CHAPTER 1

GENERAL INTRODUCTION

1.1. Natural products as medicinally useful agents

Natural products are those chemical compounds derived from living organisms viz., plants, animals, insects, etc. Drugs derived from natural products are usually secondary metabolites and their derivatives. Allelochemicals generally refer to the secondary metabolites released by intact living plants into their surrounding (Rice, 1984; Dayan et al., 2000; Einhelling, 2004). These metabolites exert inhibitory or stimulatory effects on other plants, fungi, bacteria, etc., in the surrounding environment, including the rhizosphere. These chemicals include flavonoids, tannins, alkaloids and aromatic acids have been observed to be active against weeds, pathogens and insects (Inderjit, 1996 and Duke et al., 2000). They also play an important role in protecting plants from certain pathogens.

Throughout history, mankind has always been interested in naturally occurring compounds from natural sources. Various extracts of flowers, plants and insects have been used for isolating compounds whose taste, color and odour could be used for various purposes (Ikan, 2007). Recently, there has been a literary expression of Thornton Wilder in his book, The Eighth Day, “Nature never sleeps. The process of life never stands still (Calvin, 1969).

The use of natural products as medicinal agents presumably predates the earliest recorded history as the earliest humans used various, but specific plants to treat illness (Ballick and Cox, 1996). In India, herbal medicine dates back several thousand years to the Rig-Veda, the collection of Hindu sacred verses. This has led to a system of health care known as Ayurvedic medicine. One useful plant from this body of knowledge is snakeroot, Rauwolfia serpentina, used for
centuries for its sedative effects. Today, the active components in snakeroot are widely used in Western medicine to treat high blood pressure (Kong et al., 2003). Theophrastus (370-285 BC) began the scientific classification of plants, and Dioscorides De Materia Medica ("Regarding Medical Matters") (77 AD) reported the uses, medicinal and otherwise, of over 600 plants. Western medicine can be traced back to the Greek physician Hippocrates (460-377 BC), known as the Father of Medicine. Hippocrates believed that a disease had a natural cause and used various herbal remedies in his treatments. Ibn al-Baitar (1197-1248) listed over 1400 drugs and medicinal plants in his Corpus of Simples. In Europe, after the tenth century, much of the medicinal lore was based in the church, particularly the monastic orders, but by the 1500’s, after the invention of the printing press, herbals available to the general public were popular, particularly in England (Ballick and Cox, 1996). By the late 1700’s, studies like William Withering’s An Account of the Foxglove and its Medicinal Uses (1785) began to appear. These were based on case histories, described specific doses and gave administration instructions for herbal remedies. The people of India use more than 10,000 plants as medicines, out of which approximately 7500 are used in folk and tribal system, 1900 in Ayurveda, 500 in Siddha, 400 in Unani and 300 in Amchi (Tiwari et al., 2004).

Until the late 1800's, organic chemistry was used almost exclusively for the study of natural products. The natural products that were studied and used tended to be the compounds that occurred in the largest amounts, mostly in plants, and were most easily isolated in a pure or sometimes not very pure form by techniques such as simple distillation, steam distillation, or extraction with acid or base. Originally, teas or decoctions (aqueous extracts) or tinctures or elixirs (alcoholic extracts) were used to prepare, administer herbal remedies and these were usually
the starting points for isolation work. Now different solvents are employed, for e.g., ethanol to extract, hexane to concentrate non-polar constituents, methanol to concentrate polar constituents, and modern isolation techniques include all types of chromatography, often guided by bioassays, to isolate the active compounds. Stereochemistry was not often determined. Now, structures are elucidated primarily by spectroscopic techniques, and the stereochemistry is an important feature of the structure.

1.1.1. The chemistry of natural products

Among the recent outstanding contributions to the chemistry of natural products, the most important one is the conformational analysis designed by Derek Barton. He used it for the structural determinations of many complex molecules such as β-amyrin and cycloartenol. In 1945, Robert B. Woodward involved in the structural determinations of penicillin, strychnine, patalin, terramycin, aureomycin and the synthesis of Vitamin B12. Extracts of toxic plants have been used for hunting and murder throughout the world for thousands of years. Thus, *Strychnos* and *Chondrodendion* (both containing strychnine) were used in arrow poisons. The Colombian arrow poison consists of toxins from the legs of frogs. When rye is infected by the fungus, *Claviceps porpurea*, the toxin ergotamine and a number of ergot alkaloids are produced. These compounds cause serious illnesses (Geissman, 1969).

The rapid and impressive development of organic chemistry in the 19th century had a tremendous effect on the discoveries of natural products. Towards the end of the 19th century, microbiology has developed enormously. In 1877, Alexander Fleming discovered penicillin which was active against Tuberculosis and was first isolated by him in 1929. In 1944, Waxman isolated streptomycin which is used for the treatment of tuberculosis. In 1952, Bloch and Woodward
suggested a mechanism for the cyclization of squalene to cholesterol. In 1962, Francis Crick and James Watson described the double helix structure of proteins (Croat, 1969).

1.1.2. The classification of natural products

The classification of natural products may follow the four schemes below:

1. **Classification based on the molecular skeletal structure**: Open-chain aliphatic, alicyclic, aromatic, benzenoid and heterocyclic.

2. **Classification based on physiological activity**: The interest in natural products is frequently initiated by attempts to isolate and clarify a physiologically active principle of plant or animal origin. Actually, many medicines currently in use are natural products, e.g. alkaloids, such as morphine and penicillin G.

3. **Classification based on chemotaxonomy**: The field of chemotaxonomy attempts to review plant constituents according to plant taxa. Namely, constituents are regarded as markers for evolution as well as the classification of plants.

4. **Classification based on biogenesis**: It has been established that the primary synthetic process in nature is photosynthesis by which green plants utilize the energy of the sun for the production of organic compounds from carbon dioxide. The initial products of photosynthesis are carbohydrates. Further metabolic alterations lead to the formation of a pool of organic compounds of low molecular weight and simple structures such as carboxylic acids and amino acids, which are vital for the living organisms.
Fig. 1 Schematic presentation of natural products formation
1.1.3. Significance of natural products in human welfare

Drug development based on natural products is an important and fact growing area due to the limitations of developing new synthetic medicines. such as aspirin, digoxin, morphine, quinine, pilocarpine etc., (Ansari and Inamdar, 2010

It is seen that every plant is associated with some useful properties and many of them molecules based on the principle of active components or pure chemicals. Clinical, pharmacological and chemical studies on these traditional medicines, which were derived predominantly from plants, were the basis of isolation of most early are present in our pharmacopeias. The most important use of secondary plant products has been associated with diseases. Drug development based on natural product is an important and fast growing

Promising and potential among the medicinal plant products such as *Digitalis* products (cardiovascular drugs); *Rauvolfia* preparations (hypertensive drugs); *Papaver somniferum* (analgesics); *Atropa* species and *Hyoscyamus* species (antispasmodics); *Colchicum autumnale* (nonsteroidal antiarthritic); *Ephedra* species (bronchodilators); *Catharathus* species and *Taxus baccata* (anticancerous) in purified and their chemically certified form remain vital members of our materia medica. Huperzine A, an alkaloid from the leaves of a Chinese herb, has the potential to improve memory and learning ability in Alzheimer’s patients (Tang and Huperzine, 1996). There were still other studies related to the anti-Alzheimer drugs, such as the study of inhibitory effect of Zeatin, which was isolated from *Fiatoua villosa* on acetylcholinesterase activity (Hoe et al., 2002). Inspite of the competition from other drug discovery methods, natural products are still providing their fair share of new clinical candidates and drugs. A recent review was done by Newman, Cragg and Snader, who analyzed the number of natural product derived drugs present in the total drug launches from 1981 to
2002. Also a total of 15 were launched which included new drug types such as antimalarial arteether, the anti-Alzheimer's drug galantamine (galanthamine) and the antibacterial lipopeptide daptomycin (Newman et al., 2003). Most ordeal poisons are tropical; the major species is the Calabar beans (*Physostigma venenosum* - Fabaceae), containing the alkaloid physostigmine. Minor ordeal poisons includes species of the plant families such as Apocynaceae, Asclepiadaceae, Euphorbiaceae, Loganiaceae and Sapotaceae. Also a number of plant products have been used locally to destroy insect pests (Kingsbury, 1964; Watt et al., 1962).

The treatment of diseases with pure pharmaceutical agents is a relatively modern phenomenon. One of the earliest success stories in developing a drug from a natural product was aspirin. Ancient Egyptians used the bark of the white willow to treat pain and inflammation (Vainio and Morgan, 1997). Among the earliest pure compounds discovered was salicin, isolated from the bark of the white willow, *Salix alba*, in 1825-26. Willow’s active chemical constituent, salicin, was identified in 1829 by the French pharmacist H. Leroux (Hedner and Everts, 1998). It was subsequently converted to salicylic acid via hydrolysis and oxidation, and proved as successful as an antipyretic (fever reducing) that was actively manufactured and used worldwide. The use of salicylic acid, however, often led to severe gastrointestinal toxicity. This was overcome when Felix Hoffmann of Bayer Company converted salicylic acid into acetylsalicylic acid (ASA) via acetylation (Robbers et al., 1996). Bayer then began marketing ASA under the trade name aspirin in 1899. Today, aspirin is still the most widely used analgesic and antipyretic drug in the world.
1.2. Alkaloids

An alkaloid is a plant-derived compound that is toxic or physiologically active, contains nitrogen in a heterocyclic ring and it is basic, has a complex structure, and is of limited distribution in the plant kingdom. Alkaloids are an important group of diversely distributed, chemically, biologically and commercially significant natural products (Cordell et al., 2001).

Many of the earliest isolated pure compounds with biological activity were alkaloids. This was due to the ease of isolation. The nitrogen generally makes the compound basic and it exists as a salt in the plant. Thus, alkaloids are often extracted with water or mild acid and then recovered as crystalline material by treatment with base.

Malaria is caused by protozoa of the genus *Plasmodium* (Sinden and Gilles, 2002), contained as spores in the gut of the *Anopheles* mosquitoes, which then spreads the spores to humans when it bites. As the Spanish and Portuguese explorers began to colonize South America, they discovered a cure for malaria known to the native Indians. This was the bark of the *Cinchona* trees. The use of *Cinchona* bark to treat malaria was first reported in Europe in 1633, and the first bark reached Rome about 12 years later. Teas made from the bark, cured people suffering from malaria. The landmark discovery of quinine from *Cinchona* bark was made by the French scientists Caventou and Pelletier (Mechoulam and Gaoni, 1967; Kong et al., 2003).
Quinine was isolated originally from *Cinchona succirubra*, the principal antimalarial compound in the plant. Malaria is still a major problem throughout the world, and, although synthetic antimalarial drugs largely supplanted quinine as the treatment for malaria during World War II, quinine is often once again the drug of choice as strains of malaria have become resistant to the synthetic drugs.

Morphine acts as an anesthetic without decreasing consciousness, and it is one of the most powerful analgesics known (Raffauf, 1996). However it also suppresses the respiratory system and high doses can cause death by respiratory failure. Its analgesic properties are related to the ability of the molecule to fit into and block a specific receptor site on a nerve cell. This eliminates the action of the pain receptor, preventing the pain signals reaching the brain. This is similar to the way in which the body's natural painkillers (endorphins and enkaphalins) work. The shape of the morphine molecule is crucial due to its ability to exactly fit into the active site on the receptor by the 'lock-and-key' mechanism. The benzene group of the morphine molecule fits snugly against a flat section of the receptor protein, whilst the bent neighboring group of carbon atoms fits into a nearby groove. This allows the positively charged nitrogen atom to attach to a negatively-charged group on the receptor, so locking the two molecules together. It was widely used for pain relief, in the beginning of 1830's, but was also recognized as an addictive. In an attempt to make morphine as less addictive, Bayer chemists
acetylated the two hydroxyl groups (by replacing the both -OH groups with OCOCH₃) of morphine with acetyl chloride to produce diacetylmorphine or diamorphine, which was called later heroin, the most notorious derivative of morphine.

This was marketed as a non-addictive pain reliever under the trade name heroin for about two years in the early 1900’s, until it was recognized to be more addictive than morphine. Other derivatives of morphine have been developed and found to be used as opiate antagonists or as animal tranquilizers.

Vincristine, one of the most potent antileukemic drug is in use today. It was isolated in a search for diabetes treatments from *Vinca rosea* (now *Catharanthus roseus*) in the 1950's along with vinblastine, a homologue in which the N-methyl group is oxidized to an aldehyde moiety (Jordan and Wilson, 2004). This is such a complex structure that it is still isolated from the plant (the Madagascan periwinkle) today, rather than prepared by synthesis. The small change in structure, however, causes a significant change in pharmacological efficacy. They work by preventing mitosis in metaphase. These alkaloids bind to tubulin, thus preventing the cell from making the spindles which are needed for division (Owellen *et al.*, 1972). This is different from the action of taxol which interferes with cell division by preventing the spindles from being broken down. Vincristine
(leurocristine, VCR) is most effective in treating childhood leukemias and non-Hodgkin’s lymphomas, whereas vinblastine (vincaleukoblastine, VLB) is used to treat Hodgkin’s disease, advanced testicular cancer and advanced breast cancer. These are administered intravenously in their sulphate forms. Both vincristine and vinblastine are used in the treatment of various forms of malignant diseases. Vincristine is superior to vinblastine for the treatment of lymphosarcoma, but this is in part counter balanced, by its greater toxicity (Lobert et al., 1996).

1.3. Terpenoids

Terpenes are natural products derived from plants that have medicinal properties and biological activity. Terpenes may exist as hydrocarbons or have oxygen containing compounds such as ketone or aldehyde groups (terpenoids). The basic structure of terpenes is repeating isoprene units \((C_5H_8)_n\). Monoterpenes contain 2 isoprene units; examples include menthol, pinene, and camphor. Diterpenes contain 4 isoprene units; examples include phytol, vitamin A1 (Lam et al., 2006).

Menthol is an organic compound made synthetically or obtained from peppermint or other mint oils. The main form of menthol occurring in nature is \((-)\) menthol, which is assigned the \((1R, 2S, 5R)\) configuration and it is widely used to relieve minor throat irritation. Pinene \((C_{10}H_{16})\) is a
bicyclic monoterpene chemical compound. There are two structural isomers of pinene found in nature, α-pinene and β-pinene and both forms are important constituents of pine resin. They are also found in the resins of many other conifers, as well as in non-coniferous plants.

Camphor is a waxy, white or transparent solid with a strong, aromatic odor. It is found in wood of the camphor laurel (Cinnamomum camphora), a large evergreen tree found in Asia. Camphor is readily absorbed through the skin and produces a feeling of cooling similar to that of menthol and acts as slight local anesthetic and antimicrobial substance. There are anti-itch gels and cooling gels with camphor as the active ingredient (Mann et al., 1994).

Gossypol is a dimeric sesquiterpene isolated from the seeds of cotton plants. Gossypol occurs as a mixture of enantiomers in cotton seed (Stipanovic et al., 2005). These enantiomers exhibit different biological activities. The (-) enantiomer is toxic to animals, but it has potential medicinal uses. It has been used clinically in China as a male contraceptive. Gossypol is a phenolic aldehyde that permeates cells and acts as an inhibitor for several dehydrogenase enzymes. It is a yellow pigment. It inhibits replication of the HIV-1 virus. It is an effective protein kinase D inhibitor (Hoshiai et al., 1982).

Lavender oil is an essential oil obtained by distillation from the flower spikes of certain species of lavender. Lavender oil, which has been used for long
time in the production of perfume, can also be used in aroma therapy. The scent has a calming effect which may aid in relaxation and reduction of anxiety. Lavender oil can be used as an antiseptic, pain reliever for minor burns, insect bites and stings. It is also said to treat a variety of common ailments, such as sunburn and sunstroke. It can also be used in massage oil mixtures, which may be effective in the relief of joint and muscle pain (Woelk and Schläfke, 2010).

Lavender oil is cytotoxic as well as photosensitizing. A study demonstrated that lavender oil is cytotoxic to human skin cells in *in vitro* (endothelial cells and fibroblasts) at a concentration of 0.25%. Linalool, a component of lavender oil, reflected the activity of the whole oil, indicating that linalool may be the active component of lavender oil (Prashar *et al.*, 2004). Limonene is a phytonutrient belonging to a class of chemicals called terpenes. Limonene is found in the oil of citrus peels, and is a by-product of the orange juice industry. Industrial uses for limonene include cleaning products and additives meant to add aroma or flavoring to a product. The best known compound in this group is camphor oil. Oil derived from the *Salvia officinalis* tree, thujone, has recently become popular because of its hallucinogenic qualities (Schultes, 1976) and it is quickly becoming a drug of abuse (Bucheler *et al.*, 2005). Thujone is a GABA<sub>₃</sub> receptor antagonist (Olsen,). By inhibiting GABA<sub>₃</sub> receptor activation neurons may fire more easily which can cause spasms and convulsions. Thujone is also a 5-HT<sub>3</sub> antagonist. Thujone is cause muscle reported to be toxic to both brain and liver cells and could cause convulsions if used in a dose too high. A toxicology study of α-thujone in mice, showed more active of the two isomers. The study also found that α-thujone was metabolized quickly in the liver (Höld *et al.*, 2000).
The carotenes are tetraterpenes. They can be thought of as two diterpenes linked in tail-to-tail fashion. The antineoplastic agent paclitaxel is a terpene derived from yew plant (*Taxus brevifolia*) bark.

**1.4. Steroids**

Asteroid is a type of organic compound that contains a specific arrangement of four cycloalkane rings that are joined to each other. Examples of steroids include the dietary fat cholesterol, the sex hormones estradiol and testosterone, and the anti-inflammatory drug dexamethasone. The three cyclohexane rings (designated as rings A, B, and C) form the skeleton of phenanthrene; ring D has a cyclopentane structure. Hence, together they are called cyclopentaphenan-threne.

Steroids offer numerous and important benefits in the world of medicine. The drugs most notably serve as part of effective treatments for cancer and other muscle deteriorating diseases, but also help to treat Alzheimer's disease, hormonal
disorders, asthma, kidney problems, Bell's palsy and facial paralysis. Corticosteroids anti-inflammatory drugs that replicate the hormones of the adrenal gland, heighten immune system response, lessen swelling and inflammation, regulate metabolism and control blood electrolyte levels. Often used in treating leukemia, corticosteroids may also decrease and control tumors and manage the complications of cancer treatment.

1.5. Flavonoids

Flavonoids are "the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants"(Spencer, 2008). In vitro studies of flavonoids have displayed anti-allergic, anti-inflammatory (Yamamoto and Gaynor, 2001), antimicrobial (Cushnie and Lamb, 2005) and anti-cancer (de Sousa et al., 2007) activities. They also inhibit platelet aggregation (Bucki et al., 2003) and inhibit low density lipoprotein (LDL) oxidation by free radicals.

Because of the antioxidative property, it is suggested that flavonoids may delay or prevent the onset of diseases (such as cancer) induced by free radicals. The increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is not caused directly by flavonoids themselves, but most likely is due to increased uric acid levels that result from metabolism of flavonoids. Flavonoids are most commonly known for their antioxidant activity (Williams et al., 2004). The antioxidant abilities of flavonoids in vitro are stronger than those of vitamin C and E (Manashi et al., 1999). Flavonoids were found to be strong topoisomerase inhibitors and induce DNA mutations in the MLL gene, which are common findings in neonatal acute leukemia (Thirman et al., 1993; Strick et al., 2000). Flavonoids could also induce mechanisms that may kill cancer cells and inhibit tumor invasion.
The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Subramani Sellappan and Akoh, 2002). Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity. The potent antioxidant flavonoid types are quercetin, catechin and xanthohumol. Flavonoids, such as catechins found in strawberries and green and black teas; kaempferol from brussel sprouts and apples; and quercetin from beans, onions and apples, may have reduced risk of obtaining lung cancer (Verena et al., 2006).
1.6. Sapotaceae

The family Sapotaceae consists of large evergreen trees and less commonly shrubs. Plants are distributed throughout the tropic of Asia, Africa and America. The family consists of about 40 genera. The important ones are Argania, Butyrospermum, Colacarpum, Chrysophyllum, Mimusops, Payenne, Sarcosperm and Sideroxylon. Mimusops (Linn) is a genus of only trees and comprises of 30 species. Among these, Mimusops hexandera, Mimusops elengi and Mimusops manilkara (=Achras sapota) belongs to the same genus. All the three trees are of medium height 12 to 15 meters and grow throughout India and Pakistan (Hill, 1952).

Mimusops elengi is an ornamental garden tree with sweet scented flowers (Gupta, 1954). Mimusops manilkara (=Achras sapota; = Manilkara zapota) is distributed in West Indies and introduced to the moisture tropics. It is cultivated all over the world for its edible fruits A. sapota is also found in Phillipine, West Indies, Malaysia, Ceylon, India, Pakistan and Bangladesh

1.6.1. Economic Importance, Industrial and Medicinal Uses

Fruits of Achras sapota, when quite ripe, are very delicious and sweet in taste. Jams, sherbets and Syrups have also been prepared from the fruits industrially (Ginai, 1968). The parts of the these plant species are reported to have more or less similar curative properties in folk medicine (Dymock, 1891). The Mimusops hexandra bark is reported to be febrifuge and general tonic. It retards fermentation. Seeds oil is considered demulcent and emollient. The ripe fruits of Mimusops elengi are not so tasty. Bark and the fruits are astringent and tonic. These are used in diarrhoea and dysentery. The decoction of the bark cures off obstinate constipation especially in children. The Achras sapota bark (aqueous extract) contains tuberculostatic principle (Mirimanoff and Thanez, 1961). The
coagulated resinous latex (cuticle gum) obtained from the bark is used for making chewing gum.

1.7. Identification of natural products by spectroscopic and Chromatographic techniques

Recently, natural products chemistry has undergone explosive growth due to advances in isolation techniques, synthetic and biosynthetic approaches as well as spectroscopic and chromatographic methods. The advent of computers and Fourier transform completely revolutionized the detection and identification of organic compounds. Modern automated instruments allow very small samples in the nanogram (10^{-9} g) range to be characterized in a very short time. The application of Fourier transform nuclear magnetic resonance (FTNMR) and Fourier transform infrared (FTIR) allows recovery of the sample in contrast to mass spectrometric (MS) determination which is a destructive but quite often a necessary technique (Bruneton, 1999).

Modern methods used to separate complex organic mixtures utilizing gas-liquid chromatography (GLC), high-pressure liquid chromatography (HPLC), and droplet counter-current (DCC) chromatography can separate samples rapidly and efficiently in the picogram range. This has been impossible until recently. Coupling the chromatographic instruments to spectrometers enables a partially automated analysis in an even shorter period of time. The following coupling of chromatographic instruments are mostly used: GC-MS, GC-FTIR, GC-MI-FTIR, GC-UV-VIS, HPLC-MS, HPLC-FTIR, HPLC-FTNMR and MS-MS.

1.8. Objectives of the study

➢ To evaluate the in vitro antioxidant properties of methanol crude extract and its fractions of leaves of Manilkara zapota Linn.
➢ To evaluate the antiangiogenic activity of methanol crude extract and its fractions of leaves of *M. zapota*.

➢ To isolate and characterize the active constituents from the methanol crude extract of the leaves of *M. zapota*.

➢ To evaluate the pharmacological activities of the isolated compounds

➢ To evaluate the anticancer activity of the methanol crude extract by *in vitro* cell line based MTT assay method.

REFERENCES


