Chapter # 2

REVIEW OF LITERATURE
2.1 SIGNIFICANCE OF MEDICINAL PLANTS

Aromatic and medicinal plants form a very large group of medicinally and economically important plants which serve as the basic raw materials for medicines, cosmetics, perfumes and food additives. A recent aspect of interest in plant drug research is the new concept of a non specifically increased resistance of an animal to diseases attributable to other substances, besides the active principles responsible for specific biological activity. This will probably justify the use of many of the plant drugs as household remedies by indigenous people of many countries from ancient times and hence warrants their evaluation in more detail. The fact that only very few percent of the six lakhs species of plants on the planet has been investigated, indicates the opportunity provided and challenges thrown to phytochemists. More recently there is an emerging trend in research and development to support the bioactivities of medicinal plants.

Reports in the scientific literature indicate that plant derived compounds serve as potential sources of novel antimicrobial, anticancer, anti inflammatory, antioxidant and anti HIV agents. Paclitaxel is an important drug used in the cancer treatment. Over thirty thousand plants were screened for their anticancer properties and it was found that the bark extract from the Pacific yew tree \((\text{Taxus brevifolia})\) possessed antitumor properties. The active compound was isolated from these plants and called Paclitaxel. Currently Paclitaxel has become a potent phytomedicine to treat lung, ovarian and breast cancers. Mefloquine an alkaloid was developed as a part of biological screening of plants with antimalarial activity during Vietnam War for treating malaria. A good example of antimalarial compound is quinine. It was isolated from \(\text{Cinchona}\) bark and was used as a template for the synthesis of chloroquine and mefloquine. More recently another compound called artemisinin, isolated from the Chinese plant \(\text{Artemisia annua}\) has been successfully used against chloroquine resistant \(\text{P. falciparum}\) strains. Isoquinoline alkaloid emetine from \(\text{Cephaelis ipecacuanha}\) has been used for many years for the treatment
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of abscesses caused by *Escherichia histolytica* infections. The resin called gugulipid produced by the stem of the plant *C. mukul* has been used widely for more than two thousand years to treat inflammation in arthritis and as hepatic antioxidant defense. The active compound namely ketosteroids cis- and trans-4, 17 (20)-pregnadiene-3,16-dione have been extracted from the resin and have been found to be safer and more effective in lowering the cholesterol levels. The other medicinal uses of *C. mukul* includes the treatment of leprosy, anti inflammatory, cardiovascular diseases, antispasmodic, nervous diseases, anthelmintic, urinary disorders, skin disorders, hypertension etc. One of the promising areas of research is the chemotherapeutic use of medicinal plants in the treatment of diverse diseases including cancer which would enable scientists to explore new molecules from plants that would provide an important tool for encountering this dreadful disease. The impact of plant derived drugs is significant in anti tumor area where drugs like vinblastine, and camptothecin, vincristine and taxol have proved their efficacy in the chemotherapy of some cancers.

The anticancerous compounds derived from plants are classified into Epipodophyllotoxin lignans, Camptothecin quinoline alkaloid derivatives, Taxane diterpenoids and Vinca alkaloids. Many anticancer compounds have been identified and reported by scientists. Vinblastine and Vincristine are the two major naturally occurring active compounds obtained from the Madagascar periwinkle, *Catharanthus roseus*. These compounds have been reported to have potent anti tumour activity against lymphocytic leukemia in mice. Vinorelbine and Vindesine are the two semisynthetic analogues obtained from these active compounds. They showed potential activity against breast cancer, leukemia, advanced testicular cancer, lymphoma and lung cancer. A compound called Podophyllotoxin is obtained from the roots of *Podophyllum* species namely *Podophyllum peltatum* and *Podophyllum emodi*. One of the isomers of podophyllotoxin is Epipodophyllotoxin. The two clinically important semi synthetic analogs generated from Epipodophyllotoxin include Teniposide and toposide which have been used to treat lymphomas, lung and testicular cancers. Curcumin also called diferuloylmethane is a polyphenolic compound, isolated from the *Curcuma longa*, commonly called turmeric now finds its application as potent anti cancer compound. About 3–5% of the yellow pigment of turmeric contains curcuminoids. Studies have shown that Curcumin is involved in modulating
the cell cycle pathway and induction of apoptosis of various cancerous cells\textsuperscript{101}. But the exact mechanism of action is yet to be clearly understood. Clinical trials are ongoing on the effects of curcumin on multiple myeloma, pancreatic cancer and colorectal cancer. Medicinal plants are the plants that are rich in a variety of secondary metabolites that serve as potential sources of drugs. Secondary metabolites are mainly organic compounds that are not directly involved in the normal growth and development of an organism. Most of the secondary metabolites are of interest to humankind and are classified into categories based on their biosynthetic origin. The secondary metabolites mainly include alkaloids, glycosides, coumarins, flavonoids, steroids etc. Alkaloids are primarily composed of nitrogen and are widely used in medicine. They can also have highly toxic effects. The first alkaloid to be found was morphine. Morphine is isolated from \textit{Papaver somniferum}, or opium poppy. It is used in medicine as a pain reliever in patients with severe pain levels and also as cough suppressant. Cocaine is another example of an alkaloid. It can be highly addictive and dangerous. However it has also been used as an anesthetic agent.

Cocaine has long been used by the people of South America to alleviate hunger. Workers chew on the leaves while working which is not dangerous because the leaves only contain a small amount of cocaine. However cocaine derivatives are very dangerous if habitually used and can have detrimental effects. Plants serve as good candidates of biologically active molecules and lead structures for the development of modified drug derivatives with enhanced activity and reduced toxicity. The small fraction of flowering plants that have been investigated so far have yielded about one hundred and twenty therapeutic agents of known structures from about ninety species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capsaicin, allicin, curcumin, artemisinin and ephedrine among others\textsuperscript{191}. About one hundred and twenty one major plant drugs have been identified for which no synthetic ones are currently available. It has been estimated that more than over four hundred traditional plants or plant derived products have been used for the management of type 2 diabetes across the world. Galegine is a substance produced by the herb \textit{Galega officinalis}. It is an excellent example of such a discovery. The experimental and clinical evaluations of galegine
provided the chemical and pharmacological basis for the discovery of drug metformin which is the popular therapy for type 2 diabetes\textsuperscript{46}. Plants have provided humans with many of their essential needs including life saving pharmaceutical agents. It is estimated that 80\% of people across the globe rely on herbal medicines for tackling a variety of diseases. Many developing countries have made stringent efforts in recording and documenting the ethnomedical data and scientific research on medicinal plants. More than 270,000 higher plants exist on this planet, but only a small portion has been phytochemically explored and biochemically characterized. Hence it is anticipated that plants can provide potential bioactive compounds for the development of new leads to combat various diseases. As a large proportion of the available higher plant species have not yet been screened for the presence of biologically active compounds, research on drug discovery from plants should remain an essential component in the exploration of new medicines and the scientific study of traditional medicines from medicinal plants are thus of great importance\textsuperscript{54}.

2.2 PLANT POLYPHENOLS AND THEIR BIOACTIVITY

2.2.1 CLASSIFICATION OF POLYPHENOLS

The term polyphenol refers to a group of water soluble phenolic compounds having molecular weight of 500 to 3,000–4,000 Da and possess 12 to 16 phenolic hydroxyl groups and 5 to 7 aromatic rings per 1,000 Da. The original definition of polyphenols in a much broader sense includes many much simpler phenolic structures. They comprise several classes of structurally diverse entities that are essentially biogenerated through either the shikimate or phenylpropanoid or the polyketide acetate or malonate secondary metabolic pathways or both. To date, more than 4,000 types of polyphenols have been identified in nature. They can be classified into two broad categories: flavonoids and nonflavonoids. Flavonoids include anthocyanins, a pigment in brightly coloured fruit, catechins, abundant in cocoa and tea, and flavanones, found primarily in citrus fruits. The major nonflavonoids are ellagic acid, found in berries, nuts, and pomegranate, and coumarins, a mild toxin that occurs in certain legumes and grasses\textsuperscript{93}. 
Currently polyphenols occupy a unique position in research as the only class of natural products with enormous biological activity. Research findings suggest different potential mechanisms of action by which polyphenols may prevent disease. Some of the mechanisms of their action includes the stimulation of blood monocytes and tissue macrophages to produce cytokines, inhibition of bacterial replication enzymes, the stimulation of myeloperoxidase dependent iodination of neutrophils and induction of apoptosis in tumour cells. The antimicrobial effects of polyphenols has been attributed to their ability to inactivate bacterial toxins and there is an increasing interest in this area because plant polyphenols could be utilized as a source of new anti infective agents against antibiotic resistant human pathogens. Terpenoids are composed of isoprene units and are found in all plants. They constitute the largest group of secondary metabolites and are very volatile which means they evaporate easily. Isoprene is a gas that is produced in the chloroplasts and released by the leaves. Isoprene is thought to offer protection to the plant from heat. Essential oils are responsible for the fragrance of plants. In some plants the fragrance is used to deter herbivores and also protect the plants from dangerous pathogens. Essential oils are used for aromatherapy and medicine.

In aromatherapy the essential oils are thought to improve mood and mental functioning. Most of the essential oils are dangerous if consumed so they are usually applied topically or inhaled. They can be used for skin disorders, respiratory ailments and as antiseptics. Phenols have become popular due to their health benefits. The phenols comprise of a hydroxyl group (–OH) attached to an aromatic ring. Phenols are found nearly in all plant parts and in nearly every plant. The first and important group of phenols is the flavonoids. Flavonoids are water soluble pigments found in the vacuoles of plant cells. Flavonoids are further divided into three groups: anthocyanins, flavones and flavonols. The colour of anthocyanins ranges from red to blue and purple. The colour depends on the pH of the environment. Anthocyanins are most commonly found in grapes, berries etc and have a wide range of health benefits. Consumption of anthocyanins offers protection against heart diseases, diabetes and even cancer. They are also used in cosmetics and health care products to slow down the aging process. The next two groups of anthocyanins have white or yellow pigments. They are called flavonols and flavones. The phenols attract pollinators to the plants and even impact how plants interact with one
Another medically important phenol is salicylic acid, which is the active ingredient in aspirin. It is obtained from the bark of the willow tree. It has been used to effectively treat aches and fevers and also has cosmetic uses. It is used in numerous skin care products to treat acne and dermatitis\textsuperscript{108}. Another type of phenol is important to the structure of the plant and is called lignin. It gives stiffness and strength to cell walls of the plant cells. It also makes the cell wall to be waterproof and protects the plant from fungal attacks. Polyphenols can be classified into different categories: Flavonoids are important polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure into flavonols, flavones, flavanones, iso flavones, catechins, anthocyanidins and chalcones. Over 4,000 flavonoids have been identified till date. They occur mainly in fruits, vegetables and beverages. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health\textsuperscript{173}. They have been reported to have anti inflammatory, antiviral, anti platelet, anti allergic, antioxidant and antitumor activities.

The flavonoids are obtained by the lengthening of the side chain of cinnamic acids by addition of one or more C2 units that results in the mixed biosynthesis of metabolites with important biological properties. Essentially these polyphenolic compounds contain 15 carbon skeletons that is represented as the C6-C3-C6 system. The flavonoids are 1,3-diarylpropanes, neoflavonoids are 1,1-diarylpropanes and iso flavonoids are 1,2-diarylpropanes. The term flavonoid was firstly described by Geismann and Hinreiner in 1952 for the classification of compounds whose structure is correlated to the 2-phenylchroman heterocyclic system or flavan. The flavonoids of every class are mainly distinguished by the number and the stereochemistry of the hydroxyl groups and/or methoxyls on the two benzene rings and/or the heterocyclic system\textsuperscript{48}. The fundamental importance of flavonoids is their ecological role as pigments in flowers and fruits. Flavonoids are important for plants as they are the pigments that give colour to fruits and flowers and thereby help in attracting pollinators. The coumarins are the typical metabolites present in higher plants. The benzo-2-pyrone nucleus of the simple coumarins is derived from the phenylacrylic skeleton of cinnamic acids via orto-hydroxylation, trans-cis isomerisation of the side chain double bond, and lactonisation\textsuperscript{108}. 
The citrus peel obtained as a by-product of the citrus juice industry contains a large amount of flavonoids. Chalcones contain two aromatic rings in trans configuration separated by three carbon atoms of which two are connected by a double bond and the third is a carbonyl group. Some natural prenylated chalcones showed potent enzyme inhibitory activity. Three chalcone derivatives called licurside, isoliquiritin, and licochalcone A, were isolated from the roots of the *Glycyrrhiza* species and competitively inhibited the monophenolase activity of mushroom tyrosinase. Another prenylated chalcone named kuraridin was isolated from the plant *Sophora flavescens* and identified as a potent tyrosinase inhibitor. The lignans constitute a group of chemical compounds found in plants. Lignans are one of the major classes of phytoestrogens which are estrogen like chemicals that also act as antioxidants. Plant lignans are polyphenolic compounds derived from phenylalanine by dimerization of substituted cinnamic alcohols known as monolignols to a dibenzylbutane skeleton. This reaction is catalysed by oxidative enzymes and is often controlled by proteins. Many natural products, known as phenylpropanoids, are built up of C6C3 units (a propylbenzene skeleton) derived from cinnamyl. A neolignan is a structure formed by joining the two propylbenzene residues at other than the β-carbon atom of the propyl side chain. Some examples of lignans include pinoresinol, podophyllotoxin, and steganacin. Some lignans in the human diet are metabolized to form mammalian lignans known as enterodiol and enterolactone by intestinal bacteria. Examples of lignans that can be metabolized to form mammalian lignans are secoisolariciresinol, pinoresinol, hydroxymatairesinol, lariciresinol, matairesinol, sesamin and syringaresinol.

### 2.2.2 ANTIMICROBIAL ACTIVITY

Plants have enormous ability to synthesize aromatic substances, most of which include phenolics or their oxygen substituted derivatives. It comprises of secondary metabolites which help in plant defensive mechanisms that offer protection against insects, herbivores and microorganisms. Much before the discovery of the existence of microbes the idea that certain plants had healing potential and that they contained certain antimicrobial principles was well accepted. Man has used plants to treat common infectious diseases as a part of traditional medicines. For instance the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*)
to treat urinary tract infections is reported in different manuals of phytotherapy while species such as garlic (*Allium sativum*), tea tree (*Melaleuca alternifolia*) and lemon balm (*Melissa officinalis*) are described as broad-spectrum antimicrobial agents\(^{39}\). The alcoholic extract of dry nuts of *Semecarpus anacardium* showed bactericidal activity *in vitro* against three gram negative strains (*Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris*) and two gram positive strains (*Staphylococcus aureus* and *Corynebacterium diphtheriae*). Subsequent studies have shown that the alcoholic extracts of different parts of the plant (fruits, leaves, twigs) also possess antibacterial properties especially the leaf extract\(^{52}\). Essential oil obtained from the herb *Santolina chamaecyparissus* showed significant antifungal activity both *in vitro* and *in vivo* against thirteen strains of *Candida albicans*. The essential oil isolated from the leaves of *Aegle marmelos* exhibited significant antifungal activity against different fungal isolates and 100% inhibition of spore germination of all the tested fungi when evaluated using the spore germination assay.

The chloroform, petroleum ether, ethanol and acetone extracts of the leaves of the *Cassia alata* also showed significant *in vitro* antifungal activity against various fungi like *Aspergillus niger*, *Rhizobium japonicum*, *Candida albicans* and *Candida tropiathis*\(^{52}\). Some polyphenols called phytoanticipins that are compounds with a defensive role and are not synthesised in response to a pathogen attack but rather are constitutively present in plant cells. Phenolic constituents are present on the surface of plants or in the cytoplasmic fraction of the epidermal cells. On the other hand phenolic phytoalexins are secreted by wounded plants or in response to incompatible pathogens. The induced defence response includes cell death and the formation of a lesion that inhibits the growth of the pathogen. Cells surrounding the lesion accumulate polyphenols and other antibiotic compounds. Polyphenols as catechin have been reported to act on different bacterial strains like *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Bordetella bronchiseptica*, *Salmonella choleraes*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* by generating hydrogen peroxide and by altering the permeability of the microbial membrane\(^{129}\). Recently polyphenols have been reported to interfere with bacterial quorum sensing which involves the production of small signal molecules by bacterial cells that triggers the exponential growth of a bacterial population\(^{131}\). Research evidences indicate that
many plants used as folk remedies contain high concentrations of polyphenolic compounds. Plants from a broad range of angiosperm families show antibacterial activity. Many studies have shown the correlation of polyphenol contents with antimicrobial activity. Plants from different families, including Primulaceae, Asteraceae, Fabaceae, Poaceae, Lythraceae, Polygonaceae, Verbenaceae and Onagraceae have shown to have bactericidal action. Members of the Rosaceae and Geraniaceae families are also rich in polyphenolic compounds with antimicrobial activity and *Cydonia oblonga* was found to be an important source of polyphenols that are active against bacterial growth. Polyphenols with relevant antimicrobial activity have been isolated from members of other plant families and flavonoids castalagin and protodelphinidin have been identified to be effective against different bacterial and fungal strains.

### 2.2.3 INSECTICIDAL, PHYTOTOXIC AND CYTOTOXIC ACTIVITY

Insects present a problem in stored grain throughout the world because they reduce both the quantity and quality of grain. The stored pest red flour beetle, *Tribolium castaneum* is an example of a major pest in storage of grain based products. This species is for long associated with human stored food and has been found in a wide range of commodities including grain, beans, flour, peas, dried fruits, spices and nuts. A greater awareness of the hazards associated with the use of synthetic organic insecticides has led to an urgent need to explore suitable alternative products for pest management and control. Insecticidal activity of several plants against several insects has been reported by many workers.

The natural products from several floral species have been demonstrated to act as toxicants, repellents, and antifeedants against a number of coleopteran that attack stored products. Seeds and floral extracts of several plants have been reported to have toxic and potent growth reducing activity to insects. The detrimental effects of plant extracts on insects can be manifested in several manners including mortality, toxicity, growth inhibitor, anti feedant, reduction of fecundity and fertility and suppression of reproductive behavior. Exposure of toxic agents to insects can cause changes even at the molecular level. Nucleic acids (DNA and RNA) and protein contents are considered as important biomarkers of the metabolic potential of cells as they play the main role in regulating the different cellular activities. Changes in the amount of nucleic acid
can be used to detect the effect of toxic agents on cell proliferation and cell death. The harmful effects of insects and other pests such as mosquitoes, cockroaches, rodents, parasitic worms, flies has been well known and challenged by man. Pesticides are any substances or mixture of substances used for preventing, destroying, repelling or mitigating any pest. They include chemicals like lead, sulphur, mercury and arsenic. DDT (dichlorodiphenyltrichloroethane) was effectively used to control malaria and typhus diseases. It was the first synthetic organic pesticide discovered and was used for agricultural purposes. There is no doubt that the use of insecticides has immensely contributed to the increase in agricultural productivity and improvement in human health, particularly the eradication of diseases. However it has been established that use of synthetic organic pesticides particularly the chlorinated hydrocarbons such as DDT and derivatives has led to serious effects on human health, environmental pollution, and death of non target organisms including animals, plants, and fish. This situation led to the ban of DDT in 2004. Natural products from plants have attracted researchers in recent years as potent sources of new pesticides. The use of higher plants by the natives of various parts of the world as insecticidal agents has been well known.

One of the early plants to be reported as insecticidal agent was tobacco (*Nicotiana tabacum*). The use of tobacco leaves to kill aphids led to the isolation of the alkaloid called nicotine. The chemical investigation of plant, *Rhododendron hortense* showed the presence of an active component called rotenone, with considerable insecticidal activity. Plants of the genus *Chrysanthemum* are the sources of very successful insecticidal extract, *pyrethrum*, and the active constituents called pyrethrins. There is significant evidence that a number of plants possess pesticidal activity and this has been confirmed by investigations by various research groups in different parts of the world. The toxicity of the ethanol extracts of the leaves of twenty plant species from different families to *Callosobruchus maculatus* and *Callosobruchus chinensis* were studied. It was observed that mortality reached a maximum level in 72 hours of exposure to the oils from the leaves which indicated a high level of lethality. Similarly the protectant effectiveness of some plants native to Nigeria against the maize weevil, *Sitophilus zeamais* Motsch, and the cowpea weevil, *Callosobruchus maculatus*, respectively have been established. On the basis of the results of various pesticidal screenings, it has been established that a number
of plants have broad pesticidal activity and have been commonly used in traditional agricultural applications in many parts of the developing countries. Various investigations have shown that in most cases the insecticidal activity is usually distributed among the various parts of the same plant though the lethality and quantities of the active components may vary\textsuperscript{84}. In the past decades apart from the \textit{pyrethrum} which has attained international and commercial importance due to its high efficacy and broad spectrum insecticidal activity very few natural insecticides have been developed. The tropical plant \textit{Azadirachta indica}, popularly known as the neem tree is effectively used to control over twenty five different species of insect pests\textsuperscript{181}. The activity has been associated with the presence of active compound called \textit{azadirachtin} which is found to be highest in the kernel than in the leaves and other tissues of the plant. The effectiveness of nine insecticidal species of Chinese origin has been compared with synthetic insecticides against forty species of insects\textsuperscript{117}. Three plants \textit{Milletia pachycarpa}, \textit{Trpterygium forestii Loes} and \textit{Rhododendron molle} were found to be highly active against aphids, pentatomids and leaf beetles as well as against caterpillars, body lice and plant lice. Among the plants \textit{R. molle} exhibited specific toxicity against certain species of \textit{lepidopterous} larvae, \textit{pentatomids} and leaf beetles. The three plants were shown to contain rotenone.

Investigation of the Sri Lankan plants showed that extracts of three plants, \textit{Plearostylia opposita} (Celastraceae), \textit{Aegle marmelos Correa} (Rutaceae) and \textit{Excoecaria agallocha} (Euphorbiaceae) displayed admirable insecticidal activity. Three compounds possessing the \textit{daphnane orthoester} skeleton, which are constituents of the ethyl acetate extract of \textit{E. agallocha}, were found to be insecticidal. The genus \textit{Piper} (family Piperaceae) is one of the most studied. With over 1000 species, about 112 genera have been screened for insecticidal activity and more than 611 active compounds have been isolated and identified from various parts of the species. The compounds from \textit{Piper longum}, \textit{Piper retrofractum} and \textit{Piper guineense} are known to be active against \textit{Callosobruchus maculatus}, the garden insect, \textit{Zonocerus variegatus} L, and the mosquito larvae causing 96-100\% mortality rate in 48 hours mostly as solution sprays. From the chloroform and petroleum extracts of \textit{P. guineense} fruits two Piper amides, guineensine and piperine were isolated. Piperine has been shown to be a synergist rather than an insecticide in the crude extracts and a number of plants produce polyphenols called tannins which confer bitter taste on such
plants and consequently herbivores stay away from eating such plants. Brine shrimp lethality assay and phytotoxic evaluation of botanical extracts help in the prediction of various types of biological activities. The cytotoxic and phytotoxic studies of crude botanical extracts confirms their bioactivity in terms of their ability to cause cell death, uncontrolled cell proliferation or growth inhibition. This can open new ways towards discovery of anticancer drugs if the plant extracts possess antitumor activity. Growth stimulation or inhibition in phytotoxicity assay can determine herbicidal or growth stimulatory potential of plant extracts tested. A positive correlation between these assays help to determine the pharmacological importance of medicinal plants. The Euphorbiaceae are family of poisonous plants have been reported to have both medicinal and hazardous implications. The compounds responsible for the toxic properties of this family have been identified to be lectins and esters of certain diterpenes alcohols, with phorbol derivatives, tiglane, dephnane and ingenene diterpene esters. The toxic diterpenes esters cause skin inflammation with redness and formation of oedema. On exposure to the eyes they cause inflammation of the cornea and conjunctiva which sometimes leads to blindness. The consumption of these extracts leads to poisoning with severe gastroenteritis, vomiting and diarrhea.

The toxicity of *E.kamerunica* on *Oreochromis niloticus* and the compounds responsible for this toxicity were identified. Brine shrimp assay is an indicator used in determining the safety, cytotoxicity and insecticidal properties of compound and is very useful preliminary tool for isolation of bioactive compounds from plant extracts. Allelopathy seems to be an important component of plant interference capability in a variety of ecosystems. Allelopathy refers to a phenomenon where the chemicals of a plant species interferes with the germination, growth or development of other plant species and has been known for over two thousand years. Literature suggests the involvement of this phenomenon where many crop plants like barley (*Hordeum vulgare*) and gram (*Cicer arietinum*) inhibited growth of some weeds and crop plants. Allelochemicals can be present in any parts of plant including leaves, roots, rhizomes, stems, pollen, flowers and seeds which may be released into the environment by root exudation, leaching from over the ground parts, volatilization and decomposition of plant material. Aqueous extract of plants have been shown to interfere with test crop germination and seedling
growth by causing plant growth inhibition (allelopathy), causing nutrient transformation, and/or by influencing the microbial population that can affect the crop seedlings\textsuperscript{153}. The allelopathic potential of aqueous extracts of \textit{Podocarpus totara}, \textit{Dacrycarpus dacrydioides} and \textit{Prumnopitys taxifolia}, and on \textit{D. dacrydioides} and \textit{L. sativa} have been evaluated for germination and seedling elongation respectively\textsuperscript{120}. The occurrence of natural allelopathic activity in crops has significant positive and negative implications on crop plants. The allelopathic properties of some crops have been used for weed management as an alternative to usage of expensive, pollutant synthetic herbicides\textsuperscript{147}.

\subsection{2.2.4 ENZYME INHIBITORY ACTIVITY}

Natural alpha amylase and glucosidase inhibitors from the plants can be used as an effective therapy for treating post prandial hyperglycemia with minimal side effects. A wide range of plants have been shown to possess significant alpha amylase and alpha glucosidase inhibitory activity by virtue of the presence of compounds including alkaloids, glycosides, polysaccharides, peptidoglycans, guanidine, steroids, glycopeptides and terpenoids\textsuperscript{178}. \textit{Syzygium cumini} L. and \textit{Psidium guajava} L. are widely used in traditional system of medicine to treat diabetes due to their alpha amylase and alpha glucosidase inhibitory activity. The aqueous extracts from \textit{S. cumini} seeds and \textit{P. guajava} leaves both showed a concentration dependent inhibitory effect on alpha amylase activity. The seed extracts of \textit{S. cumini} also significantly decreased the levels of blood glucose in diabetic rats\textsuperscript{100}.

The methanol, ethyl acetate and hexane extracts from two varieties of \textit{Amaranthus caudatus} L. showed significant alpha amylase inhibitory activity. The extracts of several plant species namely \textit{Balanites aegyptiaca}, \textit{Camellia sinensis}, \textit{Galega officinalis}, \textit{Holarrhena floribunda}, \textit{Khaya senegalensis}, \textit{Melissa officinalis}, \textit{Mitragyna inermis}, \textit{Rosmarinus officinalis}, \textit{Securidaca longepedunculata}, \textit{Tamarindus indica}, \textit{Taraxacum officinale}, and \textit{Vaccinium myrtillus} were screened for alpha amylase and alpha glucosidase inhibitory activity and showed remarkable activity\textsuperscript{22}. Several studies reported the inhibitory effects of polyphenols on carbohydrates hydrolyzing enzymes. The green tea polyphenols have been found to inhibit the activities of alpha glucosidase and sucrase, berry polyphenols inhibit the activity of alpha glucosidase and
alpha amylase and sweet potato polyphenols inhibit the activity of α-glucosidase. Plant foods rich in polyphenolic fractions have been reported to cause insulin like effects in glucose utilization and act as good inhibitors of key enzymes linked to type 2 diabetes and lipid peroxidation in tissues. Some polyphenols such as quercetin glucoside, gallic acid, ellagic acid, ellagic acid derivative, and some other unidentified phenolic compounds have been characterized and reported to possess alpha amylase and alpha glucosidase inhibitory activity. Several workers have reported the positive correlation of polyphenol constituents of plants, its antioxidant/ radical scavenging properties, as well as its ability to prevent diseases associated with oxidative stress such as diabetes, cancer and cardiovascular disorders. Tyrosinase is an enzyme present in plant, animal and human tissues which is responsible for the synthesis of melanin pigments from tyrosine by oxidation. Tyrosinase is responsible for causing the blackening of a sliced potato due to oxidation on exposure to air. Traditional and modern scientific knowledge have generated tremendous information about the use of herbal cosmetics in today’s world. Herbal extracts possess antioxidant properties which possess metal ion chelating properties and also give protection from ultraviolet rays.

Various types of herbal formulations in the form of ointments, lotions, creams, emulsions suspensions have been used in the area of cosmetics and health care. Herbal extracts have been evaluated for their potential worldwide by researchers and phytochemical constituents isolated from the plants have been screened for their cosmetic use. Tyrosinase is also associated with neurodegenerative diseases in mammals, moulting process in insects, browning of vegetables and fruits. Hence tyrosine inhibitors in addition to their intended use in cosmetic products have also proved to be the best target sites for controlling insect pests and browning of vegetables and fruits. Some of the flavonoids were identified as tyrosinase inhibitors. This includes nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), naringin (5,7,4′-triroyxyflavanone), and neohesperidin (5,7,3′-trihydroxy-4′-methoxyflavone). But the inhibitory strength of these three inhibitors was found to have poor activity towards mushroom tyrosinase compared with kojic acid. The ethanolic extract of citrus fruit has been reported to exhibit in vitro inhibitory effects on melanogenesis in melanoma cells and in vivo prevention against UV induced pigmentation of dorsal skin in guinea pigs. The melanogenesis inhibitory activity of citrus crude extracts was
found to be mainly due to the antioxidant activity of neohesperidin in citrus fruit. The extracts from *Morus* species, which has been well known as a polyphenol rich plant and used as a non toxic natural therapeutic agent. They also have high potential in applications as skin whitening agents as many potent tyrosinase inhibitors have being isolated from different parts of the plant. 

Mulberroside F (moracin M-6,3′-di-O-β-glucopyranoside) purified from the leaves of the plant showed the antidiphenolase activity of mushroom tyrosinase to be 4.5 times higher than that of kojic acid and exhibited an inhibitory effect on melanin formation within the melanoma cells. Norartocarpetin (5,7,2′,4′-tetrahydroxyflavone), isolated from the stem bark of the plant was found to be much more active than kojic acid against monophenolase activity of mushroom tyrosinase. In addition to the leaves and stem of the plant, the roots of the *Morus* species were also found to contain many very potent tyrosinase inhibitors, including streppogenin (5,7,2′,4′-tetrahydroxy-flavavone), oxyresveratrol and norartocarpetin. The chemical structure of the flavanone streppogenin is very similar to that of the flavones norartocarpetin with the same four substituted hydroxyl groups.

Recently dihydromorin (5,7,2′,4′-tetrahydroxyflavanol and artocarpetin (5,2′,4′-trihydroxy-7-methoxyflavone were isolated from the wood of *Artocarpus heterophyllus*. For a long time the extracts from the roots and seeds of *Glycyrrhiza* species belonging to Leguminoseae family have been regarded as an effective constituent for skin whitening agents in the East Asian countries. The melanogenesis inhibitory activity of the extracts is mainly due to the isoflavonoids in the plant. Two isoflavans namely glabridin and glabrene were purified from the roots of the plant and were identified as potent tyrosinase inhibitors. Recently Glyasperin C was isolated from the same part of the plant and shown to be two times more active than glabridin. Although glyasperin C is the most active inhibitor it was seen that glabridin had the best melanogenesis inhibitory activity. Three hydroxyisoflavones including 6-hydroxydaidzein (6,7,4′-trihydroxyisoflavone), 8-hydroxydaidzein (7,8,4′-trihydroxyisoflavone), and 8-hydroxygenistein (5,7,8,4′-tetrahydroxyisoflavone) were isolated from soybean koji fermented with *Aspergillus oryzae* and demonstrated as potent tyrosinase inhibitors.
2.2.5 ANTI ARTHRITIC AND ANTI INFLAMMATORY ACTIVITY

Arthritis is the inflammation of one or more joints and involves the breakdown of cartilage. Cartilage normally protects a joint, allows it to move smoothly and also absorbs shock when pressure is put on the joint. The abnormal amount of cartilage causes rubbing of bones which leads to pain, swelling and stiffness. The usual age of onset of arthritis is between the age of 25 and 50 with a peak in the 40s and 50s. In India it is estimated that more than about 20% of total population is suffering from arthritis. The joints most commonly affected by arthritis are weight-bearing joints, such as hips, spine, feet, knees, thumb and finger joints. Symptoms of arthritis can include declined ability to move the joint, stiffness, difficulty performing daily activities, disability, chronic pain etc. The key risk factors of arthritis includes excess weight, injury, age, gender, dietary pattern, excess alcohol consumption, lifestyle, heredity, hormonal factors, lack of physical activity and environmental factors.

Inflammation is a normal protective response to tissue injury and involves a sequence of responses such as enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair. It is a complex process frequently associated with pain, increase in vascular permeability, increase of protein denaturation and membrane alterations. Inflammatory response is initiated by harmful stimuli including pathogens, irritants or damaged cells. Inflammation is a protective mechanism elicited by the organism to remove injurious stimuli as well as initiate the tissue damage healing process. However if inflammation is left untreated it leads to diseases like vasomotor rhinorrhoea, rheumatoid arthritis and atherosclerosis. The four main groups of drugs used to treat arthritis are pain killers (analgesics), non-steroidal antiinflammatory drugs (NSAIDs), disease-modifying anti rheumatic drugs (DMARDs) and corticosteroids. Because of the limitations of the existing synthetic drugs there is an enormous need to search for newer drugs. A large number of medicinal plants have been tested and found to contain active principles with curative properties against arthritis. Plants with anti arthritic and anti inflammatory activity contain a variety of chemical constituents like flavonoids, alkaloids, anthocyanins, xanthenes, phenols, coumarins, essential oils, monoterpenes, catechins, quinones, carotenoids etc. The anti arthritic activity of ethanolic extract of *Ajuga bracteosa*
was evaluated against turpentine oil and formaldehyde induced acute nonimmunological and complete Freund’s adjuvant induced chronic immunological arthritis in albino rats. The extract showed a significant and dose dependant inhibitory effect against acute and chronic models of arthritis. The anti arthritic activity of stem barks of *Alangium salviifolium* belonging to family Alangiaceae was studied in rats\(^\text{107}\). The petroleum ether, chloroform, alcoholic extracts of the *A. galanga* rhizomes were evaluated for their anti arthritic activity in rat model. Application of all the three extracts exhibited statistically significant edema inhibition when compared with the arthritic control group. Anti arthritic activity of *Anisomeles malabarica* was studied by the inhibition of protein denaturation method. It was reported that methanolic extract of *Anisomeles malabarica* is capable of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease\(^\text{107}\). Arthritis stand as one of the foremost health troubles worldwide therefore treating arthritis with plant derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive\(^\text{103}\).

### 2.2.6 PLANT POLYPHENOLS AS ANTIOXIDANTS

Many plants have been known to produce biologically active substances, some of which are related to special flavour or taste and others are found to be useful as antioxidants and/or antimicrobial agents\(^\text{13}\). Medicinal Plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments. Approximately 80% of the world population depends exclusively on plants for their health and healing. In the developed world, reliance on surgery and pharmaceutical medicine is more usual, but in recent years, more and more people are complementing their treatment with natural supplements obtained from medicinal plants. The plant kingdom holds many species of plants containing substances of medicinal interest which are yet to be investigated. Large numbers of plant are constantly being screened for their chemical and pharmacological properties. By the application of modern techniques of isolation and pharmacological evaluation, many new plant drugs find their way to medicine as purified substances\(^\text{29}\). This will probably justify the use of many of the plant drugs as household remedies by indigenous people of many countries from ancient times.
The surge in the number of publications on the investigation of antioxidant and anti-inflammatory properties of various plants and methods of finding those properties clearly indicates the importance of the antioxidants. Oxygen is necessary for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals. Free radicals are types of reactive oxygen species (ROS), which include all highly reactive oxygen containing molecules. The main endogenous (originates within the organism) sources of most of the oxidants produced by cells in different ways, including normal aerobic respiration. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, liver diseases, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress among others.

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize free radicals. Considerable amount of data have been generated on antioxidant properties of food plants around the globe. Several medicinal plants have also been extensively used in the Indian traditional system of medicine for the treatment of number of diseases. Some of these plants have shown potent antioxidant activity. However, majority of plants have not yet been screened for such activity. Chronic diseases and oxidative stress constitute a major challenge for medicine and basic biology and will certainly remain so for the next few decades. Oxidative stress is the consequence of an imbalance of peroxidants and antioxidants in the organism. This is rapidly gaining recognition as a key phenomenon in chronic diseases. It is directly associated with the pathogenic mechanism of risk factors and in the protection exerted by various environmental factors. The amount of oxidative stress in populations is a possible indicator for the magnitude of environmental risk factors. Diet plays a major role in the environmental control of oxidative stress. It has been reported that fruits, vegetables and red wine decrease oxidative stress, whereas the diets rich in fats induces oxidative stress. Hence diet is a key environmental factor and a potential tool for the control of chronic diseases.
Dietary recommendations of fruits and vegetables have been shown to exert a protective effect\textsuperscript{167}. The high contents of polyphenol antioxidants in fruits and vegetables are probably the main factor responsible for these effects\textsuperscript{185}. Polyphenols occur in a variety of plants and are utilized as important components of both human and animal diets. These include food grains such as barley, sorghum, millet, dry beans, peas, and other legumes, fruits such as peaches, apples, blackberries, cranberries, pears, plums, raspberries, strawberries and grapes; and vegetables such as onion, cabbage, parsley and celery also contain a large quantity of polyphenols. Phenolic compounds are also present in wine and tea. Diets containing an abundance of fruit and vegetables are protective against a variety of diseases, particularly diabetes, cardiovascular disease and cancer\textsuperscript{184}. The presence of polyphenol antioxidants in plant foods is largely influenced by genetic factors and environmental conditions. Other factors, such as germination, degree of ripening, variety, processing, and storage, also influence the content of plant phenolics. Studies on the flavonoid content of fruits, vegetables, and beverages showed the presence of important flavonoids namely quercetin, kaempferol, myricetin, apigenin, and luteolin.

Studies have shown that polyphenolic content of the foods change on storage, due to easy oxidation of these polyphenols\textsuperscript{40}. Oxidation reactions result in the formation of more or less polymerized substances which lead to changes in the quality of foods. Quercetin the abundant polyphenol in onion has been shown to be inversely associated with mortality from cardiovascular disease by inhibiting the expression of metalloproteinase\textsuperscript{1} and the disruption of atherosclerotic plaques\textsuperscript{159}. Tea catechins inhibit the invasion and proliferation of the smooth muscle cells in the arterial wall, that may contribute to slow down the formation of the atheromatous lesion\textsuperscript{169}. Resveratrol the wine polyphenol prevents the platelet aggregation by preferential inhibition of cyclooxygenase activity\textsuperscript{143}. The aflavins and thearubigins, the abundant polyphenols in black tea have also been proven to possess strong anti cancer property. Black tea polyphenols were found to inhibit proliferation and increase apoptosis in prostate carcinoma cells\textsuperscript{158}. Quercetin has also been reported to possess anticancer property against lung carcinogenesis in mice\textsuperscript{159}. Resveratrol prevents all stages of development of cancer and has been found to be effective in most types of cancer including prostate, lung, skin, breast, colorectal and gastric cancer. It has also been shown to suppress metastasis and
angiogenesis. Extensive studies on human cell cultures indicate that resveratrol can modulate multiple pathways involved in cell growth, apoptosis and inflammation\(^7\). All these effects have been attributed to the free radical scavenging activity of the plants.

2.2.7 ANTI DIABETIC ACTIVITY

Diabetes mellitus is a chronic endocrine disorder that affects the metabolism of carbohydrates, proteins, fat, electrolytes and water. It includes a group of metabolic diseases characterized by hyperglycemia, in which blood sugar levels are elevated either because the pancreas do not produce enough insulin or cells do not respond to the produced insulin. Impairment of glucose metabolism leads to physiological imbalance with the onset of the hyperglycemia and subsequently diabetes mellitus. There are two main categories of diabetes namely type 1 and type 2. Studies have shown that diabetic conditions alter several physiological parameters of the body\(^5\). Long term effects of diabetes is associated with progressive development of specific clinical conditions such as retinopathy which affects eyes and lead to blindness, nephropathy in which the renal functions are altered or disturbed and neuropathy which is associated with the risks of amputations, foot ulcers and features of autonomic disturbance. Therefore a therapeutic approach to treat diabetes is to decrease postprandial hyperglycemia.

Currently, there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents for the treatment of diabetes mellitus. Hence the traditional herbal medicines are mainly obtained from plants are used in the management of diabetes mellitus\(^1\). In recent years herbal medicines have started to gain importance as a source of hypoglycemic agents. It is estimated that more than thousand plant species are being used as folk medicine for diabetes. Biological actions of the plant products used as alternative medicines to treat diabetes are in relevance to their chemical composition. Herbal products or plant products are rich in flavonoids, phenolic compounds, coumarins, terpenoids and other constituents which help to reduce blood glucose levels. Several species of herbal drugs with potential antidiabetic activity have been described in the scientific literature\(^4\). Herbal drugs are prescribed due to their good effectiveness, fewer side effects in clinical experience and relatively low costs. Medicinal and natural herbal plant products are traditionally used from long time in many countries for the
treatment of diabetes mellitus. The ethnobotanical information reports about one thousand plants that may possess antidiabetic potential. Some examples of medicinal plants possessing hypoglycemic properties include *Elephantopus scaber, Gymnema sylvestre, Liriope spicata, Parinari excelsa, Ricinus communis, Sarcopoterium spinosum, Smallanthus sonchifolius, Swertia punicea, Vernonia anthelmintica, Combretum micranthum* etc\(^\text{15}\). Medicinal Plants serve as reservoirs of natural products. Medicinal plants and their products have been widely used in the Indian traditional system of medicine and have shown experimental or clinical antidiabetic activity. Medicinal plants and their products have been widely used for treatment of diabetics all around the world with less known scientific basis of their functioning mechanisms. Therefore natural products from medicinal plants need to be investigated by scientific methods for their antidiabetic activity. *Mangifera indica* stem bark and leaves have been reported to produce hypoglycemic effects in diabetic rats. Numerous studies have reported the antidiabetic effects of polyphenols\(^\text{164}\). Tea catechins have been evaluated for their antidiabetic potential\(^\text{156}\). Polyphenols may influence glycemia through different mechanisms including the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues.

The hypoglycemic effects of diacetylated anthocyanins were observed with maltose as a glucose source, but not with sucrose or glucose. The studies suggest that these effects are due to an inhibition of alpha glucosidase in the gut mucosa\(^\text{172}\). Inhibition of alpha amylase and sucrase in rats by catechin was also observed. The inhibition of intestinal glycosidases and glucose transporter by polyphenols has also been studied. Individual polyphenols such as isoflavones, catechin, epigallocatechin, epicatechin gallate and epicatechin from soybeans, glycyrrhizin, tannic acid from licorice root, chlorogenic acid and saponins also decrease S-Glut-1 mediated intestinal transport of glucose. Additionally saponins also delay the transfer of glucose from stomach to the small intestine\(^\text{58}\). Resveratrol has also been reported to act as an antidiabetic agent. Many mechanisms have been proposed to explain the antidiabetic action of this stilbene\(^\text{47}\). Modulation of SIRT1 is thought to be one of them which improves whole body glucose homeostasis and insulin sensitivity in diabetic rats. It is reported that grape seed polyphenols inhibited high glucose induced cytotoxicity and oxidative stress in cultured LLC-PK1 cells\(^\text{48}\). Resveratrol inhibits diabetes induced changes in the kidney in diabetic nephropathy
and significantly improves renal dysfunction and oxidative stress in diabetic rats. Treatment with resveratrol also resulted in decreased insulin secretion and delayed the onset of insulin resistance\textsuperscript{189}. Onion polyphenols especially quercetin is known to possess strong antidiabetic activity\textsuperscript{145}. A recent study shows the ability of quercetin to protect the alterations in diabetic patients during oxidative stress\textsuperscript{159}. Quercetin significantly protected the lipid peroxidation and inhibition of antioxidant system in diabetics. \textit{Hibiscus sabdariffa} extract contains flavonoids, polyphenolic acids, anthocyanins and protocatechuic acid. A study performed showed that polyphenols present in the extracts from \textit{Hibiscus sabdariffa} attenuated diabetic nephropathy including serum lipid profile, pathology and oxidative markers in kidney\textsuperscript{138}. Ferulic acid is another polyphenol very abundant in vegetables and maize bran. Several studies have shown that ferulic acid acts as a potent antidiabetic agent by acting at many levels. It was demonstrated that ferulic acid lowered blood glucose followed by a significantly increased plasma insulin and a negative correlation between blood glucose and plasma insulin\textsuperscript{37}.

Recently it was found that the polyphenol extracts from burs of \textit{Coriandrum sativa} exhibited antioxidant potential in DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS\textsuperscript{+} (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) radical assays and reducing power analysis\textsuperscript{46}. Similar results have been demonstrated where the phenolic extract from \textit{Coriandrum mollissima} elicited antioxidant properties both in chemical antioxidant and cellular antioxidant analysis. It was also reported that the spiny burs extracts of \textit{C. sativa} could increase rat pancreatic β-cell viability after streptozotocin (STZ) treatment by protecting DNA from oxidative damage and by enhancing the natural antioxidant system \textit{in vitro}\textsuperscript{124}. Diabetes is characterized by progressive failure of pancreatic β-cell and the direct beneficial effects of phenolic rich chestnut extracts on pancreatic β-cells indicated that the polyphenols of chestnut burs could be used as a potential therapeutics for diabetes.
2.3 SOLVENT EXTRACTION AND PRELIMINARY PHYTOCHEMICAL SCREENING OF PLANTS

The phytochemical investigation of a plant involves the following steps: authentication and extraction of the plant material, separation and isolation of the constituents of interest, characterization of the isolated compounds and quantitative evaluation. Considerable effort has been established by researchers to find efficient extraction methods in order to get high efficiency and efficacy. Efficiency refers to the yield of extraction, whereas efficacy refers to the potency or magnitude of bioactivity or the capacity to produce an effect of the extract. For isolation of bioactive components, extraction from plant is one of the most sustainable approaches. For obtaining better quality and high efficiency of extraction from plants, the optimization of methods is very important. Literature shows a strong positive linear correlation between extraction efficiency and total bioactivity of plant extracts. The analytical procedures include several critical steps like sampling, sample preparation, quantification, statistical evaluations, etc. The need for selection of most appropriate extraction methodology is evident from the fact that the application of different methods on same plant material with same solvent, extraction efficiency can vary significantly.

In addition, the standardization of the method selected as the most appropriate is needed to achieve acceptable degree of reproducibility. It should be noted that choice of appropriate solvent is of essential significance along with application of a compatible extraction method. Polar solvents will extract out polar substances and non polar material will be extracted out by non polar solvents. Solvent extraction is the most popular and method of choice for extraction. Hydroalcoholic solvent mixture, a mixture of alcohol and water in varying proportions is generally considered to give high extraction yields, owing to their expanded polarity range. Sample preparation is the first crucial step in analysis of herbs, because it is necessary to extract the desired chemical components from the herbal material for further separation. For the extraction of therapeutically desirable active constituents various solvents such as ethanol, chloroform, water, methanol, ethyl acetate are commonly used. Sometimes mixtures of solvents are also used to get better extraction efficiency. The development of modern sample preparation
techniques has significant advantages over the conventional methods in terms of reduction in organic solvent consumption and in minimizing sample degradation. They also result in elimination of undesirable and insoluble components from the extract. The modern methods includes ultrasonication assisted extraction, microwave assisted extraction, supercritical fluid extraction, solid phase micro extraction etc. Classical methods are fairly simple, standard and continue to have widespread use; however these methods can also be insufficient and slow, consume large quantities of organic solvents, and cause degradation of heat labile constituents. Using conventional methods, quality related problems like lack of safety, consistency and efficacy are also encountered. Furthermore, elimination of additional sample clean up and concentration steps before chromatographic analysis, improvement in extraction efficiency, and selectivity are also the advantages of modern processes. The purpose of standardizing extraction procedures for production of crude herbal drugs is to obtain the therapeutically desired portion and to eliminate the inert material by treatment with selective solvents and methods. With the increasing demand for herbal medicinal products and natural products for health care all over the world, herbal manufacturers focus at using the most appropriate extraction technologies to produce extracts of defined quality.

Standardization of extraction procedures contributes significantly to the final quality of the herbal drug. To have a complete idea of the bioactivity of crude extracts it becomes necessary to optimize the extraction methodology inorder to achieve the broadest possible range of phytochemicals. The selection of method to isolate active components with best yield and highest purity from plant sources is mainly dependent on the nature of compounds and raw material which is going to be processed. Phytochemical screening assay is a quick, simple, and inexpensive procedure that gives the researcher a quick solution to the various types of phytochemicals in a mixture and an important tool in bioactive compound analysis. The phytochemical interaction and trace components tend to alter the drug response in ways that cannot currently be replicated with a combination of few purative active ingredients. Pharmaceutical researchers recognize the concept of drug synergism and clinical trials may be used to investigate the efficacy of a particular herbal preparation if the formulation of that herb is consistent. The clinical and medicinal experience of more than two thousand years and integrated theory system for
diagnosis and treatment open a gateway for discovering new drugs from these natural products. In the past decades, a large number of effective analytical tools have been used for analyzing the constituents of traditional medicines in order to control the quality and discover bioactive compounds especially the chromatography methods provide impressive separation technique for complex mixtures\textsuperscript{204}. There is evidence that using some alternative medicines especially those involving herbs, metals, minerals or other materials involves potentially serious health risks including toxicity\textsuperscript{94}. In recent years, the application of chemical data to systematics has received serious attention of a large number of botanists and biochemists. The screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes. Traditional chinese medicines have a long history dating back several thousands of years. A vast majority of active components have been discovered from these Chinese medical products for anti bacterial, anticancer, antifungal, antiviral activity such as colchicine, taxol, vinblastine, vincristine, podophyllotoxin and camptothecin that exhibit antineoplastic activity, tripterygium wilfordii multiglycoside, simomenine, total glucosides of astragalus exhibiting antiinflammatory activity\textsuperscript{26}.

The combination of chromatography methods and spectroscopy techniques, such as mass spectrometry, nuclear magnetic resonance spectroscopy, infrared spectroscopy, etc, can conveniently provide a number of structural information of the separated components. Thereby it is possible to simultaneously determine the structure of separated components or other information in one program. It is still very difficult to obtain specific bioactive compounds, even from a well documented formulas for specific disease therapy. Because the amount of active compounds with a number of interfering ingredients is usually low, and their action mechanisms is not yet clear. The routine process for screening is to extract single ingredient or single distilled fraction from plant extracts and determine its bioactivity by the classic pharmacological means. The whole animal model is the most selected classic pharmacological screening model to study the efficacy, side effect and toxicity of medicines. The tissues and organs model are used to study the effect of drug under the physiological condition and even under the pathological condition. It overcomes the demerits of whole animal model as the amount of screening sample is cut down. As usual the amount of sample in the whole animal
model need more than 1-5 g, according to the dosage and the size of used animal, whereas the amount of sample used in tissues and organs model is low as one tenth as the whole animal model or less\textsuperscript{134}. Multisample screening can be performed at the same time so that the efficiency is improved and the cost is reduced. It also decreases the interference of other in vivo factors and allows obtaining the authentic evaluation of pharmacology. In recent years the field of cellular model based screening is expanding rapidly. Cell based screening is useful in providing a broad range of data on drug activity, mechanism of action and drug efficacy\textsuperscript{67}. Compared with the whole animal models, the cellular models based on different disease and mechanisms are more efficient for large scale drug screening because the resource and culture of cells are relatively economical and easy. Natural products from medicinal plants are obtained either as pure compounds or as standardized extracts and they provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is an increasing demand for chemical diversity in screening programs for seeking therapeutic drugs from natural products. This has led to the interest of researchers in edible plants throughout the world. Herbal preparations and botanicals for medicinal use contain various types of bioactive compounds.

The key focus is currently on the analytical methodologies which include the extraction, isolation and characterization of active ingredients in botanicals and herbal preparations. Extraction is the most important step in the analysis of constituents present in botanicals and herbal preparations\textsuperscript{79}. The analysis of bioactive compounds present in the plant extracts involves the applications of common phytochemical screening assays, chromatographic techniques such as HPLC and, TLC as well as non-chromatographic techniques such as immunoassay and Fourier Transform Infra Red (FTIR)\textsuperscript{68}. For several years mankind is using plant source to alleviate or cure illnesses. Plants form a source of novel chemical compounds which are of potential use in medicine and other applications. Plants contain many active compounds such as glycosides, alkaloids, steroids, tannins, resins, phenols, flavonoids and essential oils which are present in their specific parts such as leaves, flowers, bark, seeds, fruits, root, etc. The beneficial medicinal effects of plant materials are typically the result of the combination of these secondary products. Out of 255 drugs, which are considered as basic and essential by the World Health Organization, 11% are obtained from plants and a number of synthetic drugs are also obtained from natural
precursors\textsuperscript{125}. The development of drug resistance and the undesirable side effects of synthetic drugs have led to the search for new herbal agents with unique chemical structures.

\section*{2.4 \textit{IN VITRO AND IN VIVO} BIOACTIVITY SCREENING OF PLANT EXTRACTS}

Biological screening of plant extracts is carried out to evaluate their potential for various activities. This requires development of simple, rapid and inexpensive biological assays. Further the potent plant extracts can be fractionated for isolation and identification of biologically active constituents. Some of the commonly used biological assays include antimicrobial assays, toxicity assays, insecticidal assays, antioxidant assays and antidiabetic assays. The antimicrobial assays include antibacterial assay and antifungal assay. The agar diffusion test or the Kirby-Bauer disk diffusion method is a means of measuring the effect of an antimicrobial agent against bacteria grown in culture\textsuperscript{141}. The causative organism is swabbed uniformly across a culture plate. A filter paper disk impregnated with the compound to be tested is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is called the zone of inhibition. The size of the zone of inhibition is a measure of the compounds effectiveness, the larger the clear area around the filter disk, the more effective is the compound. Toxicity assays include phytotoxicity assay and Brine shrimp cytotoxicity assay\textsuperscript{95}. Herbicidal properties of plant extracts can be evaluated by using phytotoxic assays. Herbicides are chemicals used to kill weeds in crops. Herbicides can be obtained from natural sources like plants or synthesized chemically in the laboratory and used to improve the quality and yield of crops. Herbicides from natural resources are preferred because of the various side effects and environmental impacts of chemical herbicides. Seed phytotoxicity assay is described as a general pre-screening assay for phytotoxic evaluation of plant extracts. Pea seeds have been used in general toxicity studies because of their sensitivity to phytotoxic compounds and as a standard assay in allelopathic studies. The measurement of phytotoxicity is studied in terms of inhibition of
germination, root and shoot elongation. Moreover phytotoxic evaluation is necessary in order to screen plant antitumor agents because those with growth inhibitory properties cannot be used as antitumor agents. Reports show that phytotoxicity evaluation is necessary in order to screen plant antitumor agents because those with growth inhibitory properties cannot be used as antitumor agents\textsuperscript{153}. The brine shrimp cytotoxicity assay is considered as a convenient probe for preliminary assessment of toxicity, detection of fungal toxins, heavy metals, pesticides and cytotoxicity testing of dental materials. It can also be used for evaluating cell line toxicity and anti tumor activity. The brine shrimp assay is very useful for the isolation of biogenic compounds from plant extracts. The brine shrimp lethality assay is a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity and in most cases correlates reasonably well with cytotoxicity and anti tumor properties\textsuperscript{31}. The screening of insecticidal activity helps in the use of plant derivatives as an alternative to chemical insecticides and has been widely studied throughout the world.

More than 2000 plant species have been reported to possess pest control properties\textsuperscript{183}. Two of such important plants are \textit{Azadirachta indica} and \textit{Lantana camara}. Various plant metabolites have been characterized as having defensive mechanisms against insect pests. These include azadirachtin from the neem plant and lantanine from \textit{L. camara}. Extracts from the leaves of \textit{L. camara} showed nematicidal, antimicrobial, fungicidal and insecticidal activities. \textit{A. indica} has been shown to possess potent insecticidal properties\textsuperscript{63}. The insecticidal activity of plant extracts can be determined by direct contact application of the test agent using filter paper and calculating the number of survivals and percentage mortality\textsuperscript{117}. A number of methods are present for determination of antioxidant activity of plant extracts. Antioxidant assays can be used for the evaluation of natural and synthetic antioxidants. These assays differ from each other in terms of experimental condition, reagents, substrates, reaction medium and standard analytical evaluation methods. Two important methods cited in literature include DPPH and TBARS assays\textsuperscript{148}. Anti diabetic effects can be studied \textit{in vivo} using animal models or \textit{in vitro} using a variety test systems. \textit{In vitro} tests can play a very important role in the evaluation of antidiabetic activity of drugs as initial screening tools where the screening of large number of potential therapeutic candidates may be necessary. \textit{In vitro} antidiabetic assays include the
determination of inhibition of carbohydrate hydrolyzing enzymes such as α-glucosidase and α-amylase by the plant extracts\textsuperscript{22}. Inhibition of these enzymes delays the digestion of the carbohydrates and causes a reduction in the rate of glucose absorption. α-Glucosidase or α-D-glucoside glucohydrolase is a carbohydrazyme distributed widely in microorganisms, plants, and animal tissues\textsuperscript{57}. The α-amylase is mainly synthesized in plants, near the starch deposit sites and also present along the digestive tract of animals. Pancreatic α-amylase is the key enzyme in the digestive system which catalyses the initial step in the hydrolysis of starch to a mixture of small oligosaccharide consisting of maltose, maltotriose and a number of oligoglucans. These are then acted on by α-glucosidases and further degraded to glucose which on absorption enters the bloodstream. Degradation of dietary starch proceeds rapidly and leads to elevated post prandial hyperglycemia\textsuperscript{178}. Plants with inhibitory activity against α-glucosidase and α-amylase, delay carbohydrate digestion and prolongs overall carbohydrate digestion time thereby causing a reduction in the rate of glucose absorption, consequently lowers post prandial plasma glucose levels. Because of their ability to prevent starch breakdown and absorption, α-amylase inhibitors have been used for weight loss in humans.

\textit{In vivo} biological systems using live animals or whole organisms are necessary to study how data from experiments carried out in \textit{in vitro} systems can behave under clinical or pathophysiological conditions and can establish mechanisms and define toxicities\textsuperscript{22}. Many of the animal models studied apparently share similar characteristic features of type 2 diabetes and have allowed experimentation that would be impossible in humans. The animal model act as essential tool for investigating genetic, endocrine, metabolic, morphologic changes and underlying mechanisms that could also operate during the evolution of type 2 diabetes in humans. In the screening of antidiabetic compounds, it is particularly important to note that some animal models are better suited to screen particular class of antidiabetic compounds. The use of smaller animal models such as mice will reduce the expense of producing test materials while some advanced efficacy studies or toxicological examinations which require invasive procedures and large blood and tissue samples may be facilitated by using animals with large body size such as rat or other non rodents. Further, the selection of particular animal model particularly depends on the choice of the investigator whether to use inbred or out bred, availability of particular
strain, aim of scientific strategy, type of drug being sought, institutional financial and facility resources. Rodents have been widely used for better understanding of the disease mechanisms in much closely similar human situation as well as for discovering newer targets and drugs for the treatment of type 2 diabetes and its complications. Streptozotocin (STZ) is a naturally occurring broad spectrum antibiotic and cytotoxic chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals. Induction of experimental diabetes in the rat using STZ is very convenient and simple method to use\textsuperscript{124}. STZ injection leads to the degeneration of the Langerhans islets beta cells. Clinically the symptoms of diabetes are clearly seen in rats within 2 to 4 days following single intravenous or intraperitoneal injection of 60mg/kg STZ\textsuperscript{20}. The \textit{in vivo} antidiabetic assay can be carried out on STZ induced diabetic rats, by oral administration of test agents. Further, the blood glucose levels are monitored at specific intervals, various biochemical parameters and antihyperglycemic activity in STZ induced rats is compared to the standard drug Glibenclamide\textsuperscript{175}.

\subsection*{2.5 BIOASSAY GUIDED FRACTIONATION AND ISOLATION OF BIOACTIVE CONSTITUENTS}

Bioassay guided fractionation is a procedure where the extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated using a bioassay system and only active fractions are further fractionated. Bioassay guided fractionation procedures have been employed by many workers for antioxidant research to isolate active compounds from medicinal plants using Silica gel chromatography and DPPH assay. Successive fractionation technique followed by DPPH assay led to isolation of polyphenols such as methyl gallate, procyanidin B-3, (+)-catechin, gallic acid, quercetin, quercetin-3-O-\beta-D-glucoside, quercetin-3-O-\beta-galactoside, kaempferol and quercetin-3-O-rutinose which possessed strong DPPH free radical scavenging activity\textsuperscript{163}. Bioassay guided fractionation has been extensively used for antimicrobial research to isolate compounds that are capable of inhibiting the growth of several foodborne pathogens like \textit{E. coli}, \textit{Salmonella typhimurium}, \textit{Listeria monocytogenes}, \textit{S. aureus} and \textit{B. cereus} from Chinese green tea extract\textsuperscript{161}. High speed counter current chromatography method was used for the separation and
purification of active compounds, like epicatechin gallate, epigallocatechin gallate, epicatechin and caffeine. The bioactive compound can then be isolated through preparative TLC. TLC is a basic method suitable for rapid detection of drug substances. It is a method for separating individual components present in a sample by using thin layer of silica gel as a stationary phase. TLC technique is advantageous as it is selective, specific and rapid in identifying drug substances than the simple characterization methods using chemical reagents which reveal substances by colour and precipitation reaction tests. There is no interference caused by excipients in TLC and the method can be used for identification, purity test and semi quantitative estimation of the active ingredients\textsuperscript{163}. Each fraction produced can be evaluated in an \textit{in vivo} bioassay system using rats to facilitate selection of the bioactive fraction. The bioactive fraction would usually produce the desired biological effects in the experimental rats. Degradation or transformation of the active plant constituent may occur during fractionation due to oxygenation, hydrolysis, ultraviolet irradiation or esterification. Again, decrease in activity after fractionation may be due to the result of the phenomenon of synergy between the active ingredients. Biological activity guided fractionation has been the process employed to identify the lead druggable candidate from any given phytochemical mixture.

Two approaches might be followed as the design of extraction for bioactivity guided fractionation leading to compound isolation to act as a lead compound:

\textbf{i. Parallel approach}

This approach may be applied when the biological activity of the plant is known by virtue of its traditional use. The objective of this approach is to isolate compounds responsible for the activity based on their biological activity. In parallel extraction approach, different types of extracts are obtained from a crude plant. The most active fraction based on the primary screening for bioactivity is chosen for further extraction and evaluation\textsuperscript{2}.
ii. Sequential approach

This approach may be useful when the biological activity of the subject plant is not known and random selection strategy is adopted for plants. The extraction is done based on the polarity of the solvents and fractions are obtained in a sequential process using hexane, chloroform, ethyl acetate, and butanol as solvents\(^2\). Small molecules from natural sources are recognized as structures with greater likelihood than many synthetic compounds to exhibit specific bioactivities. There is substantial evidence that 73% of cancer therapeutics approved to date are either natural products or their derivatives. However, the use of natural products in drug discovery has significantly declined in the past two decades due in part to persisting difficulties in the isolation and synthesis of such molecules. One promising strategy to possibly exploit the therapeutic potential of natural products could be the use of more bio medically relevant assays on \textit{in vivo} models for the screening and bioactivity guided fractionation of plant, fungal and microbial extracts. Many currently known bioactive natural products were originally identified using \textit{in vitro} assays like cytotoxicity in tumor cells, for their activity guided isolation from extracts\(^6\).

The biological activity of many other natural products was determined only after their initial isolation on the basis of physical characteristics determined by preparative chromatography followed by mass spectrometry and NMR spectroscopy analysis\(^7\). \textit{In vivo} assay guided fractionation is currently not a widely used approach for the discovery of herbal drugs because of the low throughput of conventional \textit{in vivo} models such as mice and rats and in addition relatively large amounts of compound is required for testing in these systems. The natural sources have many useful and important bioactive compounds and many have been discovered using bioactivity directed fractionation and isolation. The pharmacognosy research or isolation of natural products is facilitated by the development of new bioassay methods. It has been reported that the bioactive compounds are mostly plant secondary metabolites, which become medicine after processing to pure compounds, some are very useful dietary supplements, and many are used as commercial products\(^8\). Further modification of the active compounds leads to enhancement of the biological profiles and a large number of such compounds are approved or undergoing clinical trials for clinical uses against different diseases like malaria, pulmonary diseases, cancer, AIDS, Alzheimer’s disease etc\(^9\).
2.6 IDENTIFICATION AND CHARACTERIZATION OF ACTIVE COMPONENTS IN PLANT EXTRACTS BY ANALYTICAL TECHNIQUES

Natural products from medicinal plants are obtained either as pure compounds or as standardized extracts with the help of analytical methodologies, which include the extraction, isolation and characterization of active ingredients in botanicals and herbal preparations\textsuperscript{62}. The analysis of bioactive compounds present in the plant extracts involves the applications of common phytochemical screening assays, chromatographic techniques such as HPLC and TLC as well as non-chromatographic techniques such as immunoassay and Fourier Transform Infra Red (FTIR). Due to the fact that plant extracts usually occur as a combination of different types of bioactive compounds or phytochemicals with different polarities their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. A common strategy in isolation of these bioactive compounds includes the use of a number of separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC to obtain pure compounds\textsuperscript{121}.

The pure compounds are then used for the determination of structure and biological activity. High performance liquid chromatography (HPLC) is a versatile, robust, and widely used technique for the isolation of natural products. Presently this technique is gaining popularity among various analytical techniques as the main choice for studying the quality control of herbal plants. Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order to fully characterize the active entity. The biologically active entity is often present only as minor component in the extract and the resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale\textsuperscript{194}. Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, flow rate, suitable detectors and columns to get an
Identification of compounds by HPLC is a crucial part of characterization technique. Many significant reviews are published on the use of HPLC methods for analysis of phenolic compounds from different materials like fruits, vegetables and medicinal plants. Reports suggest the determination of anthocyanidins and derivatives of hydroxycinnamic acid (caffeic, coumaric acids) from sweet cherries by RPHPLC. HPLC analysis of rhizome of *alpinia officinarum* showed two major bioactive flavonoids: galangin and 3-ο- methyl galangin. RP HPLC has been used for qualitative and quantitative analysis of phenols in thirty two herbs. Major phenolics identified were neochlorogenic, caffeic, ρ coumaric and ferulic acid while flavonoids like quercetin, luteolin, apigenin and Kaempferol were also identified. Reversed phase HPLC has been used in a number of studies for the analysis of flavonoids in plants. Flavonoids consist mainly of flavonols, flavones, catechins, and flavanones. Many reports on the investigations of the distribution of the major flavonoids in plant foods have been published. It has been used to distinguish species based on the quantitative variation of flavonoids among them. In another study it was used for the quantitative analysis of flavonoid aglycones.

HPLC has been widely used for separation and determination of flavonoids in a variety of foods including tomatoes and tomato juice. Liquid chromatography coupled with mass spectrometry (LC/MS) is also a powerful technique for the analysis of complex botanical extracts. It provides abundant information for structural elucidation of the compounds when tandem mass spectrometry is applied. Therefore the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs especially when a pure standard is unavailable. The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution is very important for the overall success of natural product isolation. The source material like dried powdered plant will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent like methanol, chloroform may be used as the initial extractant and following a period of maceration, solid material is then removed by decanting off the extract by filtration. The filtrate is further concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant level of strongly binding
components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. Spectroscopic Techniques like Nuclear magnetic resonance spectroscopy (NMR) and GC MS enable the structural elucidation of the isolated active components. Nuclear magnetic resonance spectroscopy or NMR spectroscopy has been named due to which the magnetic properties of certain nuclei used in this technique have been named due to which the magnetic properties of certain nuclei used in this technique.

Both proton NMR and carbon-13 NMR spectroscopy are important applications for the analysis process. NMR spectrum gives us many types of information. NMR can be applied to a wide variety of samples, both in the solution and the solid state. NMR spectra have been a major tool for the study of both newly synthesised and natural products isolated from plants, bacteria etc. The drugs in clinical use are mostly either synthetic or natural products. NMR spectroscopy has been mainly used for the elucidation and confirmation of structures of active components isolated from these resources. NMR methods have been introduced to quantitative analysis in order to determine the impurity profile of a drug, to characterise the composition of drug products, and to investigate metabolites of drugs in body fluids. For pharmaceutical experts solid state measurements can provide information about conformation of drugs in tablets, polymorphism of drug powders etc. NMR is also used to the mixtures of analytes. Several reports suggest that it can also be used to understand the dynamic effects like temperature and reaction mechanism and can also provide useful information regarding protein and nucleic acid structure and function.

2.7 AIM AND OBJECTIVES OF THE PRESENT STUDY

The present study was carried out to screen the bioactive potential of the plants *Artocarpus heterophyllus*, *Artocarpus altillus* and *Piper betle* for specific biological activities, isolate and identify the bioactive components using analytical techniques. The principal objectives of the study were: Selection of plant material, extraction and preliminary phytochemical screening of plant extracts, isolation of flavonoid fractions by chromatographic techniques, *in vitro* biological screening of fractions by simple bioassays, *in vivo* bioactivity screening of fractions using animal models, identification and characterization of bioactive components in fractions by HPLC and NMR spectroscopy.