CHAPTER I
INTRODUCTION

India is one of the world’s 12 megadiversity countries (Singh and Chowdhery, 2002) with 10 biogeographic regions (Rodgers and Panwar, 1990); in addition, it has over 40 sites which are known for their high endemism and genetic diversity (Nayar, 1996). Myers et al. (2000), in their updated list of world’s biodiversity hotspots, included two from India. Climatic and altitudinal variations, coupled with varied ecological habitats of our country, have contributed to the development of immensely rich vegetation with a unique diversity in medicinal plants, which provides an important source of medicinal raw materials for traditional medicine systems as well as for pharmaceutical industries in the country and abroad (Bhattacharyya et al., 2006).

The history of medicinal plants dates back to the origin of human civilization on earth. Humans have benefitted from natural sources of drugs for thousands of years (Small and Catling, 1999). There are at least 110,000 known, and possibly as many as a million uncharacterized, natural plant products serving ecological functions, such as protection against predators and disease. These products can act within the human body against microorganisms and other causes of illness (van Seters, 1995). The World Health Organization (WHO) has defined herbal medicines as herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients singly or in combinations (WHO, 2000). These active ingredients include terpenes, aromatics, proteins and alkaloids (Kaufman et al., 1998). Today, there are over 120 plant-derived drugs in professional use worldwide, three-quarters of which
were discovered through scientific investigations of traditional medicines (Marles, 1996).

India has a rich culture of medicinal herbs and spices, with high potential for use in Ayurvedic, Unani and Siddha medicinal practices. According to the WHO, most populations still rely on traditional medicines for their psychological and physical health requirements (Rabe and van Stoden, 2000), since they cannot afford products of Western pharmaceutical industries (Salie et al., 1996), together with their side effects and lack of healthcare facilities (Griggs et al., 2001). People inhabiting the rural areas of many developing countries still rely on traditional medicine for their primary healthcare needs and many such medicines find a place in their day-to-day lives.

Herbal medicines have great demand in both developed and developing countries as a source of primary healthcare with wide biological and medicinal activities, high safety margins and lesser costs (Iwu et al., 1999; Idu et al., 2007; Mann et al., 2008; Ammara et al., 2009). Herbal medicines are safe and are better capable of overcoming the resistance of the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell (Lai and Roy, 2004; Tapsell et al., 2006). Balick and Cox (1996) have noted that a number of modern drugs have also been derived from plants, used by indigenous people.

Traditional use of medicine is recognized as a way to learn about potential future medicines. People living in rural areas, from their personal experience, know that traditional remedies are valuable for maintaining human health, even though they
may not understand the science behind these medicines; they also seem to know that some medicinal plants are highly effective only when used at therapeutic doses (Maheshwari et al., 1986; van Wyk et al., 2000). Researchers have identified a number of compounds used in mainstream medicine derived from ‘ethnomedical’ plant sources (Fabricant and Farnsworth, 2001).

A plant is said to be medicinal when at least one of its parts possesses therapeutic properties (Dhiman, 2003). With the recent advancements in research in the field of medicinal plants, it has become apparent that many of the species utilized by indigenous people and the knowledge of traditional healers are possible avenues for finding ways for combating diseases (Jäger et al., 1996). Scientific evidence has brought about the possibility of utilization of plant extracts in the treatment of fungal infections and development of antibacterial and antifungal products (Farnsworth, 1994; Fox, 1999).

Contribution of higher plants to medicine and pharmaceutical industry due to nature’s ability to present highly diverse chemicals has prompted increasing interest of researchers in searching for natural product sources (Topçu et al., 2009). Research efforts could be directed at a number of diseases for which suitable drugs are not available in the modern system of medicine and where herbal drugs have a possibility of offering new drugs. Systemic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories. The search for components with antimicrobial activity has gained importance in recent times due to growing worldwide concern about the alarming increase in the
rate of infection by antibiotic-resistant microorganisms. Hence, there is a constant need for new and effective therapeutic agents.

1.1. Phytochemistry

Plant chemicals are classified as primary or secondary metabolites. Primary metabolites are widely distributed in nature, occurring in one form or another in all organisms. In higher plants, such compounds are often concentrated in seeds and vegetative storage organs. Plants generally produce many secondary metabolites which are biosynthetically derived from primary metabolites and constitute an important source of microbicides, pesticides and pharmaceutical drugs. For long medicinal plants and their secondary metabolites have been directly or indirectly playing an important role in the human society to combat diseases (Wink et al., 2005).

Secondary metabolites (compounds) have no apparent function in a plant’s primary metabolism, but often have ecological roles, as pollinator attractants, and represent chemical adaptations to environmental stresses or serve as chemical defense against microorganisms, insects and higher predators and even other plants (allelochemicals). Secondary metabolites are frequently accumulated by plants in smaller quantities than primary metabolites (Karuppusamy, 2009; Sathishkumar and Paulsamy, 2009).

A survey of current pharmaceutical use revealed that, of the total prescription drugs dispensed, 25% are plant-derived (Ogundipe et al., 1998). Plant compounds are highly varied in structure; many are aromatic substances, most of which are phenols or their oxygen-substituted derivatives. However, there is an increasing attention on extracts and biologically active compounds isolated from plant species used in herbal
medicine, due to the side effects and the resistance that pathogenic microorganisms build against antibiotics (Essawi and Srour, 1999). New compounds inhibiting microorganisms such as benzoin and emetine have been isolated from plants (Cox, 1994). Of the various pharmaceuticals used in modern medicine, aspirin, atropine, ephedrine, digoxin, morphine, quinine, reserpine and tubocurarine serve as examples of drugs discovered through observations of indigenous medical practices (Gilani, 2005). Eloff (1999) stated that antimicrobial compounds derived from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of antibiotic-resistant microbial strains.

Plant constituents may be isolated and used directly as therapeutic agents or as starting materials for drug synthesis or may serve as models for pharmacologically active compounds in drug synthesis. The general research methods include proper selection of medicinal plants, preparation of crude extracts, biological screening, detailed chemopharmacological investigations, toxicological and clinical studies, and standardization and use of active moiety as the lead molecule for drug design (Wink et al., 2005).

1.2. Antioxidant potential

In living systems, oxidation is a basic part of the normal metabolic process, in which reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$), hypochlorous acid and many free radicals (hydroxyl radical (OH) and superoxide anion) are generated (Finkel and Holbrook, 2000; Halliwell, 2000; Pietta, 2000; Vijayabaskaran et al., 2010). Rapid production of free radicals may cause alteration in the structure as well as function of cell constituents and membranes, resulting in
disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular diseases, neurodegenerative diseases, and premature ageing (McLarty, 1997; Young and Wood, 2001; Yang et al., 2001; Sun et al., 2002; Bimal et al., 2011). Antioxidants or free radical-scavenging molecules should be present in higher levels so as to combat the above-mentioned diseases.

Antioxidant substances and free radical-scavenging molecules present in plants are in the form of phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, tannins), nitrogen compounds (alkaloids, amines), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites (Zheng and Wang, 2001; Naruthapata and Supaporn, 2009). So to maintain a healthy body, one should always increase the intake of foods rich in antioxidant compounds that lower the risk of chronic health problems associated with the above disease conditions (Halliwell, 2000; Klipstein et al., 2000).

Naturally occurring antioxidants can be used in foods and also for prevention and treatment of free radical-related disorders (Middleton et al., 2000; Kumar and Kumar, 2009); they can also be replaced by commercially available, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are quite unsafe to use and is restricted due to their carcinogenic effects (Velioglu et al., 1998; Vinay et al., 2010). Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell-mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger and vasodilator, and has
antimicrobial and antitumour property (Shreejayan and Rao, 1997; Hagerman et al., 1998; Balakrishnan et al., 2009).

The most commonly used methods for measuring antioxidant activity involve the generation of free radicals which are then neutralized by antioxidant compounds. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a well-known radical and a trap (scavenger) for other radicals (Husain et al., 1987; Visioli et al., 2000; Solai et al., 2010). The DPPH method is rapid, simple and independent of sample polarity, and hence very convenient for quick screening of many samples for radical-scavenging activity.

Superoxide anion is a reduced form of molecular oxygen that is generated during normal metabolic processes mainly in mitochondria. It is known to be destructive to cellular components as a precursor of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical or singlet oxygen (Stief, 2003), contributing to tissue damage and various chronic diseases (Halliwall, 1991). Therefore, studying the scavenging activity of plant extracts on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity.

Hydroxyl radical is an extremely reactive oxidizing free radical formed in biological systems and has been implicated as a highly damaging species in free-radical pathology (Wang et al., 2007). It has an extremely short half-life but is capable of causing damage within a small radius of its site of production. A single hydroxyl radical can result in formation of many molecules of lipid hydroperoxides in the cell membrane, which may severely disrupt its function, and lead to cell death.
ABTS (2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonate) is a stable free radical, bluish-green in colour. The antioxidant assay is based on the reduction of ABTS by plant extracts. This assay is based on the inhibition of the absorbance of the cation, ABTS\(^+\), which has a characteristic wavelength at 734 nm, by antioxidants. In the presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS (Sreejayan and Rao, 1996).

1.3. Antidiabetic activity

Diabetes mellitus, referred to simply as ‘diabetes’, is a disorder characterized by the presence of excess glucose in the blood and tissues of the body. It has been estimated that the global burden of diabetes for 2011 was 285 million, which has been projected to increase to 438 million in 2030 (WHO, 2002). Management of diabetes without any side effects is still a challenge in the medical field, as presently available drugs for diabetes have one or more adverse effects (Bohannon, 2002). Since the existing drugs for the treatment of diabetes do not satisfy our need completely, the search for new drugs continues. In recent years, herbal remedies for the unsolved medical problems have been gaining importance in the research field. Apart from the currently available therapeutic options, many herbal medicines have been recommended for the treatment of diabetes. Herbal drugs are prescribed widely because of their effectiveness, less side effects and relatively low cost (Devaki et al., 2011).

Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. Presently, there is ethnobotanical information about 800 plants that may possess
antidiabetic potential (Alarcon-Aguilara *et al*., 1998). Several such herbs have shown antidiabetic activity when assessed using presently available experimental techniques (Jafri *et al*., 2000). India has a rich history of using various potent herbs and herbal preparations for treating diabetes. In India, indigenous remedies have been used in the treatment of diabetes since the time of Charaka and Sushruta (Grover *et al*., 2001). There have been several reviews on Indian botanicals having hypoglycaemic activity (Saxena and Vikram, 2004; Sudha *et al*., 2011). Many Indian plants have been investigated for treating different types of diabetes and are reported in numerous scientific studies (Joy and Kuttan, 1999; Nagarajan *et al*., 2005; Verma *et al*., 2010).

Although hundreds of plants are used all over the world to prevent or to cure diseases, scientific evidence for such activity as in the case of modern medicine is lacking in most cases. However, today it is necessary to provide scientific proof to justify the use of plant as well as its active principles.

### 1.4. Anticancer activity

Cancer is one of the most life-threatening diseases with more than 100 different types. Due to lack of effective drugs, expensive cost of chemotherapeutic agents and side effects of anticancer drugs, cancer can be a cause of death. Cell death can occur through several different mechanisms, of which the most widely described are apoptosis and necrosis (Sengupta *et al*., 2004; George *et al*., 2010). A significant physiological consequence of cell death by apoptosis is that the apoptotic cells are immediately phagocytosed by macrophages. Therefore, the release of intracellular molecules that cause secondary disturbance to the surrounding tissue is much limited compared with necrosis, which causes further tissue destruction and inflammation (Earnshaw, 1995). Now people have started realizing the importance of natural
bioactive substances found in fruits, vegetables and herbs as powerful antioxidants and ‘functional foods’ (Wang et al., 1997; Kitts et al., 2000; Lee and Lim, 2001). Some of these substances are believed to be potential chemopreventive or therapeutic agents for cancer (Pezutto, 1997; Christou et al., 2001; Mukherjee et al., 2001). Most of these substances exert their chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, inducing apoptosis in tumour cells has become an indicator of the tumour-treating ability of naturally derived bioactive substances (Smets, 1994; Paschka et al., 1998).

Apoptosis or programmed cell death is a highly organized physiological process to eliminate damaged or abnormal cells. It also plays a major role in embryogenesis where apparently normal cells undergo apoptosis. It is involved in maintaining homeostasis in multicellular organisms (Perandones et al., 1993). Apoptosis is triggered by the activation of the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, and results from activation of members of the cysteine protease family called ‘caspases’ (Miller, 1999; Wolf et al., 1999; Fan et al., 2005). Mitochondria are involved in a variety of key events, including release of caspase activators, changes in electron transport, etc. (Zamzami et al., 1996; Green and Reed, 1998; Gottlieb, 2000). Alterations in mitochondrial structure and function have been shown to play a crucial role in caspase-9-dependent apoptosis (Green and Kroemer, 1998) by releasing apoptotic factors from mitochondria including cytochrome C. In this manner, released cytochrome C interacts with Apaf-1 and procaspase-9 to form the apoptosome. Then caspase-9 cleaves and activates caspase-3, the executioner caspase, which cleaves poly (ADP-ribose) polymerase (PARP) and
activates endonucleases leading to DNA fragmentation (Cai et al., 1998; Cecile et al., 2004).

In addition to monitoring caspase activity, some of the biochemical features of apoptosis such as loss of membrane phospholipid asymmetry and DNA fragmentation can also be used to identify apoptosis (Williamson et al., 2001). The outstanding feature of an apoptotic cell is its remarkably stereotyped morphology showing cytoplasmic condensation, plasma membrane blebbing and nuclear pycnosis, leading to nuclear DNA break down into multiples of ~200 bp oligonucleosomal size fragments (Kerr et al., 1972; Telford et al., 1991; Williamson et al., 2001). In addition, apoptotic cells cultured in vitro will eventually undergo ‘secondary necrosis’. After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium (Riss and Moravec, 2004). Therefore, cells that have initiated apoptosis may exhibit some of the morphological phenotypes associated with necrosis. In recent decades, apoptosis-inducing compounds are being studied to assess their potential for treating cancer (Smets, 1994; Watson, 1995; Panchal, 1998). Apoptosis itself also plays an important role in the development of various diseases including cancer (Fisher, 1994; McConkey et al., 1996).

Recent studies of tumour inhibitory compounds of plant origin have yielded an impressive array of novel structures. Epidemiological studies suggest that consumption of diets containing fruits and vegetables, which are major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer. Certain products from plants are known to induce apoptosis in neoplastic cells but not
in normal cells (Chiao et al., 1995; Hirano et al., 1995; Jiang et al., 1996; Shaikh et al., 2011). It has become increasingly evident that apoptosis is an important mode of action for many anti-tumour agents, including ionizing radiation (Radford et al., 1994) and alkylating agents such as cisplatin and camptothecin. Camptothecin has been effective against a broad spectrum of tumours. Camptothecin is a quinoline-based alkaloid found in the bark of the Chinese camptotheca tree. It has been used for treating psoriasis, leukaemia and diseases of liver, gall bladder, spleen and stomach. Even though there are number of synthetic antitumour agents available, efforts are still on to search for effective naturally occurring anticarcinogens that would prevent, slow or reverse cancer development. Plants have a special place in the treatment of cancer. It is estimated that plant-derived compounds constitute more than 50% of anticancer agents (Newman et al., 2003; Nipun et al., 2011). Cell viability assays can be combined with apoptosis assays to provide more information about mechanisms of cell death through multiplexing assays on a single sample (Dive et al., 1992).

1.5. Antimicrobial activity

Medicinal plants have always been considered as a source for healthy life for people. Therapeutical properties of medical plants are very useful in healing various diseases and the advantages of these medicinal plants are natural. In many parts of the world, medicinal plants have been used for their antibacterial, antifungal and antiviral properties for hundreds of years (Ali et al., 1998; Barbour et al., 2004; Yasunaka et al., 2005). Researchers are increasingly turning their attention to natural products and looking for new leads to develop better agents against microbial infections (Ibrahim, 1997; Towers et al., 2001; Koshy et al., 2009). Several synthetic antibiotics are employed in the treatment of infections and communicable diseases
caused by harmful microorganisms and this has resulted in the emergence of multiple drug-resistant bacteria. In general, bacteria have the genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents (Murray, 1992; Madunagu et al., 2001; Koshy et al., 2009; Senthilkumar and Reetha, 2009). Therefore, actions must be taken to reduce this problem, such as to minimize the use of antibiotics, develop research into resistance among microorganisms and to continue studies to develop new antibiotics and immune-modulating compounds with diverse chemical structures and novel mechanisms of action, either synthetic or natural, to control pathogenic microorganisms because there has also been an alarming increase in the incidence of new and re-emerging infectious diseases (Ikenebomeh and Metitiri, 1988; Rojas et al., 2003).

Generally, antimicrobial studies have shown that Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria. This may be due to the variation in the cell wall structures of Gram-positive and Gram-negative bacteria. More specifically, a Gram-negative bacterium has an outer membrane that is composed of high-density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics (Paz et al., 1995; Vlietinck et al., 1995; Kudi et al., 1999). Although hundreds of plant species have been tested for antimicrobial properties, the vast majority have not been adequately evaluated (Onwuliri and Dawang, 2006; Mahesh and Sathish, 2008).

Indian flora offers great possibilities for the discovery of new compounds with important medicinal applications in combating infection and strengthening the immune system. The antimicrobial compounds found in plants may prevent bacterial
infections by different mechanisms than the commercial antibiotics and therefore may have clinical value in treating resistant microorganism strains (Eloff, 1999). The indiscriminate use of antibiotics has resulted in many bacterial pathogens rapidly becoming resistant to a number of originally discovered antimicrobial drugs (Barbour et al., 2004). There is, thus, a continuous search for new antibiotics, and medicinal plants may offer a new source of antibacterial agents. This is indeed very important because *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* are some of the important human pathogens that have developed resistance to antimicrobials (Barbour et al., 2004).

### 1.6. Larvicidal activity

Control of vector-borne diseases is getting difficult mainly due to the lessened efficacy of chemical insecticides due to the development of resistance. Mosquito is the most indisputable medicinally significant arthropod vector of diseases. Vector-borne diseases caused by mosquito are some of the major health problems in most of the countries. The diseases affect the socioeconomic status of many nations (Govindarajan et al., 2005). Vectors transmit parasites and pathogens which continue to have disadvantageous impact on human beings (Maheswaran et al., 2008a). Filariasis, dengue, yellow fever, malaria, Japanese encephalitis and chikungunya are some of the deadly diseases spread by mosquitoes. *Culex quinquefasciatus* is an important vector of bancroftian filariasis in tropical and subtropical regions. In India alone, 25 million people harbour microfilaria and 19 million people suffer from filarial diseases (Maheswaran et al., 2008b). Ill-maintained drains are excellent breeding places for mosquitoes (Komalamisra et al., 2005). Even though vector control programmes based on chemicals have been conducted for long, mosquito
vectors are still prevalent, causing many diseases because they have developed resistance to most synthetic chemicals used (Das et al., 2007). Hence, there is a need for developing biologically active natural chemical constituents which act as larvicides (Maheswaran et al., 2008a; Prabakar and Jebanesan, 2004). This has necessitated the need for research and development of environmentally safe, biodegradable, and low cost indigenous methods for vector control, which can be used with minimum care by individuals and communities in specific situations (Kumar et al., 2012).

1.7. Pharmacognostical studies

Standardization is an important tool for herbal drugs in order to establish their identity, purity, safety and quality. In order to standardize a drug, various macroscopic, microscopic, fluorescence analyses are generally done. Microscopic method is one of the cheapest and simplest methods to correctly identify the source material (Khandelwal, 2007). The quantitative determination of some pharmacognostical parameters is useful for setting standards for crude drugs. Stomatal number, stomatal index value and palisade ratio, vein islet and vein termination value determination are equally important in the evaluation of crude drugs. These values help in the evaluation of purity of drugs. Morphological and microscopic studies of leaves act as a reliable aid for detecting adulteration. These simple but reliable standards will be useful to a lay person in using the drug as a home remedy. These studies can also help in the identification and selection of the raw material for drug production. Hence these characteristics could be used to identify and to know the adulterants if any with this species.
Objectives

*Hyptis suaveolens* (L.) Poit., a member of the family Lamiaceae, commonly called ‘bush mint’ or ‘pig mint’, is one of the traditionally valuable plants considered as a remedy for various diseases. Even though the weed is used in traditional medicine, its efficacy has not been scientifically evaluated. Hence there is an urgent need to intensively analyse this plant using all the latest available methods. This study was conducted to evaluate the phytochemical and pharmacological properties of different solvent extracts of *H. suaveolens* leaves with the following objectives:

1. To screen the phytochemical constituents of different solvent extracts of *H. suaveolens* leaf samples.
2. To determine the functional groups using Fourier transform infrared spectroscopy (FT-IR).
3. To identify the minor as well as major components from the ethanolic leaf extract as well as the essential oil by using gas chromatography-mass spectrometric analysis (GC-MS).
4. To study the antioxidant activity of *H. suaveolens* leaf extracts.
5. To investigate the antimicrobial properties of *H. suaveolens* leaf extracts against some human pathogenic organisms.
6. To determine the anticancer, anti-diabetic and larvicidal activity of *H. suaveolens* leaf extracts.
7. To study the pharmacognostic properties of the stem and leaves of *H. suaveolens*. 