Chapter 1

Introduction

Almost in the beginning was curiosity.

Isaac Asimov
The survival of living beings including human relies on plants, which fuels the creatures by converting carbon dioxide and water to sugars and nitrogen to amino acids. Plant derived medicines are used since prehistoric times in all civilizations and flourishes even today as the primary form of medicine for perhaps as much as 75-80% of the world's population, for the treatment of common ailments and diseases (Farnsworth et al., 1985; World Health Organization, 2002). Herbal drugs constitute a major share of all the officially recognised systems of health in India viz. Ayurveda, Yoga, Unani, Siddha, Homeopathy, Naturopathy and even Allopathy. More than 70% of India’s 1.5 billion population still use these non-allopathic systems of medicine (Vaidya and Devasagayam, 2007).

A novel approach of pharmaceutical research like chemoprevention, seeks to inhibit progression of cancer. Cancer chemopreventive agents block the transformation of normal cells and suppress the promotion of premalignant cells to malignant cells (Kelloff et al., 1999). The anticancer agents presently used, including the antimetabolites, DNA interactive agents, and tubulin inhibitors, are cytotoxic in nature. The selective toxicity of these agents may be attributed to the fact that they have greater effect on tumor cells as these cells usually divide more rapidly than healthy cells. However, cells of the bone marrow, GI tract and hair follicles also divide rapidly, which explains the consistent pattern of side effects accompanying chemotherapy, that are dose-limiting in practice (Serrano et al., 2004; Popiołkiewicz et al., 2005; Schweizer, 2009). For the development of more effective chemotherapeutic agents, more consistent and clear biochemical differences between normal and tumor cells need to be analyzed and studied. Such differences should allow a more rational approach to drug design rather than relying on the empirical manner in which many of the present-day drugs have been discovered and developed (Sporn and Suh, 2000; Sharma et al., 2001; Vlastos et al., 2003).

The development of drug resistance is another significant problem encountered in cancer chemotherapy. It has been reported that upto 50% of tumors have either de novo drug resistance or otherwise develop resistance to anticancer drugs after initial treatment (Clynnes et al., 1998; Heffeter et al., 2008; Brunelle and Zhang., 2010). Therefore, preliminary selective cytotoxicity toward a tumor can sometimes be followed by a rapid recovery in the rate of tumor growth, and the degree of resistance may increase after each subsequent administration to the point where the
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A chemotherapeutic agent becomes completely ineffective (Seeram *et al*., 2003; Coley, 2009). In current drug discovery approaches, combination drugs and multi-targeted molecules are preferable choices to control complex diseases like cancer, diabetes and inflammation (Kohno *et al*., 2005; Zhang and Meir, 2006; Shapira *et al*., 2011). The combinational drugs are less prone to drug resistance because that influences multiple targets simultaneously.

Literature survey has revealed that the exposure of cell to pro-oxidant and pro-inflammatory insults, trigger a multitude of signal transduction pathways wherein cell responds to potentiate the antioxidant/anti-inflammatory mechanisms to minimize cellular damage (Owuor and Kong, 2002; Vanden Berghe *et al*., 2006; Lin and Karin, 2007; Castor *et al*., 2010; Dorman and Hiltunen, 2011). Transient changes in redox status, communicated via a series of cellular signaling systems, initiate *de novo* synthesis of a distinct set of cytoprotective or stress-responsive proteins and enzymes that are responsible for adaptive cellular responses to noxious stimuli (Sinha and Mamnaugh, 1990). Certain stress-activated protein kinases or other upstream signaling enzymes can activate the redox-sensitive transcription factors, thereby upregulating the expression of early-response genes to adapt and survive the subsequent injury (Powis *et al*., 1995; Kim *et al*., 2004; Hail Jr. *et al*., 2008).

Inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel diseases, cancer, diabetes, multiple sclerosis, psoriasis and asthma, provide tremendous challenge to current drug discovery. The precise cause of these diseases is not known, but they, in general, can be considered as ‘gene expression diseases’ in which the proinflammatory gene program of the organism is aberrantly activated (Baeuerle, 1998; Lu and Xu, 2006). A large number of therapeutic agents are now being examined for autoimmune disorders by targeting single target. But, in most of the cases it becomes difficult to maintain the redundant side effects and drug resistance with monotherapy. Synthesis or modification of known drugs continues as an important aspect of research. However, a vast amount of synthetic work has contributed relatively small improvements over the prototype drugs. There is a continued need for new prototypes and new templates to be used in the design of potential chemotherapeutic agents (Taraphdar *et al*., 2001; Abel *et al*., 2002; Wetzel *et al*., 2010).
Botanicals are thought of offering strong therapeutical efficacy with minimal side effects particularly against autoimmune and metabolic disorders, since most of their efficacies are from a mixture of active molecules acting at the same time (Muthusamy et al., 2008). The plants develop complex chemical arsenals to survive against the myriad of attackers such as insects, fungi, viruses and bacteria, and thus become rich source of secondary metabolites, that have medicinal value (Newman, 1994). The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body. They have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. Ancient literature also mentions herbal medicines for age-related diseases namely memory loss, osteoporosis, diabetic wounds, immune and liver disorders, etc. for which no modern medicine or only palliative therapy is available (Kamboj, 2000).

Although traditional medicines have been used for thousands of years, but for most of them, neither the active component nor their molecular targets have been well identified (Goel et al., 2008). Complex nature, indistinct mechanism of action, herbal-drug interaction and quality assurance are the main disputes of herbal therapy. However, ethnopharmacological observations in the past have resulted in the discovery of several important drugs- for example, taxol, derived from the yew, is an important drug. In pharmaceutical research, ranging from Digoxin (cardiac glycoside) to Prostratin (for the treatment of HIV), the ethnobotanical approach to drug discovery has proved successful (Muthusamy and Lakshmi, 2010). A vast majority of modern drugs are though synthetic analogues but are built on prototype compounds isolated from plants (Newman et al., 2003).

The present era is witnessing a fascinating rejuvenation in the traditional system of medicine. Currently, there is an increasing focus on global search for new drugs derived from natural plant resources like alkaloids, terpenoids, glycosides, amines, steroids, polyphenols etc. (Balunas and Kinghorn, 2005). Among these natural constituents, polyphenolic compounds derived from plants, like flavonoids, tannins, curcuminoids, galloallocatechins, stilbenes and anthocyanidins have been reported to possess wide range of pharmacological properties (Fukuchi et al., 1989; Masuda et al., 1992; Cook and Samman, 1996; Simon et al., 1998; Heim et al.,
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2002; Torres *et al.*, 2003; Khan *et al.*, 2006; Choi *et al.*, 2007; Aslam *et al.*, 2009). A great many reports have established that the polyphenolic compounds are potent antioxidants (Rice-Evans *et al.*, 1996; Cao *et al.*, 1997; Korkina and Afanas’ev, 1997). Phenolic compounds such as catechin, quercetin and curcumin, normally acting as antioxidants are recognized to exhibit prooxidant properties under appropriate conditions such as in the presence of transitional metal ions e.g. copper, an important metal ion present in chromatin and closely associated with DNA bases, particularly guanine (Rahman *et al.*, 1990; Yamashita *et al.*, 1998; Ahsan *et al.*, 1999; Wang *et al.*, 2008). The constitutive intracellular redox environment dictates a cell's response to an agent that alters this environment. The prooxidant action of phenolic compounds may be an important mechanism for their anticancer and apoptosis-inducing properties, as reactive oxygen species (ROS) can mediate apoptotic DNA fragmentation (Hadi *et al.*, 2000; Oikawa *et al.*, 2001).

Triterpenoids is another class of natural products, with more than 20,000 members occurring naturally and includes betulinic acid, boswellic acid, celastrol, diosgenin, madecassic acid, maslinic acid, momordin, saikosaponins, platycodon, pristimerin, ursolic acid, and withanolide (Baas, 1985; Mahato *et al.*, 1992). Polycyclic triterpenoids offer important platforms for drug development because of their anti inflammatory and anticancer properties. These properties may be attributed to their ability to disrupt mitochondrial membrane potential ($\Psi_{mt}$) and thus release of cytochrome $c$ and apoptosis inducing factor (AIF) and Smac from mitochondria (Chen *et al.*, 2008a; Mullauer *et al.*, 2009; Tang *et al.*, 2009; Aggarwal *et al.*, 2009; Wang *et al.*, 2010). A similar situation pertains with the therapeutic and pharmacological activities of alkaloids, natural compounds which are mostly basic in nature and having one or more nitrogen atoms (usually in a heterocyclic ring). As most alkaloids are extremely toxic, plants containing them do not feature strongly in herbal medicine but they have always been important in the allopathic system where dosage is strictly controlled and in homoeopathy where the dose-rate is so low as to be harmless (Du, 2003; Aniszewski, 2007; Yan *et al.*, 2008).

It has been shown that natural compounds can exert modulatory action in cells by interacting with a wide spectrum of molecular targets central to the cell signalling machinery. The molecular mechanisms responsible for the bioactivities of different natural compounds include (1) inhibition of pro-inflammatory enzymes, such as cyclooxygenase (COX-2),
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lipoxygenase (LOX) and inducible nitric oxide synthase (iNOS), through the activation of peroxisome proliferators activated receptor gamma (PPAR γ); (2) inhibition of pro-inflammatory cytokines like IL-1, IL-2, IL-6, and TNFα, chemokines like IL-8, MIP-1α and MCP-1, phosphoinositide 3-kinase (PI3-kinase), tyrosine kinases, nuclear factor-kappa B (NF-κB), c-JUN, adhesion molecules like ICAM, VCAM, and E-selectin, acute-phase proteins, immunoreceptors, growth factors, and inducible enzymes such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs); (3) activation of phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), serin/threonin protein kinase Akt/PKB and (4) modulation of several cell survival/cell-cycle genes (Haridas *et al.*, 2001; Takada and Aggarwal, 2003; Kaminska, 2005; Oak *et al.*, 2006; Santangelo *et al.*, 2007; Tokuda *et al.*, 2007; Xu *et al.*, 2007; Rabi *et al.*, 2008; Yun *et al.*, 2008; Mulabagal *et al.*, 2009; Sogno *et al.*, 2009; Deeb *et al.*, 2010; Yadav *et al.*, 2010; Yoon *et al.*, 2010).

The rapid growth of robust biotechnological and analytical techniques gives a ray of hope in plant based drug discovery approach (Kroll and Cordes, 2006). The potential of conventional herbal remedies, enriched with vast and diverse array of phytochemicals are still not fully explored scientifically and can only be determined by active research in the area of natural products (Chitravadivu *et al.*, 2009). Understanding the mode of action of these natural products will provide useful information for their possible application in cancer prevention and perhaps also in cancer therapy. The present study is a step in this direction, which involves the evaluation of antiproliferative and antioxidative potential of *Schleichera oleosa*, a plant found widespread in tropical Himalayas and least explored for its active constituents and bioactivities.

*Schleichera oleosa*, belonging to family sapindaceae, is a well known medicinal plant in the teak forest of East Java (Indonesia) and is also a prominent bee plant for production of nectar, in parts of Southern India. Prior chemical investigations have established the presence of polyphenolic and triterpene constituents, in the plant (Dan and Dan, 1986). Pettit *et al.* (2000) isolated seven cancer cell growth inhibitory hydroxylated sterols designated schleicherastatins 1-7 and two related sterols, schleicheols 1 and 2, by bioassay (P-388 lymphocytic leukemia cell line)-guided separation of an extract prepared from the bark and stem of the Sri Lankan tree *Schleichera oleosa*. In Ayurvedic system of medicine, the ancient Indian therapeutic measure,
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The bark of *S. oleosa* has been used as an astringent and antipyretic and useful in the cure of leprosy, skin diseases, inflammation and ulcers. It is reported to cure “kapha” and “vata” doshas (Mhasker *et al*., 2000).

Though the plant has been mentioned in Ayurveda and used in traditional system of medicine yet it has not been explored for its bioactivities. Literature survey showed that in spite of reported active constituents like polyphenols and terpenes, no information or report is available with regard to antioxidative and antiproliferative properties of *S. oleosa*. Keeping this fact in view, the present study was planned to meet the following objectives:

- Preparation of different extracts from bark, leaves and roots of *Schleichera oleosa* using sequential extraction method.
- Evaluation of total phenol and flavonoid content in different extracts of *S. oleosa* as per methods given by Yu *et al.* (2002) and Kim *et al.* (2003).
- Evaluation of antioxidative properties employing assays that involve different mechanism of action:
  - Hydrogen and electron donating potential using diphenylpicrylhydrazyl free radical scavenging (DPPH) assay (Blois, 1958), reducing power assay (Oyaziu, 1986).
  - Free radical scavenging potential using non-site and site specific deoxyribose degradation assay (Halliwell *et al*., 1987; Arouma *et al*., 1987), lipid peroxidation assay (Halliwell and Gutteridge, 1989), plasmid DNA nicking assay (Lee *et al*., 2002).
  - Ferrous ion chelation potential using chelating power assay (Dinis *et al*., 1994).
- Determination of *in vitro* cytotoxicity against a panel of human cancer cell lines, from different tissues, using Sulphorhodamine B (SRB) dye assay (Skehan *et al*., 1990) and MTT assay (Heckenkamp *et al*., 1999).
- Evaluation of antiproliferative properties employing assays that involve various mechanistic parameters typical of apoptosis:
  - DNA fragmentation analysis (Bhushan *et al*., 2007).
Flow cytometric analysis using Annexin V/FITC-PI assay (Vermes et al., 1995), TUNEL assay (Li and Darzynkiewicz, 1995), cell cycle analysis (Singh et al., 2007), analysis of Mitochondrial Membrane Potential (MMP) (Bhushan et al., 2006), Reactive Oxygen Species (ROS) generation (Rothe and Valet, 1996), PARP cleavage (Bhushan et al., 2007), cytochrome c analysis (Campos et al., 2006).

Colorimetric analysis for determination of Caspases activity (Sun et al., 1999).

Immunoblotting for TNF-R1 and BAX proteins (Findley et al., 1997; Wang et al., 2002; Lin et al., 2005; Kumar et al., 2008).

Determination of Topoisomerase inhibitory activity (Wang, 1971; Osheroff et al., 1983; Shelton et al., 1983).

Isolation and characterization of bioactive constituents in most active extracts/fractions, following bioactivity guided fractionation employing a panel of human cancer cell lines and using chromatographic and spectroscopic techniques (Column chromatography, Thin layer chromatography, NMR, Mass spectroscopy and FTIR).