Chapter 1
Introduction

"The last thing that we find in making a book is to know what we must put first."

-Blaise Pascal

Ancient civilization to present humans, every culture all over the world has been using plants as a source of natural medicine for their survival. This dependency led the indigenous people living in harmony with nature to evolve a unique system of medicinal plant practices (Teron and Borthakur, 2014). This new branch of science is known as "Ethno-botany". The description of the traditional knowledge of medicinal plants has been written in different ancient literatures of India including Veda, Purana and Upanishad (Ahmad *et al.*, 2006). In ancient Egypt, "Mummy" the dead body was preserved by using some plant extracts which is unknown till date. Dioscorides wrote *De Materia Medica*, a catalogue of more than 500 medicinal plants which remained an authoritative reference from the first century into the seventeenth century and became the prototype for modern pharmacopoeias (Stockwell, 1988).

In Africa, Asia and Latin America traditional medicine and medicinal plants have continued to play a vital role in their health care systems. According to World Health Organization (WHO), a variety of drugs are obtained from different medicinal plants and about 80% of the world's developing populations depends on traditional medicine for their primary health care needs (Singh *et al.*, 2012). In 2001, it was estimated that approximately 25% of prescribed drugs originated from plants of which 121 active compounds were then in use (Houghton, 2001; Rates, 2001). In addition, of the 252 medicines considered as basic and essential by the

WHO, 11% originated exclusively from plants and a significant number were synthesized from naturally occurring precursors. Different plant species are used as main ingredient for the preparation of modern phyto-medicines which has explored in the last few years. They are still being collected from the nature and also play an important role in drug development programs of pharmaceutical industry. A significant number of drugs were obtained from different plant precursors namely, digitoxin (*Digitalis* spp.), vincristine and vinblastine (*Catharanthus roseus*), quinine (*Cinchona* sp.), atropine (*Atropa belladona*), morphine and codeine (*Papaver somniferum*) etc.

However, the difficulty in the use of medicinal plants is that they are usually used without any standardization. This makes it difficult to document and introduce a system of verification or assessment for the efficacy of the treatment. Plants may contain constituents that can be used to treat diseases such as infections, inflammatory conditions and cardiovascular diseases, but the scientific information on most of these medicinal plants in use are lacking. Therefore, as part of the efforts to promote the use of medicinal plants either as an alternative or an adjunct to conventional medicine, it is necessary for scientists to carry out investigations into herbal medicines. A bridge will help to minimize the gap between conventional and herbal medicines.

India, one of the richest floristic regions of the world, has diverse socioeconomic, ethnic, linguistic and cultural areas. There are about 54 million indigenous people of different ethnic groups colonizing various regions of the country. The aboriginal groups have their own distinctive culture, religious rites, food habit and a rich knowledge of plant utilization (Mahishi *et al.*, 2005; Boro and Sarma, 2013) which pass orally generation to generation. Therefore, the traditional knowledge of medicinal plants and their use in treating several ailments might reasonably be expected in India due to its rich floristic vegetation (Shil *et al.*, 2014). Chandel et al. (1996) have reported that nearly 70% of tribal and rural inhabitants of India are to a large extent depended on medicinal plants for their primary healthcare management due to either insufficient or inaccessible or less availability of modern healthcare system. Virtually, ethnobotanical survey may be regarded as one of the most reliable approaches towards new drug discovery and it is a prerequisite for any developmental planning concerned with the welfare of tribal and their environment (Lokho and Narasimhan, 2013). Nonetheless, in recent times medicinal plants became the backbone of herbal drugs being used over the world wide.

Medicinal plants are also well-documented and long been utilized as the basis of many traditional medicine exerting protective effects against several diseases in human body due to their antioxidative properties (Goyal *et al.*, 2011). Medicinal plants are rich in polyphenols which have the antioxidant and redox properties that act against reactive oxygen species (ROS) and reactive nitrogen species (RNS) including hydroxyl radical ($OH^{\bullet -}$), superoxide anions ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite radicals ($ONOO^-$) etc. (Justesen and Knuthsen, 2001). ROS are mainly produced in the cell by the mitochondrial respiratory chain which imparts an oxidative stress producing super oxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) etc. during endogenous metabolic reactions. ROS are also produced by myeloperoxidase (MPO) - Halide - H_2O_2 system where, in the presence of chloride ion H_2O_2 is converted to hypochlorous acid, a potent oxidizing agent (Nimse and Pal, 2015). ROS in low concentration is essential for our physiological functions like cellular growth, gene expression and providing defense against infection. However, inability to detoxify excess ROS by our body may cause oxidative stress. The oxidative stress may in turn increases the risk of various diseases like diabetes, cancer, obesity, rheumatoid arthritis, cognitive disorders and is largely involved in ageing (Durackova, 2010, Poyton *et al.*, 2009). The mitochondrial respiratory chain also produces nitric oxide (NO) under hypoxic condition, which can generate reactive nitrogen species (RNS) (Durackova, 2010). RNS induces excessive lipid peroxidation which may lead to the production of other reactive species like reactive aldehydes and malondialdehyde. The reaction between nitric oxide and super oxide is resultant of peroxynitrate, a potent and very active oxidant that can attack a wide array of biological functions. These ‘oxyradical overload’ may lead to a variety of diseases including

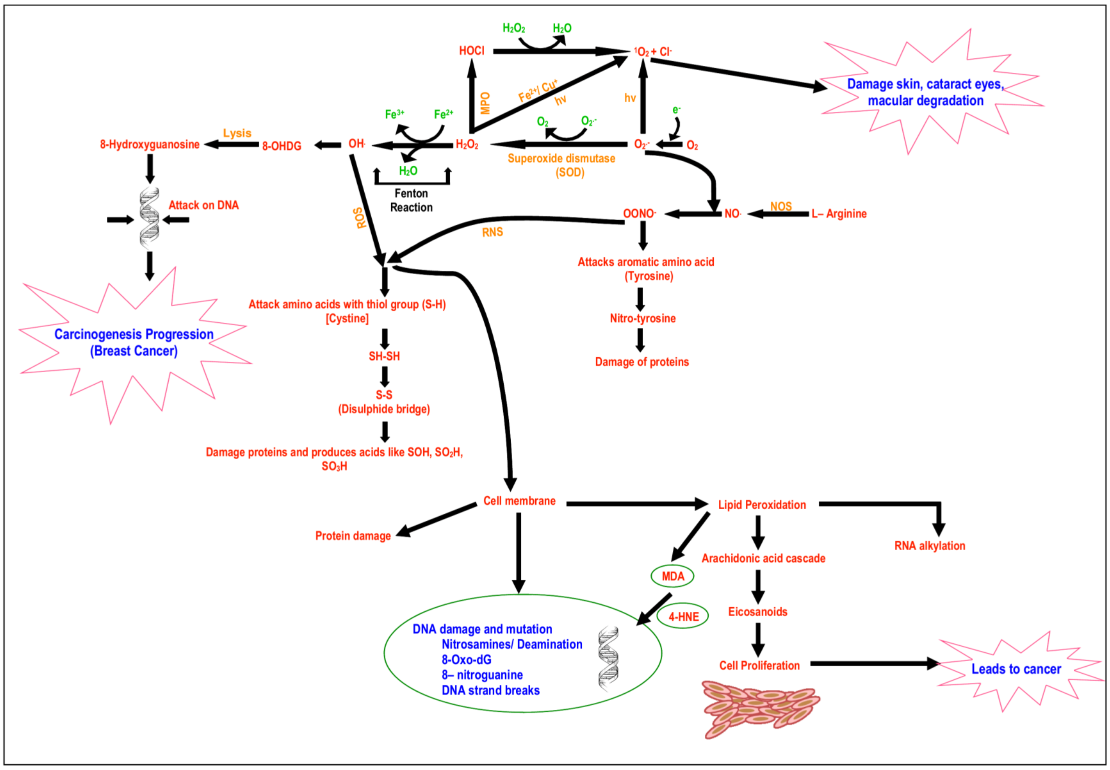

Fig. 1.1. Schematic representation showing the free radical generation followed by the chain of by-product (ROS/RNS) formed due to oxidative stress and how they affect biological systems by cellular stress and even leading to carcinogenesis. [HOCl: Hypochlorous acid; H_2O_2: Hydrogen peroxide; $^1O_2$: Singlet oxygen; $O_2^{\cdot-}$: Superoxide; OH•: Hydroxyl radical; 8-OHdG: 8-hydroxy2-deoxy guanosine; $OONO^-$: Peroxynitrate; $NO^\cdot$: Nitric oxide; Fe^{2+}: Iron ion; Cu^+: Copper ion; Cl^-: Chlorine ion; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; NOS: Nitric oxide synthase; MPO: Myeloperoxidase; MDA: Malondialdehyde; 4-HNE: 4-hydroxynonenol].

cellular inflammation and cancer by way of DNA damage, protein modification and changing transcriptional parameters (Durackova, 2010).

Accumulation of ROS by soluble mediators like arachidonic acid, cytokines and chemokines secreted by inflammatory cells, activate several signal transduction cascades including changes in transcription factors such as NF-κβ, AP-1, Nrf2, p53, SP1, HIF-1α, STAT3 and PPARγ. The activation of these transcription factors by ROS may lead to sustained inflammation or an oxidative environment which in turn might lead to damage of healthy neighbouring epithelial and stomatal cells over a long period of exposure and might lead to cancer (Fig. 1.1) (Perwez Hussain and Harris, 2007; Reuter *et al.*, 2010). Besides, FasL, a type II membrane protein belonging to the tumor necrosis factor (TNF) family, has also been reported to be associated with ROS generation causing necrotic cell death (Medan *et al.*, 2005; Vercammen *et al.*, 1998) while NADPH oxidase, a membrane bound enzyme complex was also involved to yield different ROS (Han *et al.*, 1998; Pham-Huy *et al.*, 2008). Simultaneously, toll like receptors (TLR) can also induce the ROS production (Marcato *et al.*, 2008) which trigger the signals for cell apoptosis.

A plethora of evidences suggested that herbal plants, vegetables and fruits own antioxidative compounds such as phenolics, flavonoids, tannins and proanthocyanidins which alleviate or neutralize the free radicals. Moreover, the intake of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders (Gulcin, 2012). However, several synthetic antioxidants namely, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) etc. available in the market that has been reported to show adverse health effects (Shahidi and Zhong, 2005). Hence, there is an emerging tendency to shift towards naturally occurring antioxidants for the prevention and treatment of diseases as well as maintenance of human health (Halliwell and Gutteridge, 1981).

Oxidative stress is an imbalance between the production of free radicals (reactive oxygen species, ROS and reactive nitrogen species RNS) and the inability to detoxify them through protective mechanisms. The brain is very much sensitive to oxidative stress because of its extensive O_2 consumption ability, lipid rich constitution and modest antioxidant defence mechanism (Ng *et al.*, 2008; Halliwell, 2006). The oxidative stress in brain can cause several brain disorders including neurogenerative diseases (Reynolds *et al.*, 2007) and depression (Belmaker and Agam, 2008). Neurodegenerative disorders are one kind of abnormalities that affect brain's capability to remember and process information at late age, could be balanced by the antioxidative defence system (Trivedi, 2006). Whereas, depression is a mood disorder characterized by persistently low mood, loss of interest and a feeling of sadness. ROS generated oxidative stress has been linked with several diseases such as schizophrenia, bi-polar disorder and major depressive disorder (Fig. 1.2) (Valko *et al.*, 2007; Delattre *et al.*, 2005; Bilici *et al.*, 2001). Hence, neurodegenerative disorder has emerged as a great public

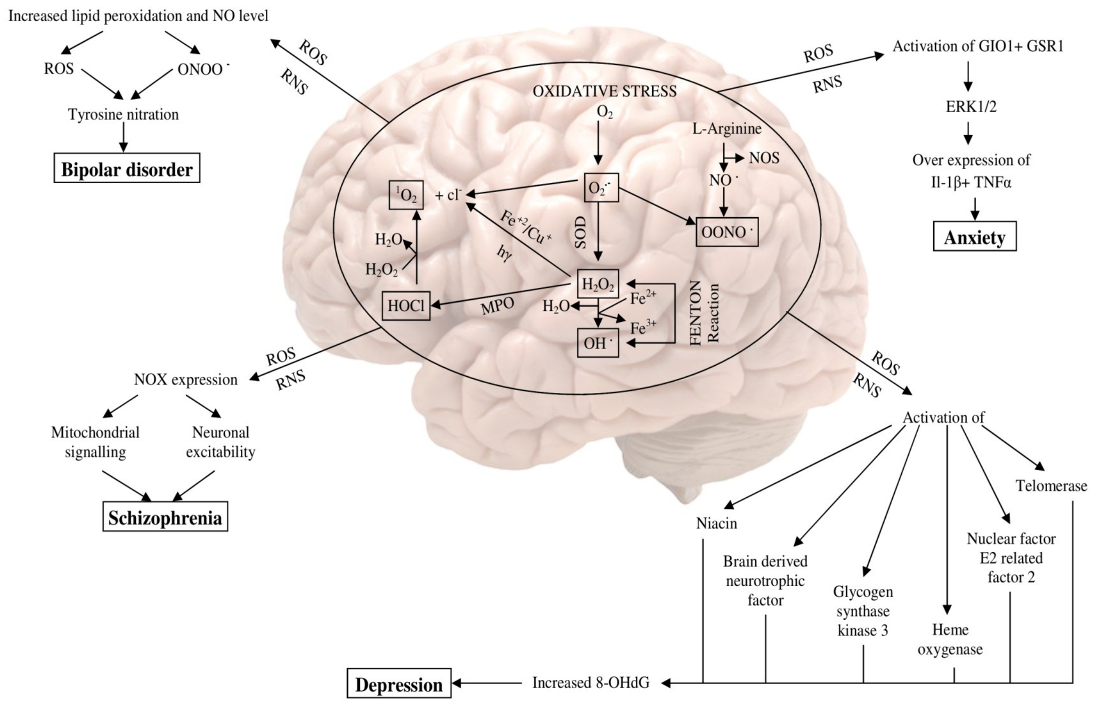

Fig. 1.2. Schematic representation showing the free radical generation followed by the chain of by-product (ROS/RNS) formed due to oxidative stress and how they affect biological systems by cellular stress and even leading to different types of mental disorders. [NOX: Nitrous oxide; TNF-α: Tumor necrosis factor α; ERK: Extra cellular signal regulated kinases; SOD: Super oxide dismutase].

health concern, thereby demand intervention to ameliorate oxidative stress. Several approved drugs including donepezil, tacrine, rivastigmine, galanthamine etc., to some extent alleviate the symptoms of neurogenerative diseases. However, their chronic use is often associated with exerting side effects (Chattipakorn *et al.*, 2007). Herbal formulations on the other hand have been documented effective against several cognitive disorders so far (Mathew and Subramanian, 2014).

In our biological system, excessive reactive species derived from oxygen and nitrogen may lead to tissues and organ damage. Oxidative stress can be considered as a combined pathological mechanism and it induces the initiation and progression of liver injury. The liver is associated with most of the physiological and metabolic functions in our body system. Liver fibrosis is the gateway to several liver related ailments and among all the reasons for liver dysfunctions, the exposure to toxic reagents and drug is prime (Cichoż-Lach and Michalak, 2014).

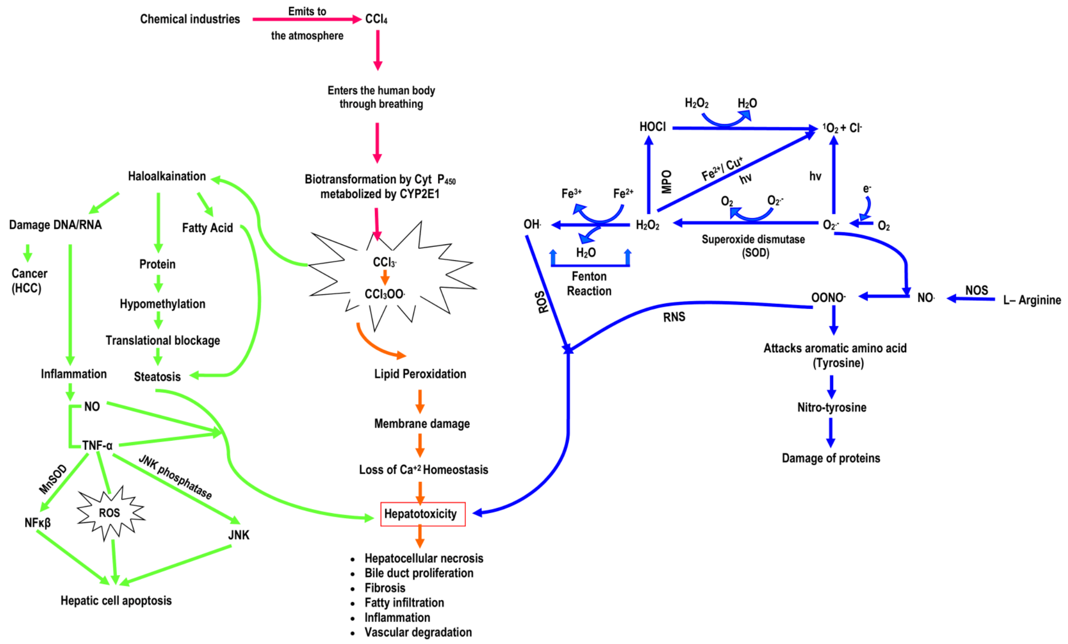

Fig. 1.3. Schematic representation showing the free radical generation followed by the chain of by-product (ROS/RNS) formed due to oxidative stress and how they affect biological systems by cellular stress and CCl_4 induced hepatotoxicity. The pathway demonstrates the mechanism of CCl_4 induced hepatotoxicity which is predominantly mediated by oxidative stress and inflammatory injury due to the formation of reactive metabolic intermediates and the free radical formation cascade during xenobiotic induced hepatotoxicity causing oxidative and nitrosative stress. Cyt P450: Cytochrome P450, $CCl_3^{\bullet}$: Trichloromethyle radical, $CCl_3OO^{\bullet}$: Trichloromethylperoxy radical, TNF-α: Tumor necrosis factor-α.

Carbon tetrachloride (CCl_4) is considered as a haloalkene which is extensively used to generate oxidative stress and cause the liver injury (Weber *et al.*, 2003). Liver damage by CCl_4 is primarily associated with free radicals mediated tissue injury, inflammatory damages and activation of intracellular signaling cascade that intensify pro-inflammatory gene expression (Dutta *et al.*, 2018; Anderson *et al.*, 1994) (Fig. 1.3). Correspondingly, kidney disorder is progressively affecting a large number of populations and fatally reducing their quality of life (Fig. 1.4). Utilization of conventional medicines in liver and kidney injury is quite expensive as well as unreasonable to most of the patients. Furthermore, these drugs exert adverse side-effects due to chronic use. Inclusion of herbal remedy into conventional healthcare system may considerably improve the overall healthcare system. The advantage of herbal remedy over modern medicines is that most of the

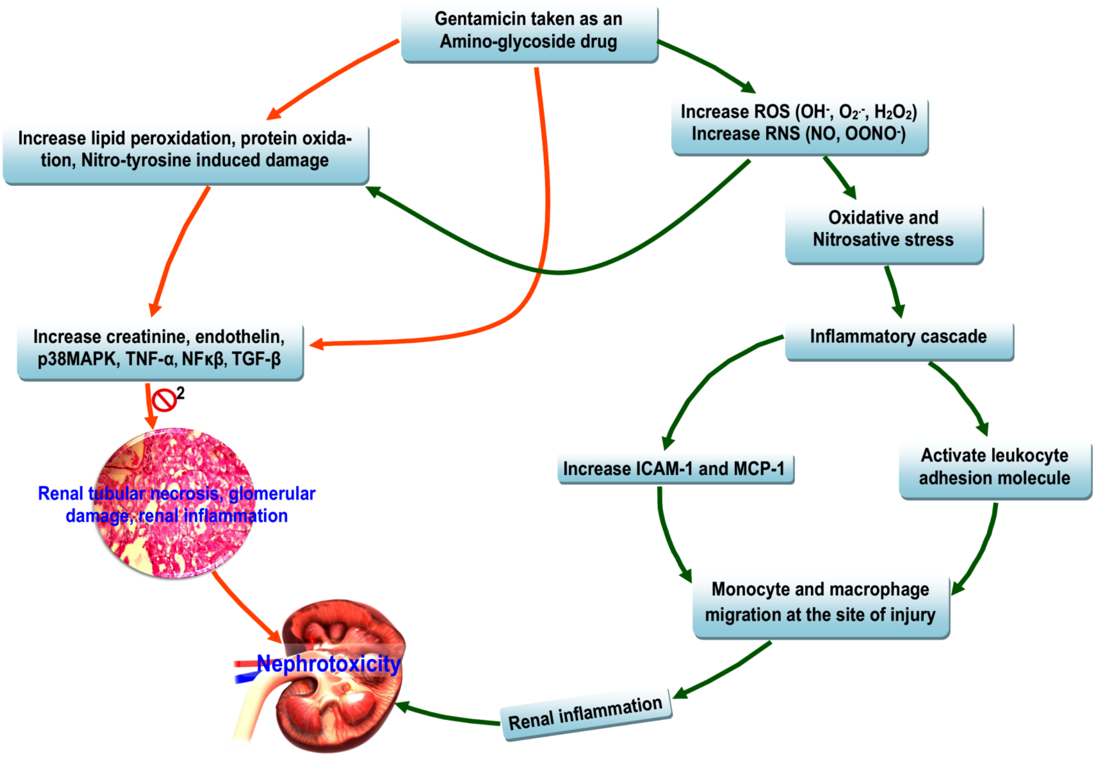

Fig. 1.4. Schematic representation showing role of gentamicin in the induction of nephrotoxicity. MAPK: Mitogen activated protein kinase; TNF-α: Tumor necrosis factor-α; NFκβ: Nuclear factor kappa β; TGFβ: Transforming growth factor-β; ICAM-1: Intercellular cellular adhesion molecule-1; MCP-1: Monocyte chemoattractant protein-1; O_2^-: Superoxide radical; OH^-: Hydroxyl radical; H_2O_2: Hydrogen peroxide; NO: Nitric oxide; $OONO^-$: Peroxynitrate; ROS: Reactive oxygen species; RNS: Reactive nitrogen species.

herbal medicines are plant-based and comparatively cheaper, possess fewer or negligible side-effects owing easy acceptability. Therefore, the study on medicinal plants strongly supports the idea that plant constituents (i.e. phenolics, flavonoids, tannins, proanthocyanidins etc.) along with antioxidant properties are capable of exerting protective effects against oxidative stress, kidney disorder, cancer, neurodegeneration, aging etc.

Approximately 3,000 plants species of more than 200 families were identified to have medicinal properties in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island of India (Prakasha *et al.*, 2010). Among them there are few species which have good medicinal properties. During literature survey it was found that the genus *Clerodendrum* has excellent medicinal values as well as prominent traditional knowledge. Therefore, an attempt has been taken to explore this as a studied plant.

It was in 1753, Linnaeus first describe the genus *Clerodendrum*, with the identification of *C. infortunatum*. Adanson changed the Latin name "*Clerodendrum*" to its Greek form '*Clerodendron'* in the year 1763; and after a decade Moldenke(1942), changed the Latinized name '*Clerodendrum*', which is currently used for the classification and description of the genus and species (Rueda, 1993; Hsiao and Lin, 1995; Moldenke, 1985; Steane *et al.*, 1999). In Greek 'Klero' means chance and 'dendron' means tree (Shrivastava and Patel, 2007).

Shrivastava and Patel (2007) reported that, *Clerodendrum* is a very large and diverse genus with about 580 identified species is distributed throughout the world. But according to 'The Plant List', 701 plants are enlisted under *Clerodendrum* with 327 accepted, 345 synonym, 9 unplaced and 20 unresolved (http://www.theplantlist.org/browse/A/Lamiaceae/*Clerodendrum*). Rajendran and Daniel (2002) recorded 23 species in India of which 16 were recorded from Arunachal Pradesh by Srivastava and Choudhary (2008). There is some controversy of the genus *Clerodendrum* for its systematic position. Previously, Fletcher (1938), Kochummen (1978), Liang and Gilbert (1984) and Munir (1989) placed *Clerodendrum* in the family Verbenaceae but with the use of modern molecular techniques *Clerodendrum* was shifted to the family Lamiaceae based on morphological and molecular phylogenic evidence (Cantino *et al.*, 1992; Harley *et al.*, 2004). Recently, chloroplast DNA (cpDNA) restriction site data (Steane *et al.*, 1997), nuclear ITS sequences (Steane *et al.*, 1999) and the four relatively fast evolving chloroplast DNA regions (trnT-L, trnL-F, trnD-T and trnS-fM) (Yuan *et al.*, 2010) were used to carry out the phylogenetic studies of the genus *Clerodendrum* and its related genera. From this study, it was strongly indicated that *Clerodendrum* is not monophyletic but rather is separated into three major clades that are in general associated with their geographical distribution: an Asian clade, a Pantropical Coastal clade and an African clade.

A number of species from this genus have been reported to have several medicinal properties including rheumatism, asthma, malaria, anti-diabetic and anti-hypertensive properties (Hazekamp *et al.*, 2001; Kang *et al.*, 2003; Chaturvedi *et al.*, 1984; Nath and Bordoloi, 1991). In fact, these species are characteristically used by the ethnic people as a source of polyherbal formulations in the management of several diseases in different parts of India (Ahmed, 1996; Nath and Bordoloi, 1991). Botanically, *Clerodendrum* is classified as follows (Cronquist, 1981):

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Lamiales
Family: Lamiaceae
Sub-family: Teucrioideae
Genus: *Clerodendrum* L.

The different species of this genus are usually small trees, shrubs, herbs or climbers. Taxonomically the genus is characterized by simple opposite decussate leaves with most of them being petiolated. There is a wide variability in the sizes of the leaves. In *C. aculeatum*, the leaves range from 0.9-4 cm in length and 0.3-1.4 cm in wide while *C. paniculatum* has leaves that are 6-35 cm long and 6-30 cm wide. The leaf shape is also variable with *C. walichii* having a lanceolate shape but *C. japanicum* being ovate shaped. The inflorescences of members of this genus are placed both axillary and terminal position. The inflorescence ranges from 1-39 cm long to 1-25 cm wide. They may be cymes, panicles or solitary flowers which may be crowded or sparsely arranged. Almost all the species of *Clerodendrum* have foliaceous bracts and linear lanceolate bracteoles. The calyx is usually gamosepalous, commonly green but sometimes red or white. They are almost always campanulate, rarely elliptic, truncate, 5 -lobed, glabrous or pubescent. The size of the corolla ranges from 0.6-4.0 cm long to 0.3-2.0 cm wide. The corolla is hypocrateriform and may be white, red, pink or purple. The corolla is glabrous, pubescent or glandular puberulent. The fruits are drupaceous, mostly subglobose, ovoid or glabrous usually separating at maturity (Rueda, 1993).

Evidences suggested that a few preliminary works including antioxidant activity, anti-aging, anti-diarrhea, anti-asthmatic, hypoglycemic, anti-tumor etc. have been done with selective species of *Clerodendrum*. However, no attempt was made to support the ethnopharmacological claims. Even though, an in-depth medicinal property showing comparative and comprehensive information of *Clerodendrum* is still missing.

More surprisingly, it has further been observed that there has no sufficient information illustrating the comprehensive genetic variation among the different medicinal species of *Clerodendrum*. Hence, an initiative step was carried out to explore the genetic variations of some species of *Clerodendrum* through DNA fingerprinting techniques. Of the various, random amplified polymorphic DNA (RAPD) and microsatellite DNA marker like inter simple sequence repeat (ISSR) were recently shown to be sensitive for detecting variations among individuals between and within species, cultivars and varieties (Williams *et al.*, 1990; Godwin *et al.*, 1997). Besides, restriction fragment length polymorphism (RFLP) analysis is also used to investigate the phylogenetic relationships among species. Subsequently, a new modified molecular technique i.e. DNA barcoding was developed to explore the evolution, genetic relatedness and identification of unknown animal and plants species resolving various anomalies in the taxonomic levels by using a short stretch of DNA sequence (Hebert *et al.*, 2003).

Hence, based on the therapeutic appraisal as mentioned above, 11 different species of *Clerodendrum* including, *Clerodendrum indicum* (L.) Kuntze, *C. inerme* (L.) Gaertn. (Syn. *Volkameria inermis* L.), *C. japonicum* (Thunb.) Sweet, *C. splendens* G. Don, *C. speciaosum* Dombrain, *C. thomsoniae* Balf. f., *C. infortunatum* L. (Syn. *C. viscosum* Vent.), *C. serratum* (L.) Moon (Syn. *Rotheca serrata* (L.) Steane & Mabb.), *C. colebrookianum* Walp., *C. chinense* (Osbeck) Mabb. (Syn. *C. fragrans* Willd.), *C. bracteatum* Wall. ex Walp. found in different parts of North Bengal and Assam were employed in the present study. Hence, keeping all these in mind an initiative was undertaken to explore the varied medicinal properties of these 11 species with the following objectives:

- Survey and collection of species of *Clerodendrum* from different parts of North Bengal and Assam.
- Maintenance of the germplasm in the experimental garden of the Department of Botany.
- Assessment of antioxidant properties of selected *Clerodendrum* extracts.

- Determination of cytotoxicity of extracts.
- Measurement of ROS (Reactive Oxygen Species) generation in human hepatic cell line (WRL-68), human liver cancer cell line (Hep-G2) and human embryonic kidney cell line (HEK-293).
- To study the antimicrobial activity of selected species of *Clerodendrum*.
- To study the therapeutic properties of selected species of *Clerodendrum*.
  (i) In-depth study of medicinal properties of selected plant including hepatoprotective activity on mice model.
  (ii) Evaluation of neuromodulatory activity of selected plant on mice model.
  (iii) In-depth study of medicinal properties of selected plant including nephrotoxicity on rat model.
- High throughout phytochemical analysis using FT-IR and GC-MS analysis.
- Selection of target compounds for novel drug discovery designing using *in-silico* approach.
- Study of separation of compounds through column chromatography of selected species of *Clerodendrum* and its bioactivity.
- Study of regeneration of *Clerodendrum* through tissue culture techniques.
- Detection of somaclonal variation among the *in-vitro* regenerated plantlets.
- Molecular Documentation
  (i) Detection of genetic variability and phylogenetic relationship among the 11 species of *Clerodendrum* by different PCR based DNA fingerprinting methods like Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR).
  (ii) To study the matK, rps16 and trnL-trnF region of chloroplast genome of *Clerodendrum* using PCR-RFLP.
  (iii) Exploitation of DNA barcode analysis of chloroplast genome of 11 species of *Clerodendrum* using matK, rps16 and trnL-trnF primers.