products that served as alkylating agents like 4-oxobutanediazohydroxide, 2-hydroxytetrahydrofuran (2-OH-THF) and crotonaldehyde. These agents are responsible for causing various DNA adducts of dThd, dAdo, dGuo in liver (Wang *et al.*, 1988; Wang *et al.*, 2007; Sharma and Singh, 2014). These adducts formation directly leads mutations in DNA and cause hepatic cancer.
Nitrosamines especially NPYR have capability to form free radical or reactive oxygen species (ROS) by liver microsomes (Floyd et al., 1978). Excessive free radicals induced oxidative damage have major role in development of HCC that advances the proliferation of cancerous cells which are reported by several investigations (Poljsak et al., 2011; Kabel, 2014; Valluru et al., 2014; Poillet-Perez et al., 2015). During various metabolic processes, numbers of reactive species of O₂ and their derivatives are produced as the byproduct in which some contain unpaired valence shell electrons like nitric oxide (NO), nitrogen dioxide (NO₂), peroxynitrite, hydroxyl (OH˙), superoxide and peroxyl radicals whereas some basically are not free radicals but acts as a reservoir to generate other highly efficient ROS products that are highly reactive in nature and largely depends on cascades of redox system, mitochondrial ETC (electron transport chain) and also on various metabolic reactions like lipid peroxidation (Bhattacharjee, 2012). ROS level is controlled by various ROS-scavenging pathways in aerobic living beings but its disproportion may give rise to severe damage to cell functioning including DNA damage, metabolic dysfunctioning and cell death (Karuppanapandian et al., 2011) which are collectively known as “oxidative stress.”

Extensive research work has been already done and still are undergoing for early diagnosis and effective treatment of liver toxicity and cancer. Cancer suffered persons nowadays additionally burdened by allopathic drug-induced side effects and now force to us to develop a better alternative medicine. At present, numbers of treatments like chemotherapy, radiation therapy, hormonal therapy etc. are in hand to treat various cancers. Some boosting methods to improve cancer therapies are recently invented like nanotechnology approach in which nanogold particles are used to boost up the radiation therapy (http://phys.org/). This method has no side effects but is too expensive for poor ones to afford it. In chemotherapy, various chemotherapeutic agents such as Doxorubicin (DoxR), Resveratrol (RV), 3-Berbamine (3-BB), 3-Bromopyruvate (3-BP) etc. have used to treat HCC (Wang et al., 2009; Bishayee et al., 2010; Ganapathy-Kanniappan et al., 2010; www.macmillan.org) but extreme side effects of these agents limit their usage. So, nowadays, the attraction towards drug discovery specifies some things like it should be specific, better from previous one, cost effective and possess no or less side effect in patient’s body.

Main goal to diminish side effects of allopathic medicines is to use medicinal plant derived effective herbal drugs in place of synthetic drugs. Herbs are an excellent
source of various phytoconstituents like flavonoids, polyphenols, tocopherols, tocotrienols, ascorbic acid, carotenoids etc. that impart to exert antioxidant efficacy (Zheng and Wang, 2001). Medicinal plants are known for their powerful pharmacological activities. Herbal drugs have reported a marked spectrum of potent biological functions and also used in medicinal system in folklore to treat several diseases. Keeping powerful medicinal values of herbs in mind, a historical medicinal plant “Indigofera tinctoria” was selected to see its potent anticarcinogenic effect.

Indigofera, a large genus that contain approximately 700 species, distributed geologically in various tropical regions. Indigofera tinctoria Linn. (True Indigo) belongs to family ‘Fabaceae’ is a historically important shrub. This plant is cultivated in South East Africa, Tropical Africa and also in Tropical America whereas in India, this plant is cultivated in all parts but especially in Southern India; TamilNadu (Nadkarni et al., 1991; National Institute of Industrial Research, 2004). This is a deciduous shrub, 1-2 m in height and may be annual, biennial or perennial.

I. tinctoria is a nitrogen fixing legume that induces soil fertility. Historically, this plant is known for their usage in production of blue color dye i.e. Indigo. Indigotin, the colorless glycoside present in this plant, is responsible for the blue color of the dye and thought to have antiseptic and astringent properties (http://herbs-treatandtaste.blogspot.in). This plant possess several medicinal properties and used in treating several health problems since ancient times. As mentioned in Ayurveda, I. tinctoria is helpful to cure various disorders such as constipation, liver disease, heart palpitation and gout (Singh, 2006), useful in naturopathy, splenomegaly, echolalia, cardiopathy, chronic bronchitis, asthma (Savithramma et al., 2007), ulcers, skin diseases, diuretic and are also helpful for promoting the growth of hairs (Motamarri et al., 2012). This plant also possesses anti-toxic property (Warrier et al., 2007), antimicrobial activity (Selvakumar and Kaunakaran, 2010; Renukadevi and Sultana, 2011; Vijayan et al., 2012) and antiepilepticus property (Asuntha et al., 2010). Decoction of I. tinctoria leaves is helpful in case of venomous insects bite and also for burns and scalds (Stepp, 2004).

Protective effect of I. tinctoria against Alzheimer’s disease, a neurodegenerative disorder, was already evaluated by Balamurugan and Muralidharan, 2010. Anti-proliferative activity of this plant against B16F10 melanoma cells strong its anticarcinogenic role (Dash et al., 2011). Inhibitory effect of isolated compound i.e.
Indirubin and flavanoidal fraction of this plant was also studied against human Breast cancer cell lines MCF-7 and lung cancer respectively (Aobchey et al., 2007; Kameswaran and Ramanibai, 2008).

Keeping the aforementioned problems and medicinal values of *I. tinctoria* plant, the present study was designed to elaborate antioxidant and free radical scavenging potential of crude hydroethanolic extract of *Indigofera tinctoria* (HEIT) followed by assessment of antioxidative, antihepatotoxic and antihepatocarcinogenic role of crude HEIT and its isolated isothiocyanate derivative (ITC−1) in *N*-Nitrosopyrrolidine (NPYR)-CCl₄ induced HCC bearing Swiss albino male mice (*Mus musculus*).

**OBJECTIVES OF THE PRESENT STUDY**

The overall research work was carried out in various phases which are as follows-

**Phase 1:** Pharmacognostical analysis, microscopic studies, phytochemical screening, quantification of phytoconstituents and *in vitro* antioxidant and radical scavenging potential of experimental plant (*Indigofera tinctoria*)

**Phase 2:** Isolation, purification and structural characterization of bioactive compound from the experimental plant extract

**Phase 3:** Antioxidative, antihepatotoxic and anticarcinogenic potential of one of the potent crude extract of experimental plant and its isolated compound in NPYR-CCl₄ intoxicated male mice.

**Phase 4:** Histological analysis of liver tissues.