CHAPTER 2

Review of Literature

The chapter 2 provides an overview of previous researches and supporting data in relation to the present project. The chapter is broadly divided into two parts. In the first part, description about the ITS region, its properties as well as its evolution as the DNA barcode and benefits over the other barcoding loci has been discussed. Application of the ITS region for medicinal material identification has also been reviewed with appropriate examples. The second part describes about the various aspects of the plants selected for the project. The work, done previously, related to the identification and adulteration detection of these plants has also been reviewed.

2.1 Internal transcribed spacers (ITS) and its properties

ITS or the internal transcribed region is the non-translational unit of the rRNA genes array which encodes ribosomal RNA. These spacers lost during the maturation of ribosome, but they essentially required for the ribosome formation. A single repeat unit of rRNA gene array consist of ITS1, presents between the 18s ribosomal gene and 5.8s rRNA gene, and ITS2 region which exists between the 5.8s rRNA gene and 28s rRNA gene sequence **(Figure 2.1)**.

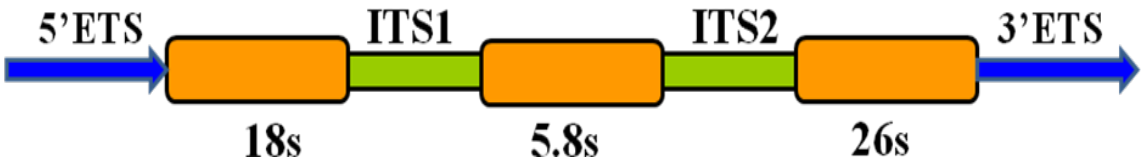

Figure 2.1 nrDNA gene showing Internal Transcribed spacers (ITS), External Transcribed spacers (ETS)

Copies of this cistron reiterate at one or many chromosomal loci in the whole genome (Vijayan and Tsou 2010). ITS1 and ITS2 are the intronic regions collectively called as the ITS which have higher evolutionary rates than the flanking rRNA genes. High degree of conservation in the genic part resulted into proper alignment, even above the family level and hence, can be applied to infer phylogenetic relations at a higher rank of taxa. On the other hand, rapid evolution in the ITS

region is helpful to infer such relationships at species or even at the population level (Álvarez & Wendel 2003; Baldwin et al. 1995).

Copy number of rRNA gene array varies even at the individual level of same population (Booy 2000). Length of ITS region also differs among the different plant domains. In angiosperms and fungi the length of complete ITS region fluctuates between a narrow range from 500 to 700 and 600 to 800 bp respectively. In gymnosperms the complete ITS region ranges from 1500-3700 bp. This vast variation in length is the result of variability in the length of ITS 1 which can ranges from 610 to 3100 bp (Liston et al. 1996; Maggini et al. 1998).

ITS region is very prone to form secondary structure. Secondary structure of the transcript also potentially used to reconstruct the phylogenetic relationship. Due to stringent role in protein synthesis the secondary structure shows high degree of conservation dominantly in ITS2 region. Presence of compensatory base mutations at the paired positions responsible for the preservation of pairing and hence, the secondary structure (Schultz et al. 2005). Suitability of the ITS region was well experienced in phylogenetic reconstruction (Álvarez and Wendel 2003; Baldwin et al. 1995) and is also endorsed to be used for DNA barcoding. Advantageous properties of the ITS loci to be used as plant DNA barcode, include biparental inheritance, universality, high intragenomic uniformity due to concerted evolution, high interspecific polymorphism, lower functional constraint and high copy number of the cistron (Álvarez and Wendel 2003; Feng et al. 2010)

2.2 Plant DNA barcoding and Internal transcribed spacers

The concept of DNA barcoding has ignited the new era of species resolution, since the origin the concept by Paul Hebert. It gives new scopes to the conventional taxonomic approaches. It provides universal standardized system to identify a particular species. The technique is more rapid, accurate and has benefits of being automated. DNA barcoding can facilitate the species identification in diverse range as well as quality of samples, without the need of expert taxonomist (Chen et al. 2014).

The concept of DNA barcoding has originated with the idea of developing a single universal sequence as a marker to identify a particular species. One sequence should represent one species.

This concept has been working effectively in animals but in plants where species boundaries are less defined than the animals, agreement on a single locus as plant DNA barcode has been found difficult. Paraphyly, reticulate evolution, hybridization, incomplete sorting of ancestral polymorphisms are the events makes the plants with less cleared species boundaries (Hollingsworth et al. 2011). Different requirements for plants, drive the researchers to focus more on plastid and nuclear genomic regions for species identification. The regions proposed include both protein coding and intergenic spacers. The regions recommended as plant barcode from plastid genome are rbcL, matK, rpoB, rpo C1, trn L-F, trnH-psb A, atpH-F, psbK-I and a nuclear region that is Internal transcribed spacers (Hollingsworth et al. 2011).

Researches belong to hunt for the marker for plant DNA barcoding; can be divided into two epochs. One era encompassed all the opinions before the decision of CBOL plant working group (Chase et al. 2007; Fazekas et al. 2008; Ford et al. 2009; Group et al. 2009; Kress et al. 2005; Kress & Erickson 2007; Lahaye et al. 2008; Newmaster et al. 2006; Taberlet et al. 2007) and after the recommendations made by CBOL. All of these studies before the study done by CBOL, were focused on the barcoding markers from plastome, except by Kress et al. (2005) and Chase et al. (2007), in which ITS offered as likely barcoding marker on the behalf of nuclear genome. It was mentioned that the ITS was advantageous due its higher copies which makes the amplification easy even from degraded samples, ITS1 and ITS2 can be amplified separately, the conserved 5.8s gene can provide anchor point for proper alignment to search algorithms, so the completely unknown sample can be identified at least at the order or phyla level and conserved flanking sequences (18srRNA gene, 5.8srRNA gene and 26srRNA gene) can be used to develop universal primers (Kress et al. 2005; Chase et al. 2007). Nuclear genome was suggested as a better solution due to its biparental inheritance which can handle the problem of common sharing of plastid genome sequences, reveal the speciation occurred through hybridization and have high evolution rates so possess more discriminatory power. However, in 2009 plant working group of Consortium of barcode of life (CBOL) voted for two marker systems, the combination of two genes - rbcL and matK and additionally psbA-trnH and ITS were proposed as supplementary markers (Group et al. 2009). Further, in the independent investigation done by Chinese plant consortium of DNA barcoding in 2011, they advocated the inclusion of ITS as core barcode for

non-flowering and flowering seed plants (Li et al. 2011b). The major issues related to ITS region to be used as DNA barcode were addressed by the China plant BOL group. The major reasons behind the concerns were – 1) Simultaneous amplification of fungal ITS region, as the primers used for plants and fungi have enough similarity, 2) Occurrence of paralogous copies within the single genome can make the identification ambiguous 3) Difficulty in amplification in some of the samples. The CBOL group reported that fungal contamination was found only in 2.5% of the individuals and 1.8% of the species whereas intragenomic variations were detected only in 7.4% of individuals and 9.3% of species of the total. Moreover, inverse to the predicted vulnerable nature of ITS during PCR and sequencing, success over the plastid markers (rbcL, matK, psbA-trnH) was demonstrated. Approximately in 71% individual of 75% species no need of cloning was generated during sequencing. In addition, the authors presented the backup plan in the form of amplification of ITS2, if the recovery of complete ITS region was found difficult. They assessed all the loci previously recommended by CBOL along with trnH-psbA and ITS with increment in the closely related species numbers, which is very crucial to decide the barcoding region (Li et al. 2011b; Hollingsworth 2011). The markers were evaluated on different parameters as universality, sequence quality and discriminatory power similar by CBOL and concluded that inclusion of ITS as single and in combination with other markers specially with matK would increase the discrimination, even between the closely related species and cryptic species. Inclusion of ITS with plastid markers gave more discrimination than the other plastid marker all together. They recommend rbcL+ mat K and ITS/ITS2 as three marker system, so that they could overwhelmed the flaws of each other. Earlier, Chen et al. (2010) also evaluated different plastid markers and ITS2 loci for the barcoding, targeting specifically medicinal plants which fall in wide range of taxa and included medicinally important algae, fungi, ferns, mosses, liverworts, gymnosperms and angiosperms. They found that ITS2 correctly identifies 92.7% species and 99.8% genus using BLAST as tool and 90.3% and 99.7% using nearest genetic distance method. Secondly, they also preferred psbA-trnH intergenic spacers. Supported by strong statistically analysis, ITS2 demonstrated highest intra and interspecific genetic divergence rate even between two cogeneric species. Based on the bioinformatics analysis of ITS sequences of 50,790 of plants and 12, 221 of animals, Yao et al. (2010) proposed ITS2 as universal

identification marker both for plants and animals (as complementary with mitochondrial region). They found that ITS2 successfully discriminate 76.1% of dicots, 74.2% of monocots, 67.1% gymnosperms, 88.1% ferns and 77.4% of mosses at the species level. Success rate of ITS2 in animals was found 91.7% and inclusion of secondary structure of ITS2 as an important discriminatory character was emphasized in the study. They also checked the ITS2 sequences for the presence of fungal nucleotides using BLAST and HMM. Similar to Chinese CBOL very less only 0.3% of the sequences analyzed were suspected to the presence of fungal contaminations. Regarding co-occurrence of multiple copies of ITS region within a genome, Song et al. (2012) performed pyrosequencing to evaluate the effect of paralogous copies on species identification. They discovered some intragenomic variations but they were not affecting the species identification negatively. In case of high intragenomic variability highly conserved secondary structure can be used for species resolution as suggested by Wolf et al. (2013). Tripathi et al. (2013) evaluated the potential of four barcoding candidates (rbcL, matK, psbA-trnH and ITS region) for species resolution of tree species. The authors found ITS region along with psbA-trnH the most suitable DNA barcode. These large scale studies including samples from diverse groups/ taxa boosted the acceptance of ITS/ITS2 as solitary or complementary marker.

2.3 ITS and traceability of herbals

Internal transcribed spacer is the only region from the nuclear genome, endorsed for the plant DNA barcoding. In many investigations belong to the DNA based identification of the medicinal plants the ITS is considered as the principal loci. In a systematic analysis of two databases NCBI and Medicinal material DNA database done by Li et al. (2011), in comparison to other plastid regions, ITS region was observed as the most applied DNA barcode candidate to trace herbal medicinal material originality. According to another report by Techen et al. (2014), ITS was included in majority of the research papers (from 2010-2013 in SciFinder database), related to the DNA barcoding for medicinal plants identification, which was followed by psbA-trnH, matK and rbcL. In these investigations barcode sequences have been utilized in different ways. In some of the reports species identification have been restricted to *in silico* analyses of the barcodes using BLAST searching, phylogenetic tree construction etc. Moreover, in other research reports

application of DNA barcoding including ITS has been broadened by developing many DNA based identification methods for herbal medicinal material. These methods have been developed with the assistance of advance techniques like conventional and quantitative PCR, high resolution melting curve analysis, microarrays, LAMP and NGS **(Figure 2.2)**. In the following sections, examples are mentioned, in which identification methods integrated with DNA barcoding, are developed for traceability of botanicals. More emphasis is given to the illustrations in which methods are devised from the ITS region.

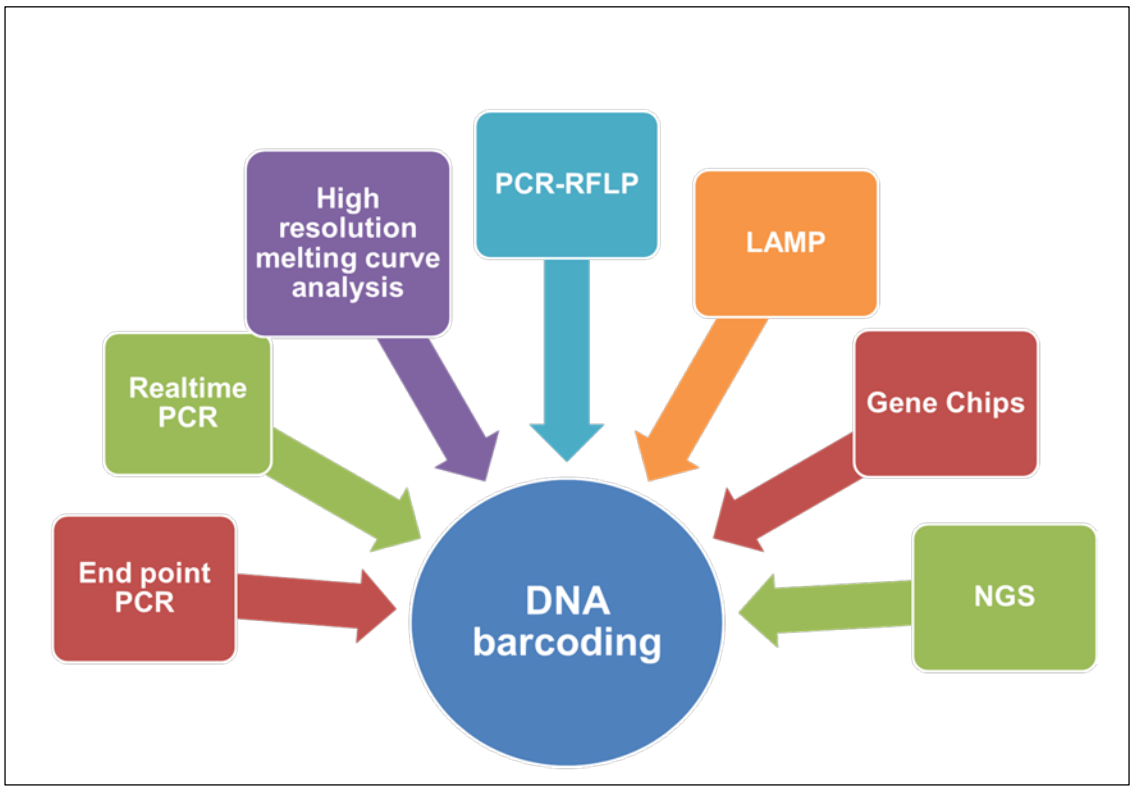

Figure 2.2 Methodological extensions of DNA barcoding for plant species identification

2.3.1 *In silico* sequence analysis

In general, sequences generated in a DNA barcoding project undergo various *in silico* analysis. Species assignment to a particular unknown sequence can be done in three ways- (1) Similarity based, (2) Tree based and (3) Character/ Diagnostic based (Bhargava & Sharma 2013; Weitschek et al. 2014). Similarity based species assignment can be done using BLAST (Altschul et al. 1990)

or the other tools as TaxonDNA. The species classification is based on the degree of similarity between the query sequence and the other sequences in the databases. Phylogenetic trees present the very convenient way to view the results of species discrimination (Group et al. 2009). In the tree based methods when two barcodes classified in similar cluster/ group are considered as cospecific. Tree can be build using various algorithm UPGMA, neighbor joining and maximum parsimony with the help of various softwares as MEGA (Tamura et al. 2011), PHYLIP (Plotree and Plotgram 1989) and PAUP (Wilgenbusch & Swofford 2002). Character based methods depends upon the sharing of particular characters between members of a particular species, which should be absent in the sister species (Bhargava & Sharma 2013; Weitschek et al. 2014). Examples of such tools are CAOS or Characteristic Attributes Organization System package (Sarkar et al. 2008), BRONX (Little 2011) etc.

2.3.2 Endpoint and realtime PCR assays

Conventional PCR is based on the hybridization of two short oligonucleotides, known as primers which are extended the DNA sequence between them and followed by agarose gel electrophoresis for detecting the amplicon (Madesis et al. 2014). DNA barcode polymorphism based SCAR markers have been developed in various investigations to ascertain the identity of a particular medicinal plant species. For this purpose primers are developed in such a way that contains polymorphic nucleotide at the 3' terminus which is corresponding to the species to be identified. ITS based species specific primers for authentication of *Angelica sinensis*, have been developed by Feng et al. (2010). These primers were also tested for amplification in seven substituents of the species but no amplification was observed in that substituents. Seethapathy et al. (2014) developed two species specific PCR assays based on the ITS region to discriminate two medicinal plant species *Aconitum heterophyllum* and its substituent *Cyperus rotundus*. Market plant samples and polyherbal formulation of the plants has been tested too. Kazi et al. (2013) have also developed the ITS based PCR method for *Hypericum perforatum* and successfully tested over the counter drugs related to the species. The market formulations included capsule, tablets and tincture. Multiplex PCR have also been developed in which ITS based SCAR markers have been combined with the SCAR markers derived from the other DNA

techniques. This is demonstrated by Babaei et al. (2014), for the authentication of saffron (*Crocus sativus* L.). Species specific primers have been developed from SCAR marker derived from both RAPD and ITS region, to use them in multiplex PCR assay.

In contrary to endpoint PCR, realtime PCR generate data in realtime mode which can be used to quantitate the amount of adulterant/allied species DNA present with the genuine herbal medicinal material (Wei et al. 2016). Quantitative PCR can also be used to detect the presence of substituent ingredients, whether present in prescribed limits or not (Madesis et al. 2014). An ITS based SYBR green realtime PCR assay coupled with ARMS technique has been developed to detect original *Dendrobium officinale* and its discrimination with other inferior *Dendrobium* species (Xu et al. 2012). Wu et al. (2015) have developed a ITS2 based TaqMan probe realtime PCR assay to detect the presence of Aristolochiaceous herbs in Chinese herbal material. Aristolochic acids found in the members of Aristolochiaceae family have been reported for their nephrotoxicity and carcinogenic properties. Species of Aristolochia are used as the substituents for the original material and reported to be caused severe adverse effects.

2.3.3 High resolution melting curve analysis of DNA barcodes (BAR-HRM)

This technique is based on the melting behavior of the PCR amplicons of a particular target region and if the target region is derived from the DNA barcode the technique is known as Bar-HRM. Melting behavior of any DNA duplex is depend upon the GC%, length of the sequence and strand complementarity (Moraes et al. 2015). Small sequence variations as single nucleotide polymorphism and indels changes the T_m of the particular target and difference in Tm can be used to discriminate authentic species from the counterfeits. The concept follows the principle of melting curve analysis, done in realtime PCR to check the purity of amplicons. It involves PCR amplification of the particular barcode region, in presence of any fluorescent dye, subsequently; PCR products are allowed to melt by increasing the temperature at the fix rate. Signals are generated when intercalated fluorescent dye start to dissociate and hence, there is decrease in the fluorescence. Fluorescence is plotted against the temperature to get the melting curve. In the various investigations this DNA barcoding has been coupled with HRM analysis (BAR-HRM). As, Osathanunkul et al. (2015) used the method to discriminate the three medicinal plant species

of the family Acantaceae (*Acanthus ebracteatus, Andrographis paniculata* and *Rhinacanthus nasutus*) using rbcL as the barcode region. Two reports are there in which Bar-HRM was used for quality control of the *Crocus sativus*. In the research by Jiang et al. (2014) melting properties of DNA barcode trnH-psbA was analyzed for *Crocus sativus*.as well as its possible adulterants *Carthamus tinctorius, Calendula officinalis, Hemerocallis fulva, Daucus carota , Zea mays , Dendranthema morifolium.* Villa et al. (2016) performed ITS and matK based HRM analysis of *Crocus sativus*. and its allied species *Crocus cartwrightianus*. ITS-HRM was reported to identify nine herbal teas as sage, Greek sage, chamomile, mountain-tea, oregano, cretan oregano, yarrow, lemon balm and rosemary (Xanthopoulou et al. 2016). In an investigation, among the various DNA barcodes, ITS1 region was suggested as the most effective barcode for HRM based species resolution of Croton species. The other barcodes used in the analysis were matK, rbcL, rpoC, and trnL (Osathanunkul et al. 2015).

2.3.4 Barcode based PCR-Restriction fragment length polymorphism (RFLP)

PCR-RFLP or CAPS (Cleaved amplified polymorphic sequence) is the method which involves amplification of the targeted region (DNA barcode in this case) and subjected to restriction digestion. The restricted PCR amplicons make specific banding patterns on agarose gel (Heubl 2010). Application of species specific RFLP patterns devised from the DNA barcodes have been demonstrated for various medicinal plants as for example *Actinidia macrosperma* (Zhao et al. 2007), 21 Korean Artemisia species (Lee et al. 2009), *Vitex glabrata* (Phoolcharoen and Sukrong 2013), etc. ITS-RFLP method was developed for authentication of *Boerhavia diffusa* and the potent adulterants *Trianthema portulacastrum* and *Trianthema monogyna* (Biswas et al. 2013). ITS-RFLP patterns were developed for molecular identification of six different species A*egle marmelos, Oroxylum indicum, Desmodium giganiticum, Solanum indicum, Solanum xanthocarpum* and *Tribulus terresteris*. These all species are used in the herbal formulation known as Dasamula which is the combination of roots of ten different plants (Biswas and Biswas 2013). ITS2 based CAPS markers have been developed to distinguish *Tetrastigma hemsleyanum* from its counterfeits (Peng et al. 2016).

2.3.5 Chip based identification of medicinal plants

DNA chip technology or microarray is a well-established method for the substantial screening of variations in large number of samples simultaneously (Kim et al. 2015). A DNA microarray is the solid surface made up of either glass or silicon, on which partial gene sequences, known as probe are adhered. These probes are used to hybridize with the complementary target sequence present in the fluorescently labeled DNA samples (Sarwat and Yamdagni 2014). For authenticating herbal medicines, species-specific probes are designed to attach on the surface in an array (Kim et al. 2015). These probes can be derived from DNA barcodes to increase the specificity of identification (Chavan et al. 2006). PCR amplified DNA barcodes from the particular species or the group of the species are used as the biological samples. Individual herbs in polyherbal formulations can be detected by designing the chip having probes corresponding to the authentic herbs used in the preparation. Chip based identification can provide a cheap and effective mean for quality control of herbal drugs. In a novel version of microarray technique probes are immobilized on the inner surface of a PCR tube cap using agarose film (Wang et al. 2015). Hybridization proceeds by simply inverting the tubes, just after the amplification of the target gene. Application of microarray for medicinal plants identification was illustrated in various researches. A gene chip was designed based on the 18s rRNA gene sequence to identify thirteen *Panax* species (Zhu et al. 2008). Gene chips, bearing species specific probes derived from 5s rRNA gene and lucine transfer RNA genes was developed to identify toxic Chinese herbal material (Carles et al. 2005). Use of ITS loci as species specific probes in a microarray, for medicinal plant identification was demonstrated for Dendrobium species (Zhang et al. 2003), for the plants in the family Umbelliferae (Kim et al. 2015),

2.3.6 Loop medicated isothermal amplification (LAMP)

LAMP is the technique in which amplification of nucleic acid is carried on the constant temperature. Amplification is carried by the means of four primers which recognize the six different sites on the target region of the nucleic acid and reaction proceed by the strand displacement method. The reaction mixture containing primers, samples, DNA polymerase with strand displacement activity and substrate is incubated at constant temperature (Notomi et al.

2015; http://loopamp.eiken.co.jp/e/lamp/) (60º C to 65º C). The polymerase enzymes have the strand displacement activity, so DNA denaturation is not required for initiation of amplification as in PCR. Moreover, contrary to PCR, chemical color reactions are used to detect the results in place of agarose gel (Notomi et al. 2015). The LAMP assays are very specific that amplification occurs only when all the primers specifically bind to the target region. Lai et al. (2015) developed a LAMP assay for authentication of *Taraxacum formosanum* which is used as the ingredient of herbal tea. Species specific primers for the assay were designed using ITS2 loci. In another report by Li et al. (2013) ITS based LAMP assay was developed for correct identification of another herbal tea ingredient *Hedyotis diffusa* and its isolation from the adulterant *H. corymbosa*.

2.3.7 Next generation sequencing (NGS)

The genome sequencing methodologies can be categorized into first, second and third generation the latter two are jointly known as next generation sequencing technologies. Next generation sequencing platforms cover most of the part of genome and generate huge data through massively parallel processing. These platforms function following different sequencing chemistries and methods (Morey et al. 2013). High scalability of the NGS platforms and capacity to process multiple samples / PCR amplicons in one run give rapidity and reliability to the DNA sequence based identification methods. The platforms generating longer reads as Roche can give more output with enough throughputs (Sarwat and Yamdagni 2014). These technologies are capable to detect both targeted and non-targeted adulteration in herbal drug preparations. Application of NGS combined with DNA barcoding was illustrated for Liuwei Dihuang Wan (LDW). It is a polyherbal formulation containing six plants *Rehmannia glutinosa* Libosch., *Cornus officinalis* Sieb. et Zucc., *Paeonia suffruticosa* Andr., *Dioscorea opposite* Thunb., *Poria cocos* (Schw.) Wolf and *Alisma orientalis* (Sam.) Juzep (Cheng et al. 2014).Various non-prescribed species were detected. ITS2 and trnL were used as the markers. Metagenomic analysis of the LDW drug collected from different manufacturers which belonged to three different batches, revealed the presence of non-prescribed species along with the authentic plants. Adulterant species were varying with the samples collected from different manufacturers and also batch to batch consistency in purity was lacking. In the another research report by Ivanova et al. (2016),

authenticity of herbal supplements was tested using the combination of DNA barcoding and NGS. These herbal supplements were related to *Echinacea purpurea, Valeriana officinalis*, *Ginkgo biloba*, *Hypericum perforatum* and *Trigonella foenum-graecum,* collected from different producer. The two barcoding markers, rbcL and ITS2 were used for the purpose.

2.4 Description of the plants

In the present project two highly valued medicinal plant species were selected- (1) Shankhpushpi and (2) *T. arjuna*. Both of the plant drugs have been included in the category having annual demand over 100 mt (Ved and Goraya 2007). In the following sections, chemical characteristics, pharmacological and biological activities of the plants are mentioned. The parameters and the methods which have been developed for the correct identification as well as for the standardization of these medicinal plants are also described.

2.4.1 "Shankhpushpi" the controversial identity

In the ayurvedic literatures the drug Shankhpushpi is known for its properties to increase intellect and memory. It is known as Medhya Rasayna (act as nervine tonic) (Amin et al. 2014). The term Shankhpushpi has plurality and equated with the four different plant species namely *Convolvulus microphyllus*, *Evolvulus alsinoides*, *Clitoria ternatea* and *Canscora decussata* (Andrade et al. 2012). However, in the Ayurvedic Pharmacopoeia of India and in the medicinal plants monographs published by ICMR, *Convolvulus microphyllus* has been approved under the name "Shankhpushpi"(Anonymus 1999; Anonymus 2005a). Overlapping and ambiguous vernacular identity provoke the need of the methods, to ascertain the identity of the botanical source of the medicinal plant material related to Shankhpushpi. In the present project DNA based methods have been developed to establish the molecular identity of *Convolvulus microphyllus* and *E. alsinoides*. These two Shankhpushpi plants are related to the same family Convolvulaceae. Market examination of crude drugs sold as Shankhpushpi, indicated the presence of *E. alsinoides* as the major substituent and mixing material (Malik et al. 2011). Description of both the plants are given below.

2.4.1.1 *Convolvulus microphyllus* Sieb. Ex Spreng.

The whole plant of *C. microphyllus* (accepted name is *Convolvulus prostratus* Forssk. syn *Convolvulus pluricaulis* Choisy) is used as drug. (Anonymus 2008; http://apps.kew.org/wcsp/namedetail.do?name_id=484749 accessed on 4.12.2015). It is a suberect, prostrate and spreading wild herb and distributed throughout the India. Flowers are white or pink in colour (Anonymus 2008; Ganie 2012).

(A) Taxonomic status

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Solanales
Family: Convolvulaceae
Genus: *Convolvulus*
Species: *microphyllus*

(B) Chemical constituents

The plant contains carbohydrates D-glucose, maltose, rhamnose, sucrose, starch etc. Alkaloids namely shankhapushpine, convolamine, convoline, convolidine, convolvine, confoline, convosine were also reported. Volatile oils, fatty acids, fatty alcohols, hydrocarbons such as myristic acids, palmitic acids and linoleic acids were also identified. Coumarines like scopoletin, scopolin and flavonoid like kampferol, phytosterols like β-sitosterol, ceryl alcohols were found in larger amounts (Sethiya and Mishra 2010; Agarwa et al. 2014).

(C) Biological activities

Traditionally the herb is reported as psychostimulant and tranquilizer, reduces mental tension. The drug is mentioned as the brain tonic in Ayurveda (Agarwa et al. 2014). Various investigations have demonstrated the nootropic, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative activities of the plant extracts as well as of the isolated compounds from *Convolvulus microphyllus*. Nootropic activity of ethanol and ethylacetate

extract of *Convolvulus microphyllus* was demonstrated by Nahata et al. (2008). Improvement in the cognitive functions was also observed during the research. Significant hypotensive activity of the aqueous extract of the species has been proved by Rizwan and Khan (2014). Ethylacetate and butanol fractions of hydromethanol extract of the *Convolvulus microphyllus* were found effective for improving Huntington disease like symptoms (body weight, locomotor activity, grip strength, gait pattern, cognitive dysfunction and oxidative stress in brain) which was induced by the neurotoxic compound 3-Nitropropionic acid (Malik et al. 2015).

Various extracts as well as individual isolated compounds from *Convolvulus microphyllus* are also tested for their therapeutic effects on memory enhancement and amnesia related to Alzheimer's disease (Rao et al. 2012). Three compounds have been isolated from the hydromethanol extract namely scopoletin, ayapanin or herniarin and scopolin. All of these compounds have been tested for attenuation of scopolamine induced amnesia in mice. The amnesia was significantly reversed by scopoletin and scopolin. These two compounds were found inhibitory for acetylcholinoestrase activity. Enhanced level of the acetylcholinoestrase enzyme reduces the acetylcholine levels in the brain leading to the memory impairment in Alzheimer's. The results are in agreement with the memory enhancement properties of the plant (Malik et al. 2015). Similarly, in a previous research, aqueous extract of plant showed the inhibition of acetylcholinesterase activity in the brain cortex and hippocampus. In addition, elevated contents of glutathione reductase, superoxide dismutase and reduced glutathione within the cortex and hippocampus indicated the neuroprotection through oxidative defense (Bihaqi et al. 2011). Attenuation of scopolamine induced expression of tau and amyloid precursor protein was reported by Bihaqi et al. (2012). These two proteins are reported to cause amnesia in Alzheimer's (Bihaqi et al. 2012). An alkaloid of *Convolvulus microphyllus* i.e convolvine exhibited properties of a sedative and nootropic agent by raising the sensitivity of acetylcholine specific receptors (Mirzaev and Aripova 1998). In a comparative evaluation of extracts of 15 medicinal plants, the root extract of *Polygonum multiflorum* Thunb. and leaf extract of *Convolvulus microphyllus* was found the most efficient in inhibition of β amyloid peptide (Liu et al. 2012).

Clinical studies were also performed using various formulations of *Convolvulus microphyllus*. Shankhpushpi syrup was found effective for symptomatic relief in the patients with anxiety and depression. Plasma cortisol level was also reduced. Syrup was found as an effective sedative in the 50% of patients undergoing gynecological surgery. A combination of *Convolvulus microphyllus, Centella asiatica* and *Acorus calamus* was found to be effective in cognitive improvement in low grade mentally retarded children. In comparison of the synthetic drugs (neomercazole, diazepam) Shankhpushpi was found effective in the patients suffering from thyrotoxicosis (Anonymus 2008; Sethiya and Mishra 2010).

2.4.1.2 *Evolvulus alsinoides* (L.) L.

It is a perennial herb. Branches are prostrate, woody and hairy. Flowers are blue in color. Plants are distributed throughout the India.

(A) Taxonomic status

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Solanales
Family: Convolvulaceae
Genus: *Evolvulus*
Species: *alsinoides*

(B) Chemical constituents

Various alkaloids were noted in the plants. Alkaloids such as betaine, shankpushpin, evolvine and four unidentified alkaloids A, B, C have been described in literatures. Various tropane alkaloids were also reported. Higher alkanes such as triacontane, pentatriacontane were also found. Other compounds as scopoletin, scopolin, umbelliferone, kaempferol glycosides, caffeic acid, β-sitosterol, 2,3,4-trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy- 2-methylbutoxy)-phenyl]-2-propenoate and 1,3-di-O-caffeoyl quinic acid methyl ester were also reported in the plants extracts (Austin 2008; Cervenka et al. 2008; Gupta et al. 2007). A chromone derivative

named as alsinoideschromone has been isolated from the aerial parts of the plants (Akhtar et al. 2009). Three new flavonol-4-O-triglycosides (Kaempferol derivative) have been isolated from the butanol fraction of the plants (Gupta et al. 2013). Recently, Gomathi et al. (2015) performed the GC-MS analysis of the ethanolic extract and reported the presence of piperine, octodeconoic acids, hexadecanoic acid and squalene.

(C) Biological activities

In the traditional and ayurvedic medicines the plant is used as brain tonic, used in asthama, amnesia, cough, cold and fever (Ganie 2012). Antistress activity of the flavonol-4-O-triglycosides from *E. alsinoides* was reported by Gupta et al. (2013). In the previous research by Gupta et al. (2007) various phenolics and flavonoids compounds were found to be effective in normalizing stress parameters, as plasma glucose, adrenal gland weight, plasma creatine kinase and corticosterone levels. Crude ethanolic plant extract was tested for its adaptogenic and antiamnesic properties in acute and chronic unpredictable stress models of rats. Plant extracts was found very effective to improve stress conditions as well as in the scopolamine induced memory impairment (Siripurapu et al. 2005). Improved learning behavior and better memory was observed in rats models when ethanol extract and its ethylacetate fraction was administrated to the rats (Nahata et al. 2010). In another study, it was found that, ethanolic extract of the whole plant effectively reduced the oxidative stress, induced by streptozotocin and potentially increased the insulin level (Gomathi et al. 2013). An antibacterial property of the ethanolic plant extract was demonstrated by (Omogbai and Eze 2011). Other activities of the plants include, antioxidant (Cervenka et al. 2008; Kumar et al. 2010) antihelmenthic (Mali and Mehta 2008), immunomodulation (Alamgir and Uddin 2010), antihyperlipidemic (Iyer and Patil 2011).

2.4.1.3 Comparative biological activities studies of *C. microphyllus* and *E. alsinoides*

This section describes the studies in which the comparative biological activities of the Shankhpushpi plants have been evaluated. Nahata et al. (2009) performed various tests to assess the anxiolytic activity of ethanolic extract and its ethylacetate and aqueous fractions of the plants. Results led to the conclusion that both the drugs own comparable significant anxiolytic effects, have equal effects on the neuromuscular coordination, and have substantial antioxidant potential.

In another investigation by Kothiyal & Rawat. (2011) alcoholic extract of *E. alsinoides* displayed superior nootropic activity than *Convolvulus microphyllus*. Contrary to this, in the study conducted by Malik et al. (2011), aqueous methanolic extract of whole plant of *Convolvulus microphyllus* exhibited profound anxiolytic and nootropic activities in comparison to *E. alsinoides* (whole plant) and *Clitoria ternatea* (roots) whereas antidepressant activity was lowest in the *Convolvulus microphyllus* extract. Herbal extracts of fifteen different plants were tested to examine their effect on the production of amyloid peptide which is directly related to Alzheimer's diseases. *Convolvulus microphyllus* and *E. alsinoides* were among those plants. The results supported the profound inhibition of amyloid peptide production by *Convolvulus microphyllus* which proved its potential role to treat Alzheimer's (Liu et al. 2012).

From the studies related to the biological activities of the individual Shankhpushpi plants as well as their comparative analysis mentioned in the previous sections, it can be concluded that *E. alsinoides* was found superior in most of the stress related studies. *Convolvulus microphyllus* was found more efficient in improving cognitive functions and memory impairment. More extensive and systematic information are needed to identify more bioactive compounds from these two plants and to check their comparative therapeutic effects for various CNS related malfunctions. Future may reveal the parallel or differential activities of both the plants. In the current scenario methods need to be developed for establishing the identity of individual plants used under the vernacular name Shankhpushpi. Various pharmcognostic and phytochemical methods were reported for standardization and to trace the botanical origin of the particular Shankhpushpi plants, which are described in the following section.

2.4.1.4 Methods for correct identification of Shankhpushpi plants

Multiplicity in the names of medicinal plants can create the confusion which can lead to the use of wrong species. Regarding, Shankhpushpi, various methods have been developed to ascertain the identity of the plant material source. Madhavan et al. (2008) presented various detailed macroscopic and microscopic characters to distinguish *C. microphyllus* and *E. alsinoides*. Different physio-chemical analysis such as total ash, extractive values, fluorescence studies, TLC fingerprinting of various extracts of both the plants were also performed. Sethiya et al. (2010)

also analyzed pharmcognostical, microscopical and phytochemical characteristics of all the four Shankhpushpi plants. When results of these reports were compared, some parameters were found similar and some had differential values. The resulted values not give the absolute indication of plant identity; they give a range. This necessitates the need of some more methods to validate the identity of Shankhpushpi plant material. Sethiya et al. (2010) also recommended the need for exploration of some more parameters to distinguish *C. microphyllus*, *E. alsinoides* and *Clitorea ternatea*, because microscopically they were found somewhat similar. Moreover, superficial similarity between *Convolvulus microphyllus* and *E. alsinoides* imposed the different taxonomist to consider both the plants as the white and blue flowered varieties of the same species (Austin 2008). Varadan et al. (1958) also mentioned about the confusion in the identity of these two plants.

Phytochemical methods have also been reported which fortify the above mentioned pharmacognostic approaches and give more testimonials to determine the identity. The chemical methods have been developed for standardization of an individual Shankhpushpi speices as well as their comparative evaluation was also performed. In case of *C. microphyllus*, in most of the phytochemical methods, one of the major coumarine namely scopoletin was used as chemical marker. In the ICMR monographs TLC method was mentioned for scopoletin (Anonymus 2005a). Further, HPTLC profile was developed for *C. microphyllus* considering scopoletin as the chemical marker by Kapadia et al. (2007). Scopoletin was also successfully quantified in the marketed samples. A HPLC method for quantification of scopoletin was developed by Vipul et al. (2013). The authors determined the scopoletin in different extracts of *Convolvulus microphyllus* and found maximum quantity in the ethanolic extract, in comparison to hydroalcohol and aqueous extracts. In another HPTLC method scopolin was considered as the marker compound for standardization of *Convolvulus microphyllus* (Lal 2014). Regarding *E. alsinoides*, in ICMR monographs TLC method for detection of luteolin 7-glucoside as the chemical marker, have been reported (Anonymus 2006). In one investigation, plolyherbal formulations containing Shankhpushpi and Brahmi as the main ingredients were analyzed using shankhpushpin and asiaticoside as the chemical markers. UV spectrophotometric method was developed for their estimation (Deshpande et al. 2014) .

In many studies, chemical methods have been developed, comparing all the four Shankhpushpi plants (*C. microphyllus, E. alsinoides, C. decussata, C. ternatea*). Sethiya et al. (2009) used scopoletin and mangiferin as chemical markers and a co-TLC method was reported to distinguish all the four Shankhpushpi species. Scopoletin was found in ethanolic extract of all the four Shankhpushpi species whereas mangiferin was detected only in *C. decussata*. In the another research, four biological markers, namely β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin were analyzed using TLC-DPPH (2,2-diphenyl-1-picrylhydrazyl) method for identification and differentiation of all the four botanical claimants of Shankhpushpi. Similar to the previous research scopoletin was found in all the four Shankhpushpi claimants. *Convolvulus microphyllus* showed presence of only scopoletin whereas other two markers as β-carotene, and chlorogenic acid were also detected in *E. alsinoides*. The other two Shankhpushpi plants *C. ternatea* and *C. decussata* showed presence of β-carotene, rutin; and β-carotene, mangiferin respectively along with scopoletin (Sethiya et al. 2013). In 2014, Sethiya and Mishra developed a co-TLC method to differentiate the Shankhpushpi claimants. The method was developed for detection and quantification of four chemical markers ursolic acid, betulinic acid, stigmasterol and lupeol. Among them stigmasterol was found in all the four Shankhpushpi plants. Betulinic acid was found specifically in *E. alsinoides*. Urosolic acid was determined in both *C. ternatea* and *C. decussate* whereas lupeol was present only in the previous one. These methods can be applied to establish the identity of plant material available as Shankhpushpi. However, concluding these reports it can be inferred that more species-specific chemical and biological markers are required for perfect authentication of plant origin.

DNA-based approaches can help more, for clear and exact justification of the source of the botanicals belongs to Shankhpushpi. Ganie et al. (2012) developed RAPD profile to differentiate *C. microphyllus*, *E. alsinoides* and *C. ternatea.* In the other study RAPD markers were developed for genetic diversity analysis of *C. microphyllus* (Ganie et al. 2014). RAPD markers were developed also for genetic diversity estimation of *E. alsinoides* (Ganie and Sharma 2014). DNA barcode-based PCR methods which are formulated in this project can overwhelm the limitations associated with the random molecular marker approaches and make the authentication more accurate.

2.4.2 *Terminalia arjuna* (Roxb.)Wight & Arn

Stem bark of *T. arjuna* is used mainly for cardioprotection and hepatoprotection. It is a perennial tree species having erect stem and papery bark.

2.4.2.1 Taxonomic status

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Myrtales
Family: Combretaceae
Genus: *Terminalia*
Species: *Arjuna*

2.4.2.2 Chemical constituents

Stem bark of *T. arjuna* contains various triterpenoids namely arjunin, arjunic acid, arjunolic acid, arjungenin, terminic acid. Glycosides as arjunetin, arjunoside I, arjunoside II, arjunaphthanoloside, terminoside A were also isolated from the bark. Several flavonoids, arjunolone, arjunone, bicalein, luteolin, gallic acid, ethyl gallate, quercetin, kaempferol, pelorgonidin, oligomeric proanthocyanidins are also present in the bark. Tannins include pyrocatechols, punicallin, punicalagin, terchebulin, terflavin C, castalagin, casuariin, casuarinin, gallic acid, ellagic acid. Phytoconstituents of roots and leaves were also analyzed. Roots also contain various triterpenoids and glycosides similar to stem bark. Luteolin was also reported in the leaves (Dwivedi 2007; Jain et al. 2009). Recently, Saha et al. (2012) have been characterized polyphenols in the stem bark of the *T. arjuna* and found extracts had 44% polyphenols by weight. The main phenolics compounds detected are catechin, gallocatechin and derivatives of ellagic acid.

2.4.2.3 Biological activities

T. arjuna has been extensively used tree species, due to its significant medicinal properties. In the Indian system of medicines, bark powder of the tree is used in case of angina and cardiovascular

ailments (Dwivedi 2007). In addition, customarily, bark is used as astringent, demulcent, expectorant, cardiotonic, styptic, antidysenteric, urinary astringent and found to be useful in fracture, ulcers, leukorrhea, diabetes, anemia, cardiopathy, and cirrhosis (Paarakh 2010; Dwivedi and Chopra 2014). Arjuna bark have anticancer, hypocholsteremic, hypolipidemic, anticoagulant, anti- hypertensive, antithrombotic, antiviral, antifungal and antibacterial properties. Triterpinoids and their glycosides are considered as the effective bioactive agents for the cardioprotection whereas flavonoids act as antioxidant, anti-inflammatory and lipid lowering agents. Tannins are responsible for the anticancer properties (Jain et al. 2009).

Therapeutic effects of the Arjuna plant on the cardiovascular system have been demonstrated in various animal models, for the treatment of many cardiac disorders like angina, myocardial infarction, hypertension, hypercholesteremia, cardiac arrest, etc. Significant inotropic effect has been revealed in many studies which are considered to be mediated through the high concentration of Ca^{++} present in the plant which increases coronary artery flow and protects myocardium against ischemic damage (Haq et al. 2012). The aqueous extract has demonstrated to cause an increase in the coronary flow in rabbit (Bhatia et al. 1998). The aqueous extract of Arjuna also increased the force of contraction of cardiac muscle in frog and rabbit heart (Verma et al. 2013). Tannin fraction of aqueous and 70% alcoholic extract of bark exhibited hypotensive effects (Nammi et al. 2003). Aqueous and alcoholic bark extract, in dogs, resulted in a decrease in blood pressure and heart rate (Dwivedi and Chopra 2014).

Ethanolic extract of the bark have been demonstrated for having significant antioxidant and hypolipidemic effect (Chander et al. 2004; Sharma et al. 2012). This hypolipidemic action is supposed to be facilitated through increased hepatic clearance of cholesterol, down-regulation of lipogenic enzymes, and inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) (Patil et al. 2011). Involvement of thyroid hormones (suppression of thyroid function), in the improvement of cardiac and hepatic lipid peroxidation, was also considered as one of the mechanism for lipid lowering effects posed by the bark extract (Parmar et al. 2006).

T arjuna have potential to prevent oxidative stress-induced damages in the heart tissues. It was found that administration of bark and alcoholic extract prevent the ischemic–reperfusion-induced

oxidative stress by boosting the level of antioxidants like superoxide dismutases, catalyze and glutathione in myocardial tissue as well as through the induction of the heat shock proteins (Gauthaman et al. 2008). Antioxidant activities were also demonstrated with methanolic and aqueous extract and high amount of phenolics were suggested as the prime reason for the potent antioxidant activity of arjuna bark (Kokkiripati et al. 2013). Among the isolated compounds arjunolic acid and casuarinin have been found to prevent oxidative stress (Sumitra et al. 2001). Ethanolic extract of *T. arjuna* have also be shown to enhance the levels of antioxidative enzymes in hepatocellular carcinoma cells (Jain et al. 2009). Different extract of bark (benzene, chloroform, acetone and methanol fractions) was found having anticancer properties mediated through the inhibition of mutagenesis (Kaur et al. 2000; Kaur et al. 2002). Various individual compounds from *T. arjuna* bark such as arjunin, arjunic acid, luteolin and casuarinin have shown significant cytotoxic activity against various cancerous cell lines (Jain et al. 2009).

Various clinical examinations of *T. arjuna* were also conducted, signifies the potential of *T. arjuna* in cardiovascular diseases (Amalraj & Gopi 2016; Dwivedi & Chopra 2014; Kapoor et al. 2014). These studies were mainly related to the cases of heart failure, anti-ischemic activity, lipid lowering activities, hypertension, endothelial dysfuction etc. Clinical improvements were noted in the patients with congestive heart failure, upon administration of *T. arjuna* bark. Hypertensive patients also demonstrated improvements when Arjuna kwataha was taken (Maulik and Talwar 2012). Significant antianginal properties have been displayed in one study. Significant decrease in ischemic mitral regurgitation and reduction in anginal frequency was observed when arjuna was used (Paarakh 2010). Hydroalcoholic extract of bark exhibited noteworthy improvements in endothelial dysfunction in chronic smokers (Bharani et al. 1995). Significant improvement was demonstrated, in left ventricular parameters as reduction in mass of left ventricle and improvement in left ventricular ejection fraction, by using arjuna (Bhawani et al. 2013; Dwivedi et al. 2005;). Dyslipidemia and the oxidative stress are the major risk factor closely associated with the heart diseases (Maulik and Talwar 2012). Administration of one gram of bark powder was found effective in improvement of lipid profiles of 21 patients with coronary heart disease (Tripathi et al. 2000). Significant reduction in triglycerides, low density cholesterol and lipid peroxide levels was observed which was attributed to the soluble fibers and sitostanol content.

Dwivedi and Kumar used the drug along with statins in patients of β-thalassemia associated with hyperlipoproteinemia and metabolic syndrome. Significant decrease in the level of lipoproteins exhibited the potential of *T. arjuna* in the syndrome caused due to elevated levels of lipoproteins (Dwivedi and Kumar 2007).

2.4.2.4 Adulterants

In the market, 13 species are being sold as arjuna. Stem bark of *Terminalia tomentosa* (*T. alata*), *T. bialata, T. manii, T. myriocarpa, T. catappa, T. calamansanai, T. travencorensis, T. pallida, T. critina, T. paniculata, T. bellirica, T. chebula* used as adulterants of *T. arjuna* (Anonymus 2005b). Among these, four most prominent adulterant/allied species namely *T. tomentosa, T. bellirica* and *T. chebula* are targeted in this project. Use of stem bark of these three species as the filler material in *T. arjuna*, can reduce its curative effects and can pose serious health risk to consumers. Stem bark of *T. arjuna* have many clinically proven biological activities as mentioned in the **section 2.4.2.3.** Very few stem bark specific therapeutic activities were reported or tested, regarding other adulterant/allied *Terminalia* species. As for example, benzene and ethanol extract of *T. bellirica* bark was tested for its effect on the functions of accessory male reproductive organs (Patil et al. 2010). Antioxidant potential of bark extract of *T. bellirica, T. chebula* and *T. alata* was also demonstrated (Shah et al. 2013; Nguyen et al. 2016). Methanolic extracts of *T. bellirica* and *T. alata* was found effective for lowering the glucose level in the diabetic rats (Nguyen et al. 2016). Alcoholic and ethyl acetate extracts of stem bark of *T. alata, T. bellirica, T. catappa, T. chebula* and *T. arjuna* was found fungicidal (Shinde and Wadje 2011). Acetone extract of *T. chebula* bark have anticancer properties (Bag et al. 2013). Methanolic extract of *T. tomentosa* bark was tested for its antidiabetic properties in the rats (Santhan et al. 2013).

These studies indicate need of extensive evaluation of the stem bark of adulterants/allied *Terminalia* species, to find their accountable therapeutic properties, similar to *T. arjuna*. More number of investigations will reveal similarity or dissimilarity in the chemical composition of the stem bark of different *Terminalia* species. Dissimilar chemical principles can work synergistically or antagonistically with the bioactive principles of *T. arjuna*. Thus, adulteration and mixing of these three species as the filler material can significantly affects commercialization

of *T. arjuna* based drugs. The concern about the use of *T. arjuna* medicinal material without proper botanical identification in preclinical and clinical studies, has also been raised by Maulik and Talwar (2012). Therefore, there is the necessity of development of methods for standardization, identification and authentication of *T. arjuna* bark, which are described in the following section.

2.4.2.5 Studies for authentication of *T. arjuna* bark

Various attempts were made to derive the parameters to ascertain the genuineness of the *T. arjuna* starting material. Dhingra et al. (2013) have been evaluate macroscopic, microscopic, preliminary phyto-chemical and physicochemical characteristics of *T. arjuna* bark for authentication purpose. For TLC fingerprinting arjunic acid, arjungenin, arjunolic acid and arjunetin were used as the reference standards. Similarly, various pharmacognostic and phytochemical parameters were established by Rupa et al. (2014). Phramacognostic parameters were also developed by various scientists in the different investigations (Patil and Gaikwad 2011; Patel and Dhanabal 2013; Dambal et al. 2014). Along with qualitative phytochemical analysis, Chaudhari and Mahajan (2015) analyzed various volatile compounds in the GC-MS analysis of the methanol extract of *T. arjuna* stem bark. In all these investigations comparison of different standardization parameters, with the stem bark of adulterant/allied *Terminalia* species are lacking. Further, Khatoon et al. (2008) evaluated seven *Terminalia* species namely *T. arjuna*, *T. bellirica*, *T. bialata, T. catappa, T. chebula, T. manii* and *T. tomentosa.* TLC fingerprints were developed to identify distinguish bands. In *T. arjuna* fluorescent blue band at RF 0.39 was obtained at 366 nm along with the band at the same RF visible after the derivatization with anisaldehyde sulfuric acid reagent. These bands can be used to differentiate arjuna bark. In the other study tannin related markers as gallic acid, corilagin, chebulagic acid, ellagic acid and chebulinic acid were quantified with HPLC in *T. chebula, T. bellirica, T. arjuna* and *T. catappa*. In *T. chebula* and *T. arjuna* all the five markers were present where as in *T. bellirica* peak of only ellagic acid was detected (Dhanani et al. 2014). In a comparative study of three *Terminalia* species (*T. chebula, T. bellirica* and *T. arjuna*), UPLC/oaTOF MSE along with multivariate statistical analysis was performed for generating chemical markers for identification and authentication. Chromatogram was generated which was further subjected to PCA analysis

(Principal component analysis). In this study, PCA analysis showed species to species chemical content variation in *Terminalia*. It was concluded that plant location, collection time and freshness of the extract were found to have negative impact and gave the differential qualitative and quantitative attributes of chemical entities even within the same species (Avula et al. 2011), which is a hitch in the chemical authentication methods.

DNA based approaches can be used to augment the identification of *T. arjuna*. Investigations, focusing on the molecular authentication of *T. arjuna* are lacking. However, DNA fingerprinting for genetic diversity analysis were reported. RAPD markers were used to diagnose genetic diversity and species relationship among *T. arjuna, T. bellirica*, *T. chebula*, *T. tomentosa* and *T. catappa*. RAPD markers clustered *T. arjuna*, *T. bellirica*, *T. chebula* in one group and the remaining two species were clustered together in another group (Deshmukh et al. 2009). Further, genetic diversity and species correlation was analyzed among *T. arjuna*, *T. bellirica* and *T. chebula*, using three marker systems AFLP, ISSR and RAPD. All the three marker systems were clearly clustered these three species in three different groups (Sarwat et al. 2011). Sequence based approaches like DNA barcoding can give more accuracy in the identification of *T. arjuna* bark.

The chapter 2 has been divided into the two parts. In the first part, main focus has been given to the ITS region as well as its evolution as the DNA barcode, in comparison to the plastid markers. In the subsequent section examples have been described in which ITS region has been used as the barcoding marker for the identification of the various medicinal plants. In addition, various methodological extension of the DNA barcoding has also been defined. In this part of the chapter more emphasis has been given to the ITS based approaches. In the second part of the chapter medicinal plants which have been selected for the project are mentioned. Their biological activities along with the problem related to use of authentic plant species of the related plants, has been described. The methods developed to ascertain the identity of the particular plant species as well as its adulterants has also been reviewed.