# 1. INTRODUCTION

From ancient times, plants have been used as an important source of medicines due to the presence of a wide spectrum of biologically active compounds. The medicinal plants are extensively utilized throughout the world in two distinct areas of health management, namely traditional system of medicine and modern system of medicine. According to the World Health Organization (WHO), about 80% of people practice traditional medicine for primary health care and more than 21,000 plants with medicinal values have been listed around the world (Mwitari *et al.,* 2013). The International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) has estimated that between 50,000 to 80,000 flowering plants are used for medicinal preparations (Mdel *et al.,* 2013).

Over centuries, a majority of cultures around the world have learnt how to use herbs as medicines to maintain health, for example the Chinese medicine and the Indian medicine. Traditional medicine is widely used because of its cultural acceptability, economic affordability, compatibility with the human body, lesser side effects and efficacy against certain types of diseases as compared to modern medicines (Tolossa *et al.,* 2013). Thus, traditional medicine forms the basis of an easily available and affordable health care regime, especially for rural populations (Adefuye and Ndip, 2013).

According to World Health Organisation (WHO), in 2003, the size of the global market for herbal and medicinal plants was about US$ 60 billion and is expected to reach US$ 5 trillion by 2050 (http://www.who.int/mediacentre/factsheets/2003/fs134/en/). In India, the herbal drug market value is estimated at US$ one billion and, about 80 million US$ worth of plant-based crude drugs is being exported (Vanerkar *et al.,* 2013). The use of herbal products has increased in developing countries due to improved knowledge about the safety, efficacy and quality assurance of ethnomedicine (Hamedeyazdan *et al.,* 2012).

India has a rich source of medicinal plants distributed in different geographical conditions and a well-practiced knowledge of traditional medicine. Herbs are used in various traditional systems of medicines such as Ayurveda, Unani, Siddha and Homeopathy (Mazid *et al.,* 2012). Ayurveda has played a major role in the process of drug discovery through the identification of active compounds from herbs (Dudhatra *et al.,* 2012).

In the modern era, the use of different chemicals, pesticides, pollutants, smoking, alcohol intake and even some synthetic medicines, has increased the occurrence of diseases due to free radicals. The diseases caused by free radicals are becoming a part of normal life.

Phytoconstituents and herbal medicine play important roles in managing the diseases caused by free radicals, since they represent a potential source of new compounds with antioxidant activity to prevent oxidative stress (Mahantesh *et al.,* 2012).

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and cellular metabolism, which possess both deleterious and beneficial effects in the body. Free radicals are highly reactive molecules that possess unpaired electrons in their outer orbits. Non-free radicals are molecules without unpaired electrons, but are highly reactive. These highly reactive free radicals and non-free radicals are collectively known as reactive species (Dawane and Pandit, 2012). Reactive oxygen species (ROS) are formed when oxygen reacts with these unpaired electrons. ROS contain both free radical and non-free radical oxygen containing molecules such as hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radical. Reactive nitrogen species (RNS) include free radicals like nitric oxide and nitrogen dioxide, and non-radical species such as peroxynitrite, nitrous oxide and alkyl peroxynitrates. Formation of the superoxide anion initiates the subsequent formation of other ROS (Poljsak *et al.,* 2013).

Under normal physiological conditions, ROS are formed by living cells as the products of cellular metabolism, where oxygen is used to produce energy. ROS are mainly produced from the mitochondrial electron transport chain (ETC). Under normal conditions, ROS plays an important role in redox-regulatory mechanisms of the cells such as the maintenance of redox homeostasis, regulation of protein phosphorylation, ion channels and transcription factors (Alfadda and Sallam, 2012). ROS are also required for biosynthetic processes such as thyroid hormone production and crosslinking of extracellular matrix. At low to intermediate concentrations, ROS act as second messengers in signal transduction for vascular homeostasis and cell signalling. But when produced in excess and not eliminated by endogenous systems, ROS become deleterious and react readily with carbohydrates, proteins, lipids and nucleic acids, thereby causing irreversible functional alterations or complete biomolecular damage (Breiger *et al.,* 2012).

Oxidative stress was first defined by Sies (1991) as "a disturbance in the prooxidant to antioxidant balance, in favour of the former, leading to potential damage". Oxidative stress can be defined as an imbalance between the production of reactive species and reduced physiological ability to readily detoxify the reactive intermediates (Zatalia and Sanusi, 2013). Mitochondria, the main source of ROS are also the targets for cellular ROS. ROS damage to mitochondria results in dysfunction that reduces the energy production in

the form of ATP and enhance ROS production and thereby aggravates the oxidative stress. Such feed-forward vicious cycle between mitochondria and ROS induces cell death (Sun *et al.,* 2013). Oxidative stress is associated with the aging process, cell death and affects all major organ systems.

ROS have been implicated as endogenous initiators in the etiology of cancer and several other degenerative or pathologic processes including Alzheimer's disease, Parkinson's disease, and virtually all cardiovascular diseases, as well as diabetes (Vetrani *et al.,* 2013). For example, the two major pathological events of the type 2 diabetes mellitus namely, the insulin resistance and beta-cell dysfunction are associated with redox imbalance (Lin *et al.,* 2012a). Oxidative damage to biomacromolecules (nucleic acids, lipids and proteins) by ROS has been implicated in the critical aspects of the aging process (Schiborr *et al.,* 2013). ROS cause damage to specific regions of the brain, which leads to neurodegeneration and resulting neurodegenerative diseases like Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Kovacic and Somanathan, 2012).

Necrosis and apoptosis of neurons and astrocytes are caused by oxidative stress. Mitochondria-mediated oxidative stress induces excitotoxic nerve cell death through calcium-related bursts of ROS production and opening of permeability transition pores (Savino *et al.,* 2013). Oxidative stress is associated with a plethora of pathological phenomena, including infection, inflammation and cancer. ROS have been implicated in the initiation of cancer by inducing DNA damage that develops into mutations, leading to the activation of oncogenes and the loss of tumour suppressor gene function (Sesti *et al.,* 2012).

In all living systems, cells are endowed with complex sets of enzyme defences called antioxidants which are designed to minimize the harmful effects caused by excessive production of ROS and to prevent, limit or repair oxidative damage. Antioxidants are defined as molecules that are present at low concentrations compared to an oxidizable substrate and prevent the oxidation of the substrate (Puertollano *et al.,* 2011). In cells, the redox homeostasis is maintained by an elaborate endogenous antioxidant defence system comprised of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and non-enzymic scavengers such as glutathione (GSH), vitamins (vitamins C, E) and phytochemicals (flavonoids, phenols), which are present in diet and medicinal plants (Halliwell, 2011).

Based on the mechanism of action, there are two classes of antioxidants namely chain-breaking antioxidants and preventive antioxidants. Chain-breaking antioxidants such

as vitamin E and β-carotene break the chain of free radical formation by donating an electron to stabilize an existing free radical, whereas preventive antioxidants are enzymes such as SOD and glutathione peroxidase, which scavenge initiating radicals before they start an oxidation chain. Other non-enzymic antioxidants include carotenoids, flavonoids and related polyphenols, lipoic acid and glutathione (Sameer *et al.,* 2013). In addition to neutralization of free radicals, the ability of the antioxidants to modulate cell signalling pathways have made them useful in the treatment of various diseases including cancer. Recent researches have shown that the antioxidants of plant origin with free-radical scavenging properties have gained great importance as therapeutic agents for the treatment of oxidative stress related diseases (Mahantesh *et al.,* 2012).

Cancer is a multifactorial disease characterized by uncontrolled growth and spread of abnormal cells. It is one of the leading causes of death in most well developed countries (Baskar *et al.,* 2012; Siegel *et al.,* 2012). According to WHO, in 2008, about 12.66 million people were diagnosed with cancer and about 7.6 million deaths (13% of all deaths) occurred due to cancer worldwide (http://www.who.int/cancer/en/). The clinical management of cancer invariably involves surgery, chemotherapy, radiotherapy, immunotherapy and gene therapy (Wang *et al.,* 2013a).

Recent evidence suggests that ROS-dependent signals are required for tumour growth and that altering levels of ROS with antioxidants or pro-oxidants could modulate tumour growth (Georgakilas, 2012). Over the past 20 years, realization of the fact that DNA damage and mutation arise from endogenous products of cellular metabolism has become the major development in carcinogenesis research (Dizdaroglu, 2012). The study on oxygen radical dependent damage to DNA has become a major thrust of carcinogenesis research (Wu, 2013). Oxygen radicals generated during reduction of oxygen can attack DNA bases or deoxyribose residues to produce damaged bases or strand breaks. Oxygen radicals can oxidize lipid or protein molecules to generate intermediates that can react with DNA to form adducts. Attempted replication of this damage leads to mutation or apoptosis, which ultimately results in cancer, the second leading cause of death worldwide (Magnander and Elmroth, 2012). Lipid peroxidation-derived adducts to DNA bases form etheno-type and propano-type exocyclic rings which are mutagenic and are elevated in several cancers (Winczura *et al.,* 2012).

Different classes of tumours exhibit multiple immunosuppressive responses to treatment and not all anticancer agents effectively give a positive response in every case.

Since the cancer morbidity will climb to around nine million worldwide by 2015 and the average survival rates have remained essentially unchanged despite such aggressive treatments, there is a critical need for new anti-cancer agents with higher efficacy, and less side effects at an affordable cost (Fadeyi *et al.*, 2013). Failure to produce satisfactory results, adverse side effects, toxicity towards normal tissues, immunosuppression and drug resistance are the major drawbacks of using systemic chemotherapy alone for cancer (Meiyanto *et al.*, 2012). Application of cancer chemoprevention is one promising solution to overcome this problem. The term chemoprevention was first introduced by Sporn and defined as the use of natural, synthetic or biological agents to inhibit, supress, or reverse the cancer development (Langner and Rzeski, 2012). A large number of natural substances present in foods, particularly antioxidative compounds in plants, such as phytochemicals and their derivatives that include vitamin derivatives, phenolics, flavonoids, saponins, tannins, terpenes, alkaloids and fatty acids are identified to be potential cancer chemopreventive agents (Tanaka *et al.*, 2012).

Development of new anticancer drugs from natural biological resources, particularly from terrestrial microbes and higher plants, has been ventured over the last five decades throughout the world (Jain and Kumar, 2012). Herbs and plant-derived products have less toxicity compared to that of synthetic chemicals, which enhances their appeal for treating cancer and for long-term preventive strategies (Harlev *et al.*, 2013). Factors such as lack of access, adverse side effects and high cost of chemical drugs encourage the use of herbal drugs instead of chemical medicines (Pan *et al.*, 2012).

The anticancer properties of plants are attributed to the ability of antioxidants in them to scavenge free radicals, inhibiting DNA damage and subsequent mutation. Since oxidative stress is the major underlying factor in carcinogenesis, chemoprevention using antioxidant substances such as phenolics has been developed for cancer formation and progression (Boulaaba *et al.*, 2013). Antioxidants terminate the free radical chain reaction by donating hydrogen ions or electrons, thereby converting the free radicals into more stable products. Antioxidants either delay or inhibit the initiation step of carcinogenesis or reverse the progressive stage by acting as growth inhibitors or by inducing apoptosis (Goyal, 2012). Antioxidants reduce the chances of carcinogenesis either by inhibiting the oxidant-induced DNA damage or by facilitating the repair of damaged DNA (El-Sayed and Hussin, 2013).

Current research in drug discovery from medicinal plants involves a multifaceted approach involving botanical, phytochemical, biological and molecular techniques. There

are at least 250,000 species of plants, among which more than one thousand plants have been identified with significant anticancer properties (Cerella *et al.,* 2013). More than 60% of all anticancer drugs are of natural origin or derived from natural compounds. About 30 plant derived anticancer compounds have been isolated and are currently under clinical trials (Manju *et al.,* 2012). There are four major structural classifications of plant-derived anticancerous compound namely vinca alkaloids (vinblastine and vincristine), epipodophyllotoxin lignans, taxane diterpenoids and camptothecin quinoline alkaloid derivatives (Nirmala *et al.,* 2011). Another class of plant-derived anti-cancer agents are topoisomerase II inhibitors such as etoposide and teniposide. Currently, paclitaxel and the semi-synthetic docetaxel are used in a large number of cancer treatments (Varma *et al.,* 2011).

Scientific validation of the traditional knowledge of the herbal remedies is required for the verification of the safety and efficacy of the treatments (Bahmani and Eftekhari, 2012). Most of the current anticancer drugs derived from plant sources act through different pathways that converge ultimately into activation of apoptosis in cancer cells, leading to cell cytotoxicity (Yang *et al.,* 2013a). *In vitro* studies using cell lines have proved the anticancer properties of many plants, but only a few have succeeded in animal experiments and clinical trials for cancer treatment (Strüh *et al.,* 2013).

Many research studies have been carried out to identify plants with significant antioxidant and anticancer potential by analysing their cytotoxic, antiproliferative, apoptotic and radical scavenging activities using both *in vitro* and *in vivo* systems (Karimi *et al.,* 2013). In accordance with this, the present study was formulated to investigate the antioxidant and anticancer properties of the flowers of the candidate plant *Caesalpinia pulcherrima*, which blooms in three different colours (orange, pink and yellow) with unique long stamens.

Earlier studies in our laboratory have shown that these flowers are rich in both enzymic and non-enzymic antioxidants (Aparna, 2000) and also exhibit both antimutagenic and antioxidant properties (Nirmala Priyadharshini, 2001). Cell viability studies *in vitro* showed that the flowers significantly reduced the survival of cancer cells, suggesting that the flowers may possess anticancer activities. They also showed protective effect against oxidative DNA damage, especially for eukaryotic DNA (Yamuna, 2004).

The protective effect of the flowers of *Caesalpinia pulcherrima* against oxidative DNA damage, which is the main cause for the cancer development, and their significant role in reducing cancer cell growth, has furthered the present study on the molecular mechanisms of the antioxidant and anticancer properties of the flowers.

## 1.1 Hypothesis

The **null hypothesis ($H_0$)** drawn for the present study was as follows:

The flowers of *Caesalpinia pulcherrima* do not possess significant antioxidant and anticancer properties.

The **alternate hypothesis ($H_A$)** was as follows:

The flowers of *Caesalpinia pulcherrima* contain components that exhibit strong antioxidant and anticancer effects.

## 1.2 Objectives

In order to test these hypotheses, the study was formulated with the following objectives:

- To study the free radical scavenging activity and biomolecule-protective effects of the flowers against oxidative stress
- To assess the antioxidant potential of the flowers on oxidative stress induced *in vitro*.
- To evaluate the differential effects of the flowers in non-transformed and transformed cells.
- To identify the chemical nature of the active components present in the flowers.

As the initial step, the vast literature pertinent to the present study was collected and studied in order to understand the present status of research in this field of study. This literature is briefly reviewed in the next chapter.