1.0 INTRODUCTION
1.1 Botanicals: Historical Perspective:

Medicine is an integral part of human life since antiquity. History of medicine goes back to the existence of human being. Man has obtained these medicines from diverse sources to cure and/or prevent diseases. Natural products including plants, animals and minerals have been the basis of treatment. Plants and products derived there of were the major source of remedies. Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago. The universal role of plants for treatment can be exemplified from their extensive use in all the major traditional systems of medicine including Ayurveda, the Traditional Indian system of medicine and Traditional Chinese Medicine (TCM). These are the two 'great traditions', which have thousand of years' experiential basis with proper documentation and have played significant role in the global health care. Ayurveda (The Science of Life) uses more than 1500 medicinal plants. Caraka Samhita (1000CE-100AD), an encyclopedia of Indian medicine comprises more than 2000 preparations mostly based on the medicinal plants. TCM is an integral part of Chinese civilization. Holistic approach of treatment, which includes acupuncture, moxibustion along with use of herbal preparations have made the system globally acceptable. Further, there exist other systems of medicine like Siddha, Unani, Homeopathy, Naturopathy, etc that uses botanical remedies.

The modern pharmaceutical industry also has its origin in natural product chemistry, which actually began with the work of Sertumer who first isolated morphine from opium. This was obtained from Opium poppy (Papaver somniferum) by process that
has been used for over 5000 years. Many such developments followed. Quinine from Cinchona tree had its origin in the royal households of South American Incas. Before the first European explorers arrived, the native people of the Americas had developed complex medical systems complete with diagnosis and treatment of physical as well as spiritual illnesses. Indigenous people derived medicines and poisons from thousands of plants. Review of some of the plants that originated from Central and South America indicate that most of them either have potentially toxic or poisonous characters or were from the food sources. Following are few examples. In the early 1500’s, Indian fever bark was one of the first medicinal plants to find appreciative consumers in Europe. Taken from the Cinchona tree (Cinchona officinalis) the bark was used as an infusion by native people of Andes and Amazon highlands to treat fevers. Jesuit missionaries brought the bark back to Europe. By the early sixteenth century, this medicine was known as “Jesuit fever bark”, quite a transformation. In 1860 a German chemist isolated the chemical responsible for anesthetic properties of Coca (Erythroxylum coca) known as cocaine. As the years passed, scientists found cocaine paralyzed nerve endings responsible for transmitting pain. As a local anesthetic, it revolutionized several surgical and dental procedures. Pot curare, an arrowhead poison used in the East Amazon is predominantly from the plant species Strychnos guianensis. Tube curare in the West Amazon is from Chondrodendron tomentosum from which curare in the modern medicine is made from and named as tubocurarine. The Jaborandi tree (Pilocarpus jaborandi) secretes alkaloid-rich oil. Several substances are extracted from this aromatic oil, including alkaloid pilocarpine, a weapon against blinding disease glaucoma. Reserpine and antihypertensive alkaloid from Rauwolfia serpentina become
available as a result of work carried out by Ciba in India. This was the beginning of developments in the modern chemistry. Even now, many pharmaceuticals have their origin in botanicals. Few more are atropine, hyosciamine, digoxin, cholchicine, taxol, vincristine, vinblastine, camptothecin, etc.

1.2 Synthetic Dominance:
Pharmaceutical research took a major leap when alongside natural products chemistry; pharmacologists, microbiologists and biochemists began to unravel the chemistry of natural processes in human, animals, plants and microorganisms. Advances in synthetic organic chemistry led to the identification of many key chemical molecules that offered more opportunities to develop novel compounds. Many new drugs emerged by this route, particularly those now being used to treat infections, infestations, cancers, ulcers, and blood pressure conditions. Many drugs were developed through random screening of thousands of chemicals synthesized as dyestuffs and the like; many others resulted from serendipity (happy chance) arising from sharp-eyed observations of physicians and scientists. Examples of such drugs include sulphonamides, isoniazid, anti-psychotics, anti-histamines and penicillin. Emergence of the modern pharmaceutical industry is an outcome of all these different activities that developed potent single molecules with highly selective activity for a wide variety of ailments. The drugs produced in many cases improved on nature, viz. a new range of local anesthetics from cocaine avoided its dangerous effects on blood pressure; chloroquine is much less toxic than quinine. These successes and many more like them resulted in reduced interest in natural products drug discovery and many major drug companies almost neglected such
divisions. Work on developing new drugs for the treatment of the world’s major diseases like malaria, trypanosomiasis, filariasis, tuberculosis, schistosomiasis, leshmaniasis and amoebiasis came almost to a standstill. In addition, although botanical medications continued to be produced in every country, the clinical efficacy of these was usually not evaluated and the composition of these complex mixtures was only crudely analyzed. Thus, herbal medicines became the domain of ‘old wives’ tales’ and quack medicine, exploitation of the sick, the desperate and the gullible. Sadly, herbal medicines continued to reflect poor quality control both for materials and clinical efficacy.

1.3 Botanical Resurgence and Global Market:

Lag phase for botanical medicine has changed for number of reasons. Some of them are:

- Emergence of drug resistant microbial strains, which makes modern drugs ineffective.
- Failure of getting lead structures to convert them into medicines.
- Potential side effects of the synthetic drugs.
- Failure to act against chronic and difficult to treat diseases like AIDS, cancer, diabetes, rheumatic disorders, etc.
- High cost of the modern drugs.

This has renewed public interest in the botanical medicine, which is clearly evident from the market analysis. Four out of ten Americans used alternative medicine therapies
in 1997; total visits to alternative medicine practitioners increased by almost 50 percent from 1990 and exceeded the visits to all US primary care physicians. Current estimates indicate that about 80 percent of people in developing countries rely on traditional medicine largely on various species of plants and animals for their primary healthcare. According to WHO estimations the populations in developing countries like India (70%), Ruwanda (70%), Uganda (60%), Tanzania (60%), Benin (80%) and Ethiopia (90%) extensively use traditional and alternative medicines for health care. In developed countries like Belgium (31%), USA (42%), Australia (48%), France (49%), Canada (70%), a significant percentage of the population has used traditional and alternative remedies at least once for health care. Pharmaceutical companies have also renewed their strategies in favor of natural product drug development and discovery. For instance, in Europe, AnalytiCon Discovery has stressed on drug discovery based on natural product chemistry. In Asia-Pacific region, MerLion Pharmaceuticals in Singapore has comprehensive structures and capabilities necessary for natural product based drug discovery. Recently, NIH has started extensive research for anti-inflammatory compounds from turmeric, ginger, and boswellia with the aid of Ayurvedic knowledge. Screening of different plants for novel anticancer compounds is also in progress with reference experiential data from traditional systems. Further, harmonization and validation of complex diagnostic and treatment processes of traditional medicine like Ayurveda is underway that can help in global promotion of such therapies. Novel efforts like Ayugenomics have begun to understand Ayurvedic concept of Prakruti from pharmcogenomics perspective to provide a base for human classification, diagnostics and customized medicine. Projects like AyuSoft aim to
develop intelligent and interactive software based on Ayurvedic classics as a decision support system. New analytical approaches like Herboprint\textsuperscript{17}, which use three-dimensional HPLC, can help activity-based standardization of botanicals.

Market statistics are also changing rapidly. The global pharmaceutical market was of worth US$550 billion in 2004\textsuperscript{18} and is expected to exceed US$900 billion by the year 2008. The herbal industry shares about US$62 billion with good growth potential (Fig.1). The World Bank reports trade in medicinal plants, botanical drug products and raw materials is growing at an annual growth rate between 5 to 15 percent\textsuperscript{19}. Within the European community, botanical medicine represents an important share of the pharmaceutical market\textsuperscript{20}; the nutraceutical sector is also growing rapidly. In year 2001, US$17.8 billion was spent in the United States on dietary supplements, US$4.2 billion of it for botanical remedies\textsuperscript{21}. In India the value of botanicals related trade is about US$10 billion per annum with annual export of US$1.1 billion\textsuperscript{22} while China’s annual herbal drug production is of worth US$48 billion with export of US$3.6 billion\textsuperscript{23}. Presently, USA is the largest market for Indian botanical products accounting for about 50 percent of the total exports. Japan, Hong Kong, Korea and Singapore are the major importer of TCM taking 66 percent share of the China’s botanical drugs export\textsuperscript{24}.
Broadly, current aim of using plants in therapeutics is:

1. To isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine;

2. To produce bioactive compounds of novel or known structures as lead compounds for semi-synthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, verapamil, and amiodarone, which are based, respectively, on galegine, 9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, khellin, and khellin;

3. To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, yohimbine;

4. To use the whole plant or its part or processed material of it as a herbal remedy, e.g., Cranberry, Echinacea, Feverfew, Garlic, Ginkgo biloba, St. John’s wort, Saw palmetto, various extracts.
The global scenario illustrates vividly both the promise and challenges presented by the botanical medicines for its quality, safety and efficacy.

1.4 Quality Control and Regulations:
Widespread and increased demands of medicinal plants and related products have generated public health challenges globally in terms of quality, which in turns is a critical determinant of safety and efficacy of medicine. There have been concerted efforts to monitor quality and regulate the growing business of herbal drugs and traditional medicine. Health authorities and governments of various nations have taken an active interest in providing standardized botanical medications. United States Congress has fuelled the rapid growth in nutraceutical market with passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994. US FDA (Food and Drug Administration) has recently published International Conference on Harmonization (ICH) guidance Common Technical Document (CTD) addressing issues related to quality of the medicines which includes herbals also. The National Center for Complementary and Alternative Medicine (NCCAM) has been inaugurated as the United States Federal Government's lead agency for scientific research in this arena of traditional medicine. Its mission is to explore complementary and alternative healing practices in the context of rigorous science, support sophisticated research, train researchers, disseminate information to the public on the modalities that work and explain the scientific rationale underlying the discoveries. The center is committed to explore and fund all such therapies for which there is sufficient preliminary data, compelling public health need and ethical justifications. World Health Organization
(WHO) is keen regarding traditional medicine and has been active in creating strategies, guidelines and standards of botanical medicines\textsuperscript{28}. Other international agencies like European Scientific Cooperation of Phytomedicine (ESCOP), European Pharmacopoeia Commission, Department of Health UK, Commonwealth of Australia and Department of Indian Systems of Medicine have also started creating new mechanisms to induce and regulate quality of botanical medicine. To meet the global standard, the botanical medicine must have desired quality that will give the desired therapeutic effect with minimum side effects.

1.5 Botanical Identification: Methods and their Limitations:

The first step in assuring quality of botanicals is identification and selection of correct plant species. It is necessary to consider various factors associated with the use of the correct botanical species. The dynamic process of evolution could have altered and affected the original identity and structure of the natural materials. Further, some of the botanical species might have been extinct or may have undergone changes due to changes in agro climatic conditions. Therefore, standardization of the botanicals will require different approach that can help in confirming their identity. Presently, the information required for authentic botanical includes the currently accepted Latin binomial name and synonyms, vernacular names, the part of plant used for each preparation and detailed information for agricultural production and collection conditions according to each country's good agricultural and collection practices. Regulatory authorities for control of raw material have suggested various methods. Most of the regulatory guidelines and pharmacopoeias suggest macroscopic and
microscopic evaluation and chemical profiling of the botanical materials for quality control and standardization. Macroscopic identity of botanical materials is based on parameters like shape, size, color, texture, surface characteristics, fracture characteristics, odor, taste and such organoleptic properties that are compared to a standard reference material. However, these parameters are judged subjectively and substitutes or adulterants may closely resemble the genuine material. It is often necessary to substantiate the findings by microscopy and/or phytochemical analysis. Microscopic examination alone cannot provide complete identification; when used in association with other analytical methods it can supply supporting evidence.

Chemical profiling establishes a characteristic chemical pattern for a plant material, its fractions, extracts or the multi component botanical preparations. Thin-layer chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) are routinely used as valuable tools for the qualitative determination of small amounts of impurities. Gas Chromatography (GC) has also found its utility in quality control and standardization of botanicals. In addition, many analytical techniques such as volumetric analysis, gravimetric determinations, gas chromatography, column chromatography, high performance liquid chromatography, and spectrophotometric methods are also frequently used for quality control and standardization.

Use of chromatographic techniques and marker compounds for standardization of botanicals has its own limitations. Analysis of secondary metabolites is restricted to
those plants that produce a suitable range of metabolites, which can be easily analyzed and can distinguish between varieties. Also, the metabolite being used as marker should ideally be neutral to environmental effects and management practices. Estimating presence of a particular compound in a herb is not sufficient to determine the desired quality, since the marker compound may not necessarily be responsible for the biological activity that is attribute to the whole herb. In view of these limitations there is need for a new approach that can complement or in certain situations serve as an alternative.

1.6 Newer Techniques:

Some of the newly emerging techniques for ensuring correct botanical identity and quality include Herboprint™, which in addition to chemoprofile also considers Ayurvedic properties. Capillary electrophoresis is another technique, which is faster, precise and sensitive method and has recently been used to ascertain the botanical identity and quality of Ephedrae herba, Coptidis rhizome, Ginseng radix, and Paeoniae radix. The technique is based on simple phenomenon of electrophoresis where movement of electrically charged particles or molecules in a conductive liquid medium is studied under the influence of electric field. Some publications have presented perspective evidence that this technique may provide a superior alternative to HPLC in some cases by facilitating the reliable discrimination of the species. The molecular marker techniques, especially the DNA fingerprint analysis can certainly provide another alternative tool for quality control of the botanicals.
1.7 Molecular Markers:

Molecular markers generally refer to biochemical constituents including primary and secondary metabolites and other macromolecules such as nucleic acids. Secondary metabolites as markers have been extensively used in quality control and standardization of botanical drugs. Current focus of the study is only on DNA markers. The markers have found their utility in the fields of taxonomy, physiology, embryology, genetics etc and have several advantages over typical phenotype markers. They are reliable markers for informative polymorphisms as the genetic composition is unique for each species and is less affected by age, physiological conditions as well as environmental factors. DNA can be extracted from fresh or dried organic tissue of the botanical material; hence the physical form of the sample for assessment does not restrict detection. Various DNA based methods for species characterization and adulteration detection in medicinal plants; agricultural crops and genetically modified (GM) foods have been published.

PCR-based methods including Random Amplified Polymorphic DNA (RAPD) have been used by various researchers for authentication of traditional Chinese medicines like ginseng, *Echinacea*, *Atractylodes*. Development of more specific, sensitive and reproducible markers like RAPD based Sequence Characterized Amplified Region (SCAR) can increase industrial application of the molecular techniques and may help in identification of the correct botanical genotype in commercial preparations.
In this study, RAPD-SCAR marker for *Phyllanthus emblica* Linn. (Amla, Indian gooseberry) was developed by comparative DNA analysis between eleven *P. emblica* cultivars (NA-06, NA-07, NA-10, Kanchan, Chakaiya, Francis, Banarasi, Hhathizool, Dongri, Bansired and Anand-01) and six different *Phyllanthus* species (*P. distichus*, *P. reticulatus*, *P. urinaria*, *P. simplex*, *P. niruri* and *P. indofischeri*). Further exploration of its application for quality control and standardization of semi-processed and processed botanical preparations was done.

### 1.8 Selection Basis of *P. emblica*:

*P. emblica* fruit is one of the top selling botanicals having diverse applications in healthcare, food and cosmetic industry. It has been studied for immunomodulatory\(^7\), anticancer, antioxidant\(^8\) and antiulcer\(^9\) activities. An official drug of Ayurvedic Pharmacopoeia\(^5\), Indian Herbal Pharmacopoeia\(^2\), it forms a main ingredient of various multi-component formulations like Triphala churna and Chyawanprash. Presently, macroscopic and microscopic parameters are used for identification of the crude drug. It is difficult to identify the botanical when it is in powdered form or is present in semi-processed and processed preparations using these techniques. Chemoprofiling using gallic acid and ellagic acid is the preferred way for authentication of the drug. However, lack of specificity of these phytoconstituents for *P. emblica* limits their use. Other six *Phyllanthus* species were selected based on their therapeutic and commercial significance. Brief description for each of the plant has been provided in the next chapter.
1.9 **Aim:**

To develop and use DNA based RAPD-SCAR marker for quality control and standardization of botanical preparations.

1.10 **Objectives:**

1.10.1 Identification of species diagnostic amplicon for *P. emblica* by RAPD analysis.

1.10.2 Cloning and sequencing of the amplicon to develop SCAR marker.

1.10.3 Applicability studies of the developed marker for authentication of semi-processed and processed botanical preparations containing *P. emblica*. 