Chapter V

Discussion
During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (WHO, 1999). Popular observations on the use and efficacy of medicinal plants significantly contribute to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not always completely known (Silva and Fernandes, 2010). The phytotherapics entered the market promising a shorter and cheaper production, since basic requirements to use medicinal plants do not involve strict quality control regarding safety and efficacy compared to the other types of drugs (Niero et al., 2010).

There is a growing awareness by scientific and medical communities about the importance of medicinal plants in the health care systems of many developing countries. Scientific projects have been launched to explain the curative phenomena associated with traditional herbal remedies and to identify simple technology that could produce drugs and therapeutic agents at a low cost to alleviate suffering and disease. Plants contain a number of chemical constituents and are employed for different medicinal purposes; however, over-dosage of plant products containing medicinal compounds may cause toxic reactions when introduced into animals or human beings (Amel and Salah, 1995).

*Kirganelia reticulata* Baill., commonly known as Krishnanelli is a rich plant containing various bioactive components. The plant has been evaluated earlier for numerous activities. The present study was
undertaken to evaluate the anti-arthritis and other pharmacological effects of the plant. Osteoarthritis is a disease involving a disturbance of the normal balance between degradation and repair in the articular cartilage and subchondral bone accompanied by capsular fibrosis, marginal osteophyte formation and variable grade of inflammation of the synovial membrane. Well established limitations of NSAID therapy, however, include the risk of developing significant injury to the upper gastrointestinal tract. They do not change the bone mass or replenish the already lost bone. There is a timely need to develop new drugs of natural or synthetic origin which will possess less undesirable side effects and can substitute or reduce the need for currently used drugs (Dieppe, 1995; Fred et al., 2008).

5.1 Pharmacognosy

Few plant species that provide medicinal herbs have been scientifically evaluated for their possible medical application. Safety and efficacy data are available for even fewer plants, their extracts and active ingredients, and the preparations containing them. Furthermore, in most countries the herbal medicines market is poorly regulated, and herbal products are often neither registered nor controlled. Assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. Both the general consumer and health-care professionals need up-to-date, authoritative information on the safety and efficacy of medicinal plants. The quantitative determination of some pharmacognostic parameters is useful for setting standards for crude drugs.

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. Each monograph contains two parts. The first part consists of pharmacopoeial summaries for quality assurance: botanical features, distribution, identity tests, purity requirements, chemical assays, and active or major
chemical constituents. The second part summarizes clinical applications, pharmacology, contraindications, warnings, precautions, potential adverse reactions and posology. WHO encourages countries to provide safe and effective traditional remedies and practices in public and private health services (WHO, 1999).

The result of these investigations could therefore, serve as a basis for proper identification, collection and investigations of the plants. The macro and micro morphological features of the leaf described the fluorescence behaviour, qualitative leaf microscopy and physico-chemical studies are parameters that are unique to the plant and are required in its standardization. The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent (Ozarkar, 2005). The moisture content of the drug is not too high, thus it could discourage bacteria, fungi or yeast growth, as the general requirement for moisture content in crude drug is not more than 14% (African Pharmacopoeia, 1986).

By virtue of their photosynthetic machinery, leaves serve as a sink for several metabolites and as an important source of several bioactive compounds (Murti et al., 2010). Empirical knowledge about medicinal plants plays a vital role in primary health care and has great potential for the discovery of new herbal drugs. These findings may be useful to supplement existing information with regard to the identification and standardization of *K. reticulata*, even in the powdered form of the plant drug, to distinguish it from substitutes and adulterants. These studies also suggested that the observed pharmacognostic and physiochemical parameters are of great value in quality control and formulation development. In conclusion, the present study may be useful to supplement information with regard to its identification and standardization, and in carrying out further research and revalidation of its use in the Ayurvedic System of Medicine.
5.2 Phytochemistry

Natural products provide unique chemical diversity, distinct from that found in synthetic or combinatorial chemical libraries currently available. It is important to note however, that chemist working predominantly in the fields of either natural products or combinatorial chemistry that can demonstrate the beautiful chemical diversity of natural products (Stephen, 2000). Synthetic drugs are perceived to have certain disadvantages in relation to compounds derived from biological systems. Synthetic drugs are viewed as insufficiently complex and as having limited structural rigidity. There is increasing evidence that many synthetic drug therapies simply suppress symptoms and ignore the underlying disease process. In contrast, many natural products, including nutritional supplements, glandular products, and herbal medicines, appear to address the cause of many diseases and yield superior clinical results.

The preliminary phytochemical evaluation revealed the presence of several secondary metabolites which are known to possess various pharmacological effects. It may be attributed to the reason that the stronger extraction capacity of ethanol could have extracted a greater number of constituents. These compounds are known to be biologically active and hence aid the investigation of several activities. These observations therefore support the use of K. reticulata in herbal cure remedies. Alkaloids were detected together with flavonoids, this may be responsible for the antioxidant activity observed in the crude extracts. According to van Beek et al., (1984), who studied a large number of plant extracts against various activities, ethanolic extracts always show positive effect. In last four decades the scientists are keen to evaluate many plant drugs used in medicinal folklore, due to their specific healing properties, health action and non toxic effects (Singh et al., 2002).
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Cheminformatics is defined as the mixing of information resources to transform data into information, and information into knowledge, for the intended purpose of making better decisions faster in the arena of drug lead identification and optimization. Cheminformatics plays a vital link between theoretical design and in drug design through extraction of information from the data and convert into knowledge. Cheminformatics methods can be used proactively to design and filter the most appropriate compounds to work within the real world. The drug likeliness of the obtained compounds was studied using ChemDraw Ultra to know the chemical and molecular properties of ellagic acid, quercetin and polyrenol.

Ellagic acid is a polyphenol found at high concentrations in a number of fruits like grapes, strawberries, black currants and raspberries. Ellagic acid is a naturally occurring plant polyphenol that exhibits antioxidative properties both in vivo and in vitro. Recently dietary polyphenols are receiving increasing attention as potential protectors against a variety of human diseases like cancer and chemotherapy induced toxicity in animal models (Ahmet et al., 2010). Ellagic acid is a dietary supplement, reported to have anti-inflammatory, antinociceptive properties via cyclooxygenase inhibition. It has been found to have anticarcinogenic, antifibrosis and antioxidative properties. The effects of ellagic acid on cell cycle events and apoptosis were studied in cervical carcinoma (CaSki) cells.

Quercetin is a plant derived flavonoid used as a nutritional supplement found in fruits and vegetables. It is thought to have potent antioxidant, antidiabetic, anti tumour, antiviral and anti inflammatory benefits. It is mainly found in many often consumed foods include green apple, onion, green tea, lemon as well as many seeds, flowers, barks and leaves. There has been increasing interest of it in the sports, science and athletic communities due to scientific and clinical research results that show quercetin as antioxidant, anti-inflammatory and other properties as likely
to improve mental and physical performance. It has been promoted as being at effective against wide variety of diseases including cancer (Phani et al., 2010). Quercetin has several biological activities such as antiproliferative effect and apoptosis induction.

Polyrenols (α-unsaturated isoprenoid alcohols) occur in green tissues of many plants and have also been found in bacteria as a structural component of the membranes (Tomas et al., 2000). Polyrenols are low molecular natural bioregulators, playing a significant modulating role in the cellular process in plants referred to as biosynthesis. Polyrenols stimulate the immune system, cellular reparation and spermatogenesis, and have antistress, adaptogenic, antiulcerogenic and wound-healing activity. Experiments on mice have demonstrated that polyrenols have antiviral activity, in particular against influenza viruses.

The obtained compounds and plant extracts were screened for various pharmacological activities which are discussed below.

5.3 Pharmacological Screening

5.3.1 In vivo studies

a. Analgesic activity

Due to having adverse side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as analgesic agents have not been successful in all the cases. Therefore, analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates. During this process, the investigation of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention because they are cheap, have little side effects and according to WHO still about 80% of the world population rely mainly on plant-based drugs (Kumara, 2001).
Acetic acid induced writhing in mice attributed visceral pain finds much attention of screening analgesic drugs (Hasan et al., 2010). Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid (Ahmed et al., 2006) via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte et al., 1988). In other words, the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products (Derardt et al., 1980). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria and Abdul, 2008). The acetic acid induced writhing method was found effective to evaluate peripherally active analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte et al., 1988; Ferdous et al., 2008).

Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phase (Raoch et al., 2005). The formalin test is a very useful method for not only assessing antinociceptive drugs but also helping in the elucidation of the action mechanism. The neurogenic phase is probably a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance. While the late phase is due to the release of histamine, serotonin, bradikynin and prostaglandins. Methanol extract and Ellagic acid was able to block both phases of the formalin response but the effect was more prominent in the second phase.

The significant pain reduction of test drugs might be due to the presence of analgesic principles acting with the prostaglandin pathways. Therefore, the methanolic extract of the plant and isolated Ellagic acid must have a central activity. Again, narcotic analgesics inhibit both peripheral and central mechanism of pain, while NSAIDs inhibit only
peripheral pain (Elisabethky et al., 1995; Pal et al., 1999). Our test drugs have exhibited both types of pain inhibition. The analgesic effect of them in both the models suggests that they have been acting through central and peripheral mechanism (Sabina et al., 2009).

b. Anti-inflammatory activity

Carrageenan-induced acute inflammation is one of the most suitable test procedures to screen anti-inflammatory agents. Development of carrageenan-induced oedema is biphasic; the first phase is attributed to the release of histamine, 5-HT and kinins, while second phase is related to the release of prostaglandins (Larsen and Henson, 1983; Vane and Booting, 1987; Brooks and Day, 1991). It has been reported that the second-phase oedema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents (Katzung, 1998). Folkloric treatment of inflammation of various etiologies, using medicinal plants, is well known to masters of the art of traditional medicine practice. Pharmacological screening of plant extracts has revealed that the leaf possesses potent anti-inflammatory effect in the topical and systemic models of acute inflammation. These extracts and compounds may have inhibited the release of pro-inflammatory mediators of acute inflammation such as histamine and prostaglandin. Interestingly, the extract caused gastrointestinal irritation in rats typical of anti-inflammatory prostaglandin inhibitors such as the non-steroidal anti-inflammatory drugs NSAIDs (Rang and Dale, 1988). Thus, these extracts may exert anti-inflammatory effect by inhibiting the synthesis of prostaglandin.

Our test drug ellagic acid shows anti-inflammatory activity at various acute phases of inflammation and on formation of oedema. The presence of phenolics and tannins assumes importance since the role of free radicals in inflammation cascade is well known and phenolics are established to have free radical scavenging activity (Haslam, 1995). The
magnitude of activity obtained indicates high potency of anti-inflammatory effect and together with the array of compounds already isolated from the plant provide impetus to continue the search for novel anti-inflammatory constituents from this plant.

c. Anti-arthritis activity

Formaldehyde induced paw oedema is one of the most suitable test procedures to screen chronic anti-inflammatory agents, as it closely resemble human arthritis. The nociceptive effect of formalin is biphasic; an early neurogenic component followed by a later tissue-mediated response (Wheeler and Cowan, 1991). In formaldehyde induced arthritis model, rats developed a chronic swelling in paw volume with the influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodelling. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal (Carl, 1963). The determination of rat paw swelling is apparently simple, sensitive and one of the quick procedures for evaluating the degree of inflammation and the therapeutic effects of drugs. The chronic inflammation involves the release of number of mediators like cytokines, GM-CSF, interferon's and PGDF. These mediators are responsible for the pain, destruction of bone and cartilage that can leads to severe disability (Eric and Lawrence, 1996). However, methanol extract and ellagic acid significantly suppressed the swelling of the rat paws.

Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti-inflammatory drugs (Winder et al., 2005). As the incidence and severity of arthritis increased, the changes in the body weights of the rats also occurred during the course of the experimental period. The loss of the body weight during arthritic condition was also supported by earlier observations, (Walz et al., 1971)
on alterations in the metabolic activities of diseased rats. Earlier findings suggest that absorption of 14C-glucose and 14C-leucine in rat's intestine was reduced in the case of inflamed rats (Somasundaran et al., 1983a). Treatment with methanol extract and ellagic acid, the decrease in absorption was nullified (Somasundaran et al., 1983b) and it shows that these drugs have corrected the decreased absorption capacity of intestine during inflammation. The increased body weight during the treatment of test drugs may be due to the restoration of the absorption capacity of the intestine.

Radiographs are necessary to determine true remission of disease and for accurate evaluation of disease status. The measurement of paw or joint swelling only gives an indication of oedematous changes in this region; however the actual damage takes place in the tibiotarsals joint (Escandell et al., 2007). Reduced bone formation and increased resorption are the causes of bone loss in formaldehyde induced arthritis in rats (Auta et al., 1996; Findlay and Haynes, 2005; Makinen et al., 2007). The x-rays clearly show that ellagic acid decreased bone loss and therefore in addition to suppressing joint inflammation and retard disease progression, it also reduced bone degradation in arthritis.

5.3.2 In vitro studies

a. Antimicrobial activity

Since ancient times, plants have been used by several communities to treat a large number of diseases, including infections. Numerous studies on the pharmacology of medicinal plants have been accomplished, since they constitute a potential source for the production of new medicines and may enhance the effects of conventional antimicrobials, which will probably decrease costs and improve the treatment quality. However, several plants may present antagonistic effects during antibiotic therapy. An important aspect comprises the search for new compounds that have antimicrobial action and synergism with currently available
antimicrobial drugs, since bacteria resistant to conventional medicines are increasingly frequent; consequently, medicinal plants constitute an alternative for infection treatment. The antimicrobial activity of different plants was proven by various examples, in the form of extracts and isolated components. Thus, this property can be a promising ally in the development of medicines necessary to combat the increasing number of bacterial strains that become resistant to conventional antibiotics (Silva and Fernandes, 2010).

The result of this study showed that methanol extract and polyprenol have varied antibacterial activities against the tested organisms. This suggests that these test drugs are broad spectrum in their activities. This correlates with the observation of previous workers that plants contain substances that are antimicrobial (Olukoya et al., 1986). The antimicrobial effect of methanol extract against the organisms may be due to the ability of the methanol to extract some of the active properties of the plants and other secondary metabolites which are reported to be antimicrobial (Cowan, 1999; Okwu and Josiah, 2006). Some authors have found that highly oxidized phenols posses more inhibitory activity (Scalbert, 1991). The mechanism of action of terpenes is speculated to involve membrane disruption by the lipophilic compounds. Alkaloids are also found to have microbicidal effect. The mechanism of action of highly aromatic planar quaternary alkaloids is attributed to their ability to intercalate with DNA (Phillipson and Neill, 1987).

Many human physiological activities such as stimulation of phagocytic cells, host mediated tumour activity and a wide range of anti-infective actions, have been assigned to tannins. One of their molecular actions is to complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1995; Stern et al., 1996). Thus their mode of antimicrobial action is related to their ability to inactivate microbial adhesions, enzymes, cell envelopes transport proteins etc., they also complex with
polysaccharides (Ya et al., 1988). The polyprenol inhibited pathogens nonspecifically, may be by neutralizing the bacterial cell surface hydrophobicity and might be also mimicking the host cell receptors, thereby blocking its binding to host cell and further not by inducing pathological effect.

Existing antibiotics probably act on the plasma membrane of bacteria affecting the efflux pumps (Kristiansen and Amaral, 1997). This modification of permeability could enhance the activity of antibiotics that act within the cell, such as the aminoglycosides. Several studies have been performed to identify drugs interfering with these pumps, called resistance modifying agents (Gunicz et al., 2002). The results obtained indicate that our isolated compound polyprenol could serve as a source of plant-derived natural products with antibiotic resistance-modifying activity to be used against multiresistant bacteria as MRSA strains acquired from hospital and community. Slime production has been reported in strains of all *Staphylococcus* spp. associated with the infection of biomedical devices (O Gara and Humphreys, 2001). Investigations to understand the pathogenesis of these infections have focused upon the process of adherence of these microorganisms on these devices. Our polyprenol exhibited to be one of the strong biofilm inhibiting agents. The inhibitory effect of the methanol extract and polyprenol of *K. reticulata* against pathogenic bacterial strains can introduce the plant as a potential candidate in drug development for the treatment of ailments caused by these pathogens.

b. Antioxidant activity

Free radicals have one or more unpaired electrons; therefore, they react with substrates either by electron transfer or by transfer of hydrogen atom. Electrons usually paired one electrically charged and rotate upon themselves while inducing a magnetic field called spin. An electron doublet is more stable than two isolated electrons because the pairing of
two electrons with opposite spin cancels their reciprocal magnetic fields (Pierrefiche and Laborit, 1995). On the other hand, a free radical is a neutral or charged chemical species whose peripheral shell contains an unpaired electron called, singular electron. Free radicals thus contain an odd number of electrons (Buechter, 1988). Free radicals are produced continuously within the cells. The main free radical generators in cells are the mitochondrial electron transport system, auto-oxidized molecules such as Xanthin oxidase, aldehydic oxidase and microsomal oxidations (Gutteridge and Halliwell, 1984). Iron-oxygen complexes, hydrogen peroxide and lipid peroxides are generated by several oxidative reactions (Vuillaume, 1987).

So far as plant phenolic constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts (Cook and Samman, 1996). Flavonoids acting as a chain breaking antioxidant impairs with the formation of free radicals in the process of formation of intracellular substances throughout the body, including collagen, bone matrix and tooth dentine (Beyer, 1994; Aqil et al., 2006). Furthermore, the antioxidant activities of putative antioxidants have been attributed to various mechanisms; among these are prevention of chain initiation, binding of transition metal ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Diplock, 1997).

With the evaluation of antioxidant properties of various extracts, methanol extract has showed promising results. The antioxidant activity of methanol extracts have been correlated with their reducing powers (Duh, 1998). Ascorbic acid was used as a control in the assay and ascorbic acid has stronger activity than the methanolic extract as reported by Apak et al., (2006), as a simple and inexpensive antioxidant assay for plant material. Moreover, result of the assay is correlated with total phenolic content and ferric ion reducing power (Guclu et al., 2006;
Ozturk et al., 2007). The systemic research for useful bio actives from the plants is now considered to be a rational approach in nutraceuticals and drug research. The result obtained confirms the ethanobotanical claim of the plant to be a potential antioxidant. Hence, the plant contains good store of antioxidants and essential metabolites to support its efficiency to be a drug.

c. Anti-helminthic activity

Helminth infections are the most common health problems in India; in developing countries they pose a large treat to public. These infections can affect most population in endemic areas with major economic and social consequences. The mechanism of interference apparently occurred through reactions necessary for the generation of metabolic energy and subsequent paralysis of the parasite or neuromuscular coordination that is depression of muscular activity, which leads to paralysis of the parasite and their subsequent expulsion from the intestine (Bueding, 1969). Interference with the neuromuscular coordination in the parasite may occur by inhibiting the breakdown of excitatory neurotransmitters or by acting like excitatory neurotransmitters resulting in spastic paralysis of the parasite (Nweze and Ngongeh, 2007). The other mechanisms involve acting like an inhibitory neurotransmitters or causing hyperpolarization resulting in flaccid paralysis of the parasite. The spastic or flaccid paralysis of an intestinal helminth allows for the normal peristaltic actions of the host to expel the parasite; levamisole and piperazine are examples of drugs with this mechanism of action (Clarence et al., 1986).

The experimental evidence obtained in the laboratory model could provide a rationale for the traditional use of polyprenol from plant as an anti-helminthic. The polyprenol displays a significant anti-helminthic activity in dose dependent manner. The chemistry of nematode surface is collagen rich extracellular matrix providing protective cuticle that forms
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exoskeleton, and is critical for viability. The collagen is a class of proteins that are modified by a range co-and post -translational modification prior to assembly into higher order complexes or extracellular matrices (Page and Winter, 2003). This form of reactivity between compound and cuticle brings toughness in the skin and hence the worms become immobile and non-functional leading to paralysis followed by death. The wormicidal activity of polyrenol suggests that it is effective against parasitic infections of humans. Hence, the isolated compound polyrenol is suggestive of being good anti-helminthic agent.

d. Anti-arthritis activity

Arthritis stand as one of the foremost health troubles worldwide, leading cause of disability in western and developing countries. Therapies developed along the principles of western medicine are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world. Therefore, treating arthritis with plant-derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive (Baranwal et al., 2012). The production of auto antigen in certain arthritic disease may be due to denaturation of protein (Gutteridge, 1995; Kahkonen et al., 1999; Kris et al., 2004). Anti-inflammatory drugs used for treating chronic inflammatory diseases such as rheumatoid arthritis are typically prescribed long term to properly control the disordered immune system. Thus, there is a strong need to develop safe and effective drugs for the long-term use. Many groups have studied nonsteroidal anti-inflammatory small molecules that were derived from natural sources with the aim of developing new treatments for clinical use (Chrubasik et al., 2007).

From the results of present study it can be stated that methanolic extract and ellagic acid are capable of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease. In vitro studies on extracts and isolated compounds demonstrated suppression of both


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inflammation and arthritis. Methanol extract and ellagic acid at two different dose levels (100 and 250 μg/ml) provided significant protection against denaturation of proteins and hypotonic saline induced RBC membrane damage. It also exhibited significantly anti-proteinase activity. Studies related to active constituents on lipid derived eicosanoids, enzyme expression (COX2, lipoxygenase) and cytokines are necessary to understand the mechanism of action in relation to the observed anti-arthritic activities.

Production of auto-antigens in certain rheumatic diseases may be due to in vivo denaturation of proteins (Brown and Mackey, 1968). The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Grant et al., 1970). Protective effect on heat and hypotonic saline-induced erythrocyte lysis is known to be a very good index of anti-inflammatory activity of any agent. Since the membrane of RBC is structurally similar to the lysosomal membrane, the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal membrane (Oyedapo and Famurewa, 1995). Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinases which carry in their lysosomal granules many neutral serine proteinases. It was previously reported that leucocyte proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. This finding justifies the usefulness of ellagic acid in the management and treatment of inflammation associated diseases like arthritis.

5.4 Pathway studies of arthritis

Osteoarthritis causes loss of articular cartilage, accompanied by fibrosis of the bone marrow, as well as thickening and other abnormalities of the
subchondral bone, the layer of bone just below the cartilage. These processes are linked with the symptoms of joint pain. There are no effective medical therapies to prevent cartilage destruction and the associated bony changes in the joint in osteoarthritis. This situation reflects an insufficient understanding of the molecular mechanisms involved in the condition (Matthew et al., 2010). The combination of genetics and molecular biology has greatly facilitated the identification of candidate genes for human diseases (Childs and Valle, 2000; Botstein and Risch, 2003). Today, it is well accepted that genes within a cell do not function alone. They interact with each other to form complexes or pathways to carry out biological functions (Barabasi and Oltvai, 2004). For some diseases, it has been shown that disease candidate genes are functionally related in the form of protein complexes or biological pathways (Oti and Brunner, 2007). Therefore, pathways could be used to represent the underlying biology of diseases.

To achieve this goal, we first identified disease associated genes and proteins through literature mining. For arthritis disease, we identified pathways where there is a significant enrichment of disease associated genes. On average, over 50% of associated proteins of a disease are mapped in pathway. This finding reinforces the notion that disease genes are related to each other in a form of functional entity such as pathways or protein complexes (Loscalzo et al., 2007; Yong and Pankaj, 2009). Furthermore, it provides us with an opportunity to investigate the role of each protein in the disease development. The different proteins involved in the disease and their function (Matthew et al., 2010) are as follows:

**HIF 2a**- Hypoxia-inducible factors (HIFs) are transcription factors that respond to changes in available oxygen in the cellular environment, specifically, to decreases in oxygen, or hypoxia.

**ARNTL**- aryl hydrocarbon receptor nuclear translocator like, which facilitates positive regulation of transcription.
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**IHH**- Indian hedgehog (Ihh) is indispensable for osteoblast differentiation during embryonic development of the endochondral skeleton. In the absence of Ihh, cells of the osteoblast lineage fail to activate the expression of Runx2, a transcription factor integral to osteoblast differentiation.

**RUN X2**- DNA-binding transcription factor that is involved in normal bone development. RUNX2 is often over expressed in osteosarcoma.

**Collagen X**- expressed by hypertrophic chondrocytes during endochondral ossification.

**VEGF**- Vascular endothelial growth factor is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. When over expressed, it can contribute to disease.

**MMP 13**- Matrix metallopeptidase 13 (collagenase 3) involved in bone mineralization. Breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive pro proteins which are activated when cleaved by extracellular proteinases. The protein encoded by this gene cleaves type II collagen more efficiently than types I and III. It may be involved in articular cartilage turnover and cartilage pathophysiology associated with osteoarthritis.

**MMP 3**- Matrix metallopeptidase 3 (stromelysin 1, progelatinase), involved in the breakdown of extracellular matrix.

**Syndecan**- transmembrane domain protein, co receptor to GPCR. It helps in binding of ligands to extracellular GAG's.

**ADAMTS4**- A disintegrin and metalloproteinase with thrombospondin motifs 4, involved in degradation of aggregan- proteoglycan.
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Osteoarthritis is the most common form of arthritis, and it is an enormous, expensive public health problem. The characteristic features of this chronic, progressive, degenerative disorder of the entire joint include variable inflammation of the synovium—the thin membrane around the joint that secretes the lubricating synovial fluid—and changes in the structure of the bone beneath and bordering the joint and in the protective cushion, called articular cartilage, that allows low-friction movement between the ends of long bones (Matthew et al., 2010). The genetics of HIF-2α and other players in chondrocyte hypertrophy are likely to become fertile areas of investigation in osteoarthritis and potentially useful for preventive and medical treatment strategies involving personalized genomics. Nonetheless, these novel relationships could offer new insights into disease etiology, classification, and pathway-based design of novel therapeutic opportunities for medicines on the current market.

5.4.1 Identification of drug targets and Binding studies

From the obtained literature glucosamine-6-phosphate synthase is selected as target for antibacterial activity. In spite of the fact that glucosamine-6-phosphate synthase is present in all kinds of cells, it may be exploited as a target for potential antibacterial drugs and selective toxicity can be achieved (Chmara et al., 1984b). Obviously, glucosamine-6-phosphate, the product of this enzyme, is indispensable for microbes as well as for human cells, yet the consequences of its deficiency in both species are very different. It has been shown that even a short-time inactivation of GlcN-6-P synthase in microbial cells is lethal for the pathogen (it induces morphological changes, agglutination and lysis), while in mammals depletion of the amino sugar pool for a short time is not lethal, because of the much longer lifespan of mammalian cells, long half lifetime of GlcN-6-P synthase, and rapid expression of the mammalian gene encoding the enzyme (Bates et al., 1966; Chmara & Borowski, 1986; Milewski et al., 1986).
Our practical studies on an isolated compound polyrenol for inhibition of glucosamine-6-phosphate synthase showed that selective inactivation of this enzyme is possible due to its geometries of binding site. Particularly the distance between the area of the binding site responsible for positioning of the ligand’s carboxyl and the thiol group of the catalytic residue seems to be longer in this enzyme (Marek et al., 2005). As a more general conclusion we postulate that, binding domain of GlcN-6-P synthase seems to be quite unexplored yet and our analysis including docking and molecular dynamics calculations proved to be effective in exploring the binding site properties and their enzyme affinity. Polyrenol binding locks the residue inactive conformation. Polyrenol has been proved to be one of the potent antibacterial agents. By in silico analysis, it seems that polyrenol is promoting the remarkable antibacterial activity through the inhibition of GlcN-6-P synthase.

It has been demonstrated by several investigators that β-tubulin protein of stomach worm could be one of the major targets for the antihelmintic activity of the drug (Anderson et al., 2001). Microtubules, present in parasites as well as in other eukaryotic organisms, which are hollow cylindrical in shape, are constructed from linear chains or protofilaments of repeating subunits of α and β-tubulin. Microtubule is involved in many cellular processes including mitosis, active transport of proteins and organelles throughout the cytoplasms, the maintenance of cell morphology etc. The α and β-tubulin share 40% amino acid identity and their 3-dimensional (3D) structures are nearly identical (Dawson et al., 1984). Both the tubulins share a GTPase domain which belongs to self-polymerizing FtsZ family of proteins. FtsZ is present even in prokaryotes and plays a central role in bacterial cell division (Little et al., 1982; Gray et al., 1998; Ellis et al., 2004). Lower eukaryotes such as protozoa and helminthes have different sensitivities to microtubule perturbing agents in contrast to higher eukaryotes (Wampande et al., 2007).
Molecular docking studies have been carried out to get an insight into the inhibitory mechanism of β-tubulin by the isolated compound polyprenol. The best docked conformation of ligand–receptor complex was determined based on the lowest interaction energy and binding free energy. The binding site residues responsible for hydrogen bond interaction and Van der Waal residues are summarized. The protein backbone RMSD was analyzed for complex to evaluate the overall backbone stability over the time period. It represents negligible deviation throughout the simulation which confirms the stability of the complex (Om et al., 2011). The docking result exhibited that the binding mode of polyprenol within the active site of β-tubulin because of its structural and conformational changes. Moreover, hydrogen bonding and Van der Waals interaction data suggested that Glu 107 as most important residues for binding with ligands. Here, it is noticeable that polyprenol showed greater results and can be used as potent antihelminthic drug.

According to obtained literature, a stress-induced increase in the activity of HIF-2α, overshadowing the beneficial effects of the closely related HIF-1α, pushes the cartilage-producing cells, or chondrocytes, in the joint toward a more differentiated state known as hypertrophy, which then drives osteoarthritis. Chondrocytes are the sole cells populating joint cartilage, and they normally function to maintain, remodel and repair this nonvascularized tissue. These cells must be remarkably hardy, as they subsist on the diffusion of gaseous nutrients through cartilage in a hypoxic environment. Chondrocytes interpret and respond to biomechanical stressors, including shear, strain and compressive forces, by altering the extracellular matrix around them. These cells balance anabolic (matrix-building) processes, including the synthesis of matrix proteins such as fibrillar type II collagen, and catabolic processes, including proteases that degrade these matrix constituents. Essentially, osteoarthritis reflects an imbalance between matrix anabolic and catabolic processes. Several remarkable studies have revealed a role in osteoarthritis for increased chondrocyte expression and activation of
proteases, including matrix metalloproteinases (MMPs) and aggrecanases of the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family. However, achieving selective, safe and effective targeting of proteases to suppress osteoarthritis progression remains a challenge in part due to the needs for drug specificity with respect to possible unintended effects on physiologic functions of other members of the ADAMTS and MMP families (Flannery, 2010). The studies reveal that HIF-2α expression is induced by multiple proinflammatory cytokines produced in large part by chondrocytes, including TNF-α and interleukin-1β. HIF-2α expression is increased in the early and progressive stages of disease in human osteoarthritic cartilages, as well as in the cartilages of mouse models of knee osteoarthritis induced by surgery and in association with aging. Furthermore, increasing HIF-2α expression specifically in the joint cartilage, using either transgenic methodology or transduction using adenoviral HIF-2α, induces osteoarthritis (Saito et al., 2010; Yang et al., 2010).

Practically, polyrenbol showed good docking energy and ligand efficiency compared to standard. The polyrenbol was completely enfolded in the entire active pocket of HIF-2α. The topology of the active site of HIF-2α was similar in both polyrenbol and aspirin, which is lined by interacting amino acids as predicted from the ligplot. The biological roles of HIF-2α are clearly understood which offers design of novel inhibitors for incurable arthritic diseases (Kulkarni et al., 2006). The genetics of HIF-2α and other players in chondrocyte hypertrophy are likely to become fertile areas of investigation in osteoarthritis and potentially useful for preventive and medical treatment strategies involving personalized genomics. Lastly, an improved understanding of how chondrocytes undergo hypertrophy should pay relatively early dividends for improving cell replacement and engineering therapies to repair cartilage defects in vivo (Matthew et al., 2010). By in silico analysis, it seems that polyrenbol is promoting the remarkable anti arthritic activity through the inhibition
Discussion

of HIF-2α protein. Hence, polyprenol has been proved to be one of the potent anti arthritic agents.