Chapter – I

Introduction
Introduction

Since the beginning of human civilization, medicinal plants have been used by mankind for its therapeutic value. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. The plant-based, traditional medicine systems continue to play an essential role in healthcare, with about 80% of the world’s inhabitants relying mainly on traditional medicines for their primary health care (Owolabi et al., 2007). The use of complementary and alternative medicines has recently increased, thereby enhancing the market for herbal products worldwide (Budeker and Kronenberg, 2002; Zollman and Vickers, 1999).

However, the purity of these herbal formulations exposes the human population to multiple risks and creates major concerns for various health agencies on both national and international levels (Fang, 2002). The three main research approaches are (a) bioactivity- or mechanism of action-directed isolation and characterization of active compounds, (b) rational drug design-based modification and analogue synthesis, and (c) mechanism of action studies (Kuo-Hsiung, 2004). The use of natural products with therapeutic properties is as ancient as civilisation and, for a long time, mineral, plant and animal products were the main sources of drugs (De Pasquale, 1984). Furthermore, throughout the development of human culture, the use of natural products has had magical-religious significance and different points of view regarding the concepts of health and disease existed within each culture. Obviously, this approach was against the new modus of the industrialized western societies, in which drugs from natural resources were considered either an option for poorly educated or low income people or simply as religious superstition of no pharmacological value (Rates, 2001). About 25% of the drugs prescribed
Introduction

worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO, 1992), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank et al., 1982; Vulto and Smet, 1988; Mentz and Schenkel, 1989). This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world’s population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that “natural” products are harmless. However, the use of these substances is not always authorised by legal authorities dealing with efficacy and safety procedures, and many published papers point to the lack of quality in the production, trade and prescription of phytomedicinal products (Rates, 2001).

India has several traditional medical systems, such as Ayurveda and Unani, which has survived through more than 3000 years, mainly using plant-based drugs. The materia medica of these systems contains a rich heritage of indigenous herbal practices that have helped to sustain the health of most rural people of India. The ancient texts like Rig Veda (4500-1600 BC) and Atharva Veda mention the use of several plants as medicine. The books on ayurvedic medicine such as Charaka Samhita and Susruta Samhita refer to the use of more than 700 herbs (Jain, 1968).
Introduction

The known vegetable kingdom includes around 400,000 - 500,000 varieties of plant and only a very small portion of these have been analyzed to determine what compounds they contain. Secondary metabolites, in turn represent only a fraction of the compounds contained in plant material. The concept of a secondary metabolite and the term used for it has been known since 1891 (Karlovsky, 2008). The current interest in secondary metabolites obtained from the plant kingdom was probably stimulated by the hope of obtaining new sources of compounds potentially useful in therapeutic programs. In the United States, herbal products are mostly registered as dietary supplements since the Food and Drug Administration does not accept bibliographic evidence of effectiveness, but prefers randomized controlled trials as evidence of efficacy (WHO, 1998).

It therefore became the aim to produce the active principles of all medicinal plants as far as possible as pure substances, which could then be investigated in the same way as clearly-definable chemical compounds. A new trend developed where such native substances were looked for, designating those phytopharmaceuticals. NMR spectroscopy has been an important analytical tool for investigating natural compounds for many years. It is an excellent alternative to X-ray diffraction for compounds that are difficult to crystallize like to X-ray diffraction, NMR analysis gives good quality information about the structures of simple natural compounds obtained from plants. Combination of NMR with other experimental methods, such as HPLC, enables the control of precursors and products, the determination of compounds with biological activity, the study of the pharmacokinetics of drugs or their metabolism in body fluids, and the fast analysis of plant extracts (Lenka, 2010). As a result it became possible to elucidate the mode of action of many of the old medicinal plants, and there can be no
doubt that this brought major advances in phytotherapy. This new approach which allowed the subject to become open to scientific investigation. In addition to structural information about natural compounds, NMR drug research generally includes characterization of a range of its physical and chemical properties and the possibility of studying its interactions with biomacromolecules. The rapid development of NMR techniques has offered many new opportunities for more effective research on natural products.

Research into the use of plant-derived natural products alone in the field of medicine covers a broad spectrum of activities (Havsteen, 1983; Wrigley and Chicarelli, 1997; Yao et al., 1998; Dahanukar et al., 2000; Yu et al., 2000). Examples of such biological activity profiles would include nootropics, psychoactive agents, dependence attenuators, anticonvulsants, sedatives, analgesics, anti-inflammatory agents, antipyretics, neurotransmission modulators, autonomic activity modulators, autacoid activity modulators, anticoagulants, hypolipidemics, antihypertensive agents, cardioprotectants, positive ionotropes, antitussives, antiasthmatics, pulmonary function enhancers, antiallergens, hypoglycemic agents, antifertility agents, fertility-enhancing agents, wound healing agents, dermal healing agents, bone healing agents, compounds useful in the prevention of urinary calculi as well as their dissolution, gastrointestinal motility modulators, gastric ulcer protectants, immunomodulators, hepato-protective agents, myeloprotective agents, pancreato-protective agents, oculo-protective agents, membrane stabilizers, hemato-protective agents, antioxidants, agents protective against oxidative stress, antineoplastics, antimicrobials, antifungal agents, antiprotozoal agents, antihelminthics, and nutraceuticals (Dahanukar et al., 2000). Many frontiers remain within
the field of natural products that can provide opportunities to improve our quality of life.

Nature offers resources that include compounds which can potentially solve many of these problems. Investigation of natural products obtained from plants – the isolation of compounds and their modification, and the evaluation of their biological activities – represents an important field of biochemical and pharmaceutical research. The term natural products today is quite commonly understood to refer to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine (Holt and Chandra, 2002). While in some cases, discovery and development of drug may have been based on herbs, folklore, or traditional or alternative medicine, the research and discovery of, along with the development of, herbal remedies or dietary supplements typically present different challenges with different goals (Lang and Wai, 2001; Spainhour, 2001). So while the stories of herbs and drugs are very much intertwined, it needs to be fully appreciated that the use of herbs as natural product therapy is different than the use of herbs as a platform for drug discovery and further development. The appearance of new and more complicated diseases in recent years, along with persistence of old ones, constrains scientists to seek new and more effective methods of treatment. In addition to prevention, it is essential to develop of more effective methods to diagnose illnesses in their early stages, when treatment can be more effective.

1.1 Plant as a drug

From the earliest times, herbs have been prized for their pain-relieving and healing abilities and today we still rely largely on the curative properties of plants. Herbal medicines have a vital role in the
prevention and treatment of diseases and medicinal herbs are commonly available and comparatively economical. Of the 15,000 species of plants found in India, about 47% are considered to be of medicinal value (Nadkarmi, 1954; Jain, 1968; Pei, 2001; Singh et al., 2005). Western Ghats is one of the richest zones of India and provides more than 300 species of medicinal plants (Gaur, 1999; Parandial et al., 2005; Bhatt and Negi, 2006). This knowledge of using native plants and its associated medicinal practice have now become a part of the local tradition, culture, art, belief and folklore (Pushpangadan and Kumar, 2005). During the last few decades, there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world (Al- Quran, 2005; Hanazaki et al., 2000; Rossato et al., 1999). Documenting indigenous knowledge through ethnomedicinal studies is important for the conservation and utilization of biological resources. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases (Azaizeh et al., 2003). Due to limited means of communication, poverty and unavailability of modern health facilities, many people, especially in rural areas, still rely on traditional medicines to treat common ailments, and many of these people form the poorest link in the trade of medicinal plants (Khan, 2002). A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance (Diallo et al., 1999).

The India coordination project on ethnobiology (AICRPE, 1992-1998) reported the use of over 10,000 wild plant species by tribal communities in India to meet their primary healthcare, food and other material requirements. Out of 8,000 wild plant species used by them for medicinal purposes (with over 175,000 specific preparations and
applications), about 2,000 seem worthy of scientific scrutiny. The medicine and aromatic plants sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives of India (Bhattacharya et al., 2005).

One such medicinal plant is *Kirganelia reticulata* Baill. belonging to family Euphorbiaceae, is a monoecious scandent shrub or small bushy tree, up to 5 m tall (in Africa rarely up to 18 m tall); trunk up to 15 cm in diameter, bark rough, brown to grey, branchlets slender. Leaves differently shaped; alternate, small or moderate sized. Flowers monoecious, male and female mixed, in axillary clusters sometimes appearing racemose from the absence of leaves. Fruit is a fleshy 5-12 celled berry (Gamble, 1921). It grows along watercourses, but also in scrub and hedges, on waste places, and in mixed evergreen forest. It is found in India and Taiwan up to 2000 m altitude. This species is often common in moist places. *K. reticulata* is found throughout the tropics. In Asia it is widely distributed from India and Sri Lanka to southern China and eastern Malaysia (Irian Jaya), including the whole of South-East Asia. This species is also widespread in tropical Africa. The synonym is *Phyllanthus reticulatus* Poir. Commonly called as Sanna Kagesoppu, Krishnanelli in Kannada; Panjoli in Hindi etc.
A black ink is prepared in the Philippines from the ripe fruits. In Indonesia a decoction of stem and leaves was used for dyeing cotton black. It is also used as a mordant. In India the root is reported to produce a red dye. Stem is used for basketry, roofing huts and wood ash with glue is used for paving boats. It has numerous medicinal uses. Roots, bark, leaves, as well as fruits are used for a large number of complaints, notably to treat asthma and coughs, and for injuries of the skin. Fruit is used in dressing syphilitic sores; also as a purgative. Bark
used in rheumatism, dysentery and venereal diseases (Yoganarasimhan, 1996). Bark ingredients are used for attennuant, astringent and diuretic. Fruits are used for inflammation and blood related diseases. Leaf is used for bleeding gums, diarrhoea in infants, burns, suppuration, chapped skin, small pox and syphilis. Stem juice is used for eyes irritation.

One very important reason behind selecting this particular plant for the study is that, it is widely used in the traditional practices. The formulation of leaves of this plant is used in treating joint pain, fracture pain and other bone pain related issues in a place called Muduba, near Shivamogga district. The research carried out was focused towards providing scientific basis for the ethanobotanical claim of the plant.

Thorough review literature suggests some of the researches already done on this plant. Previous phytochemical investigations resulted in the isolation of several compounds (Ghani, 2003). The preliminary antimicrobial, analgesic, anti-inflammatory, antidiabetic activities are reported. The extracts also contains highest amount of polyphenols compounds and exhibits the greatest antioxidant activity through the scavenging of free radicals which participate in various pathophysiology of diseases including ageing. It also exerts iron chelating and reducing power activity. Overall, the plant extract is a source of natural antioxidants that can be important in disease prevention, health preservation and promotion of longevity promoter. In vitro antiplasmodial, phytochemical analysis and hepatoprotective activities of the plant have been reported.

1.2 Osteoarthritis and its Drug Target

Osteoarthritis is the most common type of arthritis, and is seen especially among older people. Sometimes it is called degenerative joint disease or osteoarthritis. Osteoarthritis mostly affects cartilage, the hard
but slippery tissue that covers the ends of bones where they meet to form a joint. Healthy cartilage allows bones to glide over one another. Between the cartilages of two bones which form a joint there is a small amount of thick fluid called synovial fluid. This fluid 'lubricates' the joint which allows smooth movement between the bones. Osteoarthritis is characterized by joint degeneration, loss of cartilage and alterations of subchondral bone. In osteoarthritis, the surface layer of cartilage breaks down and wears away. This allows bones under the cartilage to rub together, causing pain, swelling and loss of motion of the joint. Over time, the joint may lose its normal shape. Also, small deposits of bone – called osteophytes or bone spurs – may grow on the edges of the joint. Bits of bone or cartilage can break off and float inside the joint space. This causes more pain and damage. People with osteoarthritis usually have joint pain and some movement limitations. Unlike some other forms of arthritis, such as rheumatoid arthritis, osteoarthritis affects only joint function and does not affect skin tissue, the lungs, the eyes, or the blood vessels.

Fig 2: Pathway showing Osteoarthritis (Matthew et al., 2010)
In the articular chondrocytes in the synovial joint, HIF-1α promotes homeostatic pathways, and HIF-2α promotes degradative pathways that foster osteoarthritis. The articular cartilage resides in hypoxic, avascular conditions within the synovial joint. Chondrocytes, cells of the articular cartilage, are affected by various forms of stress (biomechanical, inflammatory and aging), as well as the loss of synovial fluid boundary lubricants and the increase of certain factors released from subchondral bone and synovium. Within the normal, unstressed chondrocyte (HIF-1α pathway), the hypoxic response transcription factor HIF-1α supports normal cartilage extracellular matrix synthesis and chondrocyte differentiation and promotes autophagy—all central activities in articular cartilage homeostasis. These effects of HIF-1α are antagonized by the closely related HIF-2α. HIF-2α promotes chondrocyte hypertrophy, a terminal differentiation state characterized by a unique gene expression program, including type X collagen and the type II collagen-degrading protease MMP-13. This switch to hypertrophy seems to be a relatively early signal to ignite and drive osteoarthritis in stressed cartilage. The preferential heterodimerization of HIF-2α with ARNTL creates the most potent set of partners for inducing chondrocyte hypertrophy. HIF-2α promotes hypertrophy, and complementary mechanisms (via IHH and RUNX2) stimulate increased expression of the major matrix-degrading protease ADAMTS5. The transmembrane heparan sulfate proteoglycan syndecan-4 (acting in part by inducing MMP-3 expression) stimulates activation of ADAMTS5 in hypertrophic chondrocytes. Inhibitors of ADAMTS5 and MMP-13 are already in clinical development (Matthew et al., 2010).

Proinflammatory cytokines are mediators of inflammatory state and cartilage degradation in both rheumatoid arthritis and osteoarthritis (OA). In particular, interleukin (IL)-1band tumour necrosis factor-a (TNF-
a) activate chondrocytes to produce matrix-degrading factors and promote a catabolic condition (Goldring, 2000). These cytokines up-regulate inducible enzymes such as nitric oxide synthase-2 (NOS-2) and cyclo-oxygenase-2 (COX-2) which is prominently expressed in the synovium, fibrocartilage of osteophytes and in the blood vessels in the OA knee joint (Koki et al., 2002). Heme oxygenase-1 (HO-1) is a stress-responsive protein with cytoprotective and antiinflammatory properties (Alcaraz et al., 2003). Nevertheless, the role of this enzyme in chronic inflammatory disorders has not been established. The conversion of arachidonic acid to prostaglandin-H2 (PG) H2 by COX enzymes is the rate-limiting reaction in the synthesis of prostanoids, which are responsible for normal physiologic functions and also for inflammation and pain. COX-1 is expressed constitutively in most tissues including the gastrointestinal tract, kidneys and platelets, whereas COX-2 is expressed at low levels in normal tissue, but it is strongly induced by inflammatory mediators (Vane and Botting, 1998). Selective COX-2 inhibitors are as effective as traditional nonsteroidal anti-inflammatory drugs for the treatment of arthritis and pain, with a lower incidence of gastrointestinal side effects (Hochberg, 2002).

Doctors prescribe medicines to eliminate or reduce pain and to improve functioning. Treatment recommendations for osteoarthritis may help millions of people worldwide who live in pain temporarily, but all the drugs have some or the other side effects. A medication commonly used to relieve pain, acetaminophen is often the first medication doctors recommend for osteoarthritis patients because of its safety relative to some other drugs and its effectiveness against pain. A large class of medications useful against both pain and inflammation, NSAIDs are staples in arthritis treatment. A number of NSAIDs - ibuprofen, naproxen sodium and ketoprofen are available over the counter. The U.S Food and
Introduction

Drug Administration have warned that long-term use of NSAIDs, or use by people who have heart disease, may increase the chance of a heart attack or stroke. Side effects can also include stomach upset and stomach ulcers, heartburn, diarrhea and fluid retention. Other medications include topical pain-relieving creams, rubs and sprays; mild narcotic painkillers; Corticosteroids; Hyaluronic acid substitutes; surgery etc. (U.S. Department of Health and Human Services, 2006). Hence there is a need to develop newer and safer drug which is cost effective to meet the demands of the patients in the Indian scenario.

Drug target is a key molecule involved in a particular metabolic or signaling pathway that is specific to a disease condition. Since beginning of modern biology, researchers have sought ways to better elucidate the relationships among protein sequence, structure and function. The central dogma of biology holds that DNA sequence encodes the protein sequence, which in turn determines the three dimensional structure of protein and hence its function. Currently, there are several major realms of protein study: structural studies using X-ray crystallography, nuclear magnetic resonance (NMR), or both to determine the final three dimensional shape that proteins assume in the cell; functional studies; using mass spectrometry to examine the regulation, timing and location of protein expression; and interaction studies, which examines how proteins pair between themselves and other cellular components to form more complex molecular machines. Proteomics evaluate the mechanisms present in the cell that up regulate or down regulate the products of genes (Andreas and Francis, 2006). The purpose is to reduce the number of targets for a good drug that has to be subjected to expensive and time-consuming synthesis and trialling. The obtained protein indicates a potential role in the causation of the disease.
1.3 *In silico* Drug Designing

Drug development over the years has relied only on a small number of molecular prototypes to produce new medicines (Harvey, 1999). Indeed, only approximately 250 discrete chemical structure prototypes have been used up to 1995, but most of these chemical platforms have been derived from natural sources. The overwhelming concern today in the pharmaceutical industry is to improve the ability to find new drugs and to accelerate the speed with which new drugs are discovered and developed. This will only be successfully accomplished if the procedures for drug target elucidation and lead compound identification and optimization are themselves optimized. Analysis of the human genome will provide access to a myriad number of potential targets that will need to be evaluated (Harvey, 2001; Grabley and Sattler, 2003). The process of high-throughput screening enables the testing of increased numbers of targets and samples to the extent that approximately 100,000 assay points per day are able to be generated. However, the ability to accelerate the identification of pertinent lead compounds will only be achieved with the implementation of new ideas to generate varieties of structurally diverse test samples (Harvey, 1999; Harvey, 2001; Grabley and Sattler, 2003). Natural products have revealed the ways to new therapeutic approaches, contributed to the understanding of numerous biochemical pathways and have established their worth as valuable tools in biological chemistry, molecular and cellular biology.

Drug design is the approach of finding drugs by design, based on their biological targets. Typically a drug target is a key molecule involved in a particular metabolic or signalling pathway, specific to a disease condition. The structure of the drug molecule that can specifically
Introduction

interact with the biomolecules can be modeled using computational tools. If an experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein. This approach to drug discovery is referred as structure-based drug design. Computer-assisted drug design uses computational chemistry to discover, enhance, or study drugs and related active biomolecules. Although no single drug has been designed solely by computer techniques, the contribution of these methods to drug discovery is no longer a matter of dispute. All the world's major pharmaceutical and biotechnology companies use computational design tools. It is a new approach attracting increasing levels of interest in the pharmaceutical industry as a productive and cost-effective technology in the search for novel lead compounds.

Although the principles involved to identify compounds appropriate for a given biological receptor have been pursued for several years in molecular modeling groups, the availability of inexpensive high-performance computing platforms has transformed the process so that increasingly complex and more accurate analyses can be performed on very large datasets. Automated docking combines energy evaluation through pre calculated grids of affinity potential employing various search algorithms to find the suitable binding position for a ligand on a given protein (Morris et al., 2009). Identifying the molecular interactions using bioinformatics tools before venturing into wet lab studies saves the energy and time considerably.
Objectives of the research work

➢ To build the pharmacognostic profile of the plant *Kirganelia reticulata*.

➢ To extract crude fractions from *Kirganelia reticulata* and its phytochemical screening.

➢ To determine the antimicrobial potential of the investigated extracts.

➢ To isolate bioactive compounds from the crude extract and its characterization.

➢ To evaluate the anti-arthritic property of the extracts as well as isolated compounds through *in vivo* and *in vitro* studies.

➢ To determine the pharmacological activities such as anti-inflammatory, anti-analgesic, antihelminthic and antioxidant through *in vivo* and *in vitro* methods.

➢ To study the signaling pathway of Osteoarthritis and its Proteomic analysis.

➢ To screen candidate drug molecules *in silico* for protein-ligand interaction.

➢ To compare docking studies of the obtained compounds with commercial drugs.