CHAPTER 3

Material and Methods

This chapter encompasses the description of various chemicals, biological and non-biological material, solvents, reagents, instruments as well as various methods optimized and followed for the accomplishment of the project.

3.1 Materials

This section of the chapter 3 includes all the materials utilized in the current project and classified in various categories as follows:

3.1.1 Instruments and Equipments

- ***Autoclave***

  Vertical and horizontal autoclave from Osworld, India and Mediquip was used for the sterilization of glasswares, plasticwares and buffers.

- ***Camera Lucida***

  Camera lucida was used to trace and draw the various microscopic characteristics of the plant samples.

- ***Capillary electrophoresis platform***

  Capillary electrophoresis platform from ABI was used for sequencing.

- ***Centrifuge***

  Centrifuge from Hettich (Universal 320R, 380R), Thermofischer (Legend micro 21); Eppendorff (minispin) was used for centrifugation.

- ***Compound microscope***

  Compound microscopes were used to analyze microscopic characters of plant samples.

- ***Refrigerators and Deep freezers (-20^{o}C)***

  Refrigerators from Godrej and Samsung were used to store buffers and chemicals. Plant samples, Genomic DNA samples, fine chemicals, primers were stored at -20ºC deep freezer from Samsung, India.

- ***Electrophoresis units***

  Horizontal gel electrophoresis units from Bio-Rad and Medox were used to analyze DNA samples and PCR products.

- ***Gel documentation system***

  System from Bio-Rad and Syngene was used for analysis and documentation of agarose gels. U.V transilluminator was also used for the same purpose.

- ***Hot air oven***

  Hot air oven from Cintex, Bombay was used for drying the washed glasswares and plant samples.

- ***Hot Plate***

  Hot plate from Erection and Instrumentation Engineering, India, was used in microscopy for the sample preparation.

- ***Micropipettes***

  Micropipettes of various capacities (10 µl, 20 µl, 100 µl, 200 µl and 1000 µl) from Eppendorf, Gilson and Thermo were used in various experiments.

- ***Spectrophotometer***

  UV-Visible spectrophotometer (UV 2450) from Shimadzu, Japan was used for quantification of DNA samples.

- ***Stage micrometer***

  Standard stage micrometer of total 1mm length was used in microscopic studies.

- ***Water bath & Dry bath***

  Water bath & dry bath from Yorco, India were used for incubation at varying temperatures.

- ***Water purification system***

  Elix system was used to obtain Milli Q water for the preparation of buffers, reagents and solutions.

- ***Weighing balance***

  Chemicals for preparations of various reagents and buffers were weigh using Electronic weighing balance from Mettler (Mettler AE 163 Toledo).

- ***Thermal Cycler***

  Gradient and non gradient thermal cycler of Eppendorf and Applied Biosystem was used to amplify DNA in various PCR reactions.

- ***Tube Mixer***

  Tube mixer from Tarson was used for mixing during DNA isolation.

3.1.2 Plastic wares and glasswares

Labwares included various plastic and glasswares encompassing measuring Cylinders, Beakers, Petriplates, Watchglass, Glass slides, Micro centrifuge tubes, Micro tips, Needles, Coverslips. Plastic wares as micro centrifuge tubes and tips were procured from Tarson, Thermoscientific and Axygen. Glasswares were procured from Schott Duran and Borosil.

3.1.3 Chemicals and solvents

Agarose, Chloral hydrate solution, Safranin, Tris base, Ethylenediamine tetra acetate (EDTA), Cetyl trimethyl ammonium bromide (CTAB), Mercury chloride (HgCl2), Sodium chloride (NaCl), Potassium chloride (KCl), Sodium acetate, Sodium hydroxide, Sodium dodecyl sulphate (SDS), Ascorbic acid, β mercaptoethanol, Polyvinyl pyrollidine, DMSO, Tween 20, Glycerol, Formamide, Bovine Serum albumin, Boric Acid, Activated Charocoal, Hydrochloric acid (HCl), Bromophenol blue,. All the chemicals were used of analytical grade and purchased from Sigma, Himedia, Merck, s.d.fine CHEM, Rankem, Spectrochem, Invitrogen and Fischer Scientific. Solvents include Chloroform, Isoamyl alchohol, Isopropanol, Ethanol. All the solvents were of analytical grade and procured from Merck, Qualigens, Fischer Scientific and Rankem.

3.1.4 Fine chemicals

- ***Enzymes*** as RNase, Taq polymerase along with their appropriate buffers were purchased from Fermentas (Thermo Fischer).

- ***dNTPs*** also were procured from Fermentas (Thermo Fischer).
- ***BigDye® Terminator*** reaction mixture from ABI Life Sciences was used during the sequencing.
- All the ***oligos*** used in the project were synthesized and procured from Sigma Aldrich and Xcelris Genomics lab Pvt Ltd.

3.1.5 General reagents and solutions

- **1 M Tric-Cl (pH 8.0)**

  For one litre Tris-Cl, 121.1 g of Tris base was dissolved in 800 ml of MiliQ water. pH of the solution was adjusted with HCl and final volume was made up upto one litre with MiliQ. The solution was sterilized and stored at room temperature.

- **0.5 M EDTA (pH 8.0)**

  For one litre 0.5 M EDTA, 186.1 g of compound was dissolved in 800 ml of MiliQ water. NaOH pellets were added in the solution and mixture was stir strongly on magnetic stirrer. pH of the solution was adjusted with NaOH and final volume was make up with MiliQ upto one litre. This solution was sterilized and stored at room temperature.

- **50X TAE buffer**

  For 1000 ml of 50X TAE buffer 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA were mixed, pH was set to 8 and final volume was adjusted to 1000 ml by MilliQ water. This solution was sterilized and stored at room temperature.

- **1X TE buffer (pH 8)**

  For 100 ml of 1X TE buffer, 1 ml of Tris base from stock solution of 1 M was (Final concentration 10 mM) mixed with 200 µl of 0.5 M EDTA (Final concentration 1 mM), pH of the solution was adjusted at 8 and final volume was makeup to 100 ml by MilliQ water. The solution was sterilized and stored at room temperature.

- **70% Ethanol**

  For 100 ml, 70 ml of absolute alcohol was mixed with 30 ml of MiliQ.

- **Chloroform:Isoamyl alcohol**

  Both the solvent were mixed at 24:1 ratio and used in DNA isolation protocol.

- **Chloral hydrate solution**

  50 g of chloral hydrate powder was dissolved in 20 ml of water. It was used in microscopy of the collected plant material.

- **Glycerol water solution**

  Glycerol and water were mixed in 1:1 ratio for mounting the sections on the slides.

- **6X Gel lading buffer**

  Bromophenol blue (0.25% w/v) was dissolved in 1 ml of 30% glycerol solution in water. This buffer was used to load DNA samples into the well for electrophoresis and also used as tracking of DNA running on agarose gel.

- **Ethidium bromide**

  Ethidium bromide is a intercalating dye which is used to visualize DNA bands in agarose gel. 10 mg of dye powder was dissolved in water and stored for the further use in agarose gel electrophoresis.

3.1.6 Biological Samples

- Whole plants of *Convolvulus microphyllus*, *Evolvulus alsinoides*; Stembark samples of *Terminalia arjuna, Terminalia tomentosa*, *Terminalia bellirica* and *Terminalia chebula* were collected from various locations.

3.1.7 Softwares and *in silico* tools

The following softwares were used in various analyses in the project.

- ***BLAST (Hosted by NCBI)***

  This was used to verify the resulting assembled sequences.

- ***Chromas Lite vs 2.1.1***

  This was used to visualize chromatogram/electropherogram files generated in sequencing.

- ***Codoncode Aligner (Demo version)***

  This was used to edit and assemble the raw sequence reads based on the quality values of the bases.

- ***Clustal w and Clustal omega (Hosted by Ensemble)***

  These tools were used to align multiple sequences to inspect variable sites.

- ***MEGA vs 5.1 and 6***

  This was used for characterization of the sequences.

- ***Oligo Calc***

  This online tool was used to design and characterization of primers.

- ***Primer3 vs 0.4.0 and 4.0.0***

  This online tool was used to design and characterization of primers

- ***Reverse comp***

  This online tool was used to analyze the ITS sequences and also in primer designing.

- ***Sequence analyzer vs 5.1***

  This software was provided by ABI to analyse the sequences generated through sequencing platforms.

3.1.8 DNA reference standards

All the DNA reference standards used were procured from MBI-Fermentas (Thermo Scientifc). Lambda DNA digested with HindIII (High molecular weight) was used as reference for genomic DNA and 50 bp Gene Ruler were used for the analysis of PCR products.

3.1.9 Commercial kits

DNeasy plant DNA isolation kit and QIAquick Gel extraction kit from Qiagen were used in the study.

3.2 Experimental methods

3.2.1 Collection of plant samples

Plant samples collection was the very first objective for the project. Plant samples belong to various species were collected and stored as follows.

3.2.1.1 *Convolvulus microphyllus* and *Evolvulus alsinoides*

As, whole plant of Shankhpushpi species are used as the drug, so whole plants of *C. microphyllus* and *E. aslinoides* were collected from the different locations in India with the help of expert taxonomist and individuals. All the plant material was stored at -20º C for further analysis in airtight polybags. As both the plants are annual plants, so they were collected from the month of July to December. Some of the plant material was heat dried at 45º C in hot air oven and powdered for mimicking the processed material available in the market. Representative samples the voucher specimens were submitted to Department of Pharmacognosy and Phytochemistry of B.V. Patel PERD Centre, Ahmedabad for future reference. Voucher numbers are given in the **Chapter 4.**

3.2.1.2 Stem bark of *T. arjuna* and its adulterant/allied species

Stem bark of the *T. arjuna* has the therapeutic effects, so stem bark samples of the *T. arjuna* as well as the other three adulterant/allied species *T. tomentosa*, *T. bellirica* and *T. chebula* were collected from the various sources for developing the PCR assays for their identification. The collected part stem bark tissue contains outer dead cork layer as well as inner phloem part. The material was stored in at -20º C for further analysis. Some of the plant material was heat dried and powdered for mimicking the processed material available in the market. Representative samples as the voucher specimens were submitted to Department of Pharmacognosy and Phytochemistry of B.V. Patel PERD Centre, Ahmedabad for future reference. Voucher numbers are given in the **Chapter 4.**

3.2.2 Preliminary identification and validation of the collected plant samples

Identity of the collected plant samples was verified by analyzing their morphological characters, microscopical and histological characters. The characters were compared with the reported literatures.

3.2.2.1 Morphological analysis

Morphological analyses include the characters which can be seen with the naked eyes. It gives preliminary taxonomic identification of the particular species.

(A) *C. microphyllus* and *E. alsinoides*

Various macroscopic characters of aerial parts of both the plants including leaf type, leaf margin, leaf apex, phyllotaxy, leaf surface, flower colour were examined and compared with the literatures.

(B) *Terminalia* species

Stem bark samples of all *Terminalia* species were evaluated for their outer and inner surface structure, texture, colour and compared with the reported literature.

3.2.2.2 Microscopical Evaluation

Microscopic features of each plant species were examined with the help of compound microscope. It gives information about the cell and tissue types and their organization. Free hand sections were cut wherever required and characters were drawn with the help of camera lucida.

(A) *C. microphyllus* and *E. alsinoides*

For the microscopical analysis of *C. microphyllus* and *E. aslinoides* various leaf characters as stomatal index, palisade ratio, vein islet and vein termination number were calculated for each species and compared with the reported data (Madhavan et al. 2008). These are the constants for the identification of a particular species and vary between allied or closely related species. Transverse sections of the stem were also analyzed.

Stomatal Index

Stomatal index can be defined as the percentage of stomatal numbers as compared to the total number of epidermal cells (Kokate 1994). To count the stomatal index number, upper and lower leaf epidermis of both the species were peeled off and boiled in chloral hydrate for 2-3 minutes to remove chlorophyll. Cleared epidermal peels were mounted in glycerin. Square boxes were drawn of fix area using scale calibrated with standard stage micrometer. Epidermal cells and stomata were drawn under total magnification of 400X with the help of camera lucida. Minimum 5 such observations were taken for both upper and lower epidermis for each species. Epidermal cells and stomata were counted leaving incomplete cells of any two sides and consider incomplete cells as complete cells of other two sides of each box and stomatal index were calculated using following formula and average was taken.

Stomatal index = S/S + E

S= Total number of stomata in that box

E= Total number of epidermal cells in the same box

Palisade Ratio

Palisade ratio refers to the average number of palisade cells of mesophyll, present beneath the each epidermal cell (Kokate 1994) . For palisade ratio whole leaves of each species were boiled in chloral hydrate, mounted in glycerin and observations were taken under microscope (total magnification 400X). Four continues epidermal cells and rounded palisade cells present beneath these epidermal cells were drawn. Total numbers of palisade cells were counted and the figure obtained was divided by four (number of epidermal cells) to obtain palisade ratio. Minimum 5 such observations were taken for calculation of palisade ratio. An average was calculated including all individual observations.

Vein islet and Vein termination number

Leaves in dicots contain a midrib which is further branched into small veins distributed throughout the surface. The ultimate branches, veinlets circumvent some small portions of the leaf, is known as vein-islets. Counting of total number of such vein-islets per square millimeter of

the leaf surface between midrib and the margin is known as vein-islet numbers. The ultimate free ends of veinlets are termed as vein terminations. Number of total vein terminations per square millimeter of leaf surface between midrib and the margin is known as vein termination numbers. For this purpose five boxes of fix area (1mm) were drawn for each species with scale calibrated with stage micrometer. Leaves were boiled in chloral hydrate solution and mounted in glycerol water. Vein islets and Vein terminations were drawn under microscope (Total magnification 100X) with camera lucida. Vein islet and vein termination numbers present within the square were counted. Five such observations were taken and average was calculated.

Transverse section of stems

Thin free hand transverse sections from stems of both the plants were cut with the help of blade. Selected thin sections were destained by boiling in chloral hydrate and again stained with safranin. Sections were mounted on slides in glycerin solution and observed under microscope in the magnification of 100X and 400X. Characters were drawn with the help of camera lucida.

(B) *Terminalia* species

For the microscopic examination of *Terminalia* species stem bark samples, transverse section were taken as well as powder microscopy was performed.

Histological study

Transverse sections of outer and inner bark portion were cut with the help of blade and destain the sections with chloral hydrate by heating them on hotplate. These sections were subjected to staining in petriplates using safranine. The sections mounted on the glass slides in the glycerol solution and cover with coverslips. The sections were observed under microscope using 10X (eyepiece) and 40X (objective lens) ocular lens and photographs were taken.

Powder microscopy

Some part of the collected sample was dried on 45º C in hot air oven. The stem bark was powdered with the help of morter-pestel and grinder. A pinch of powder was taken on the slide and treated with chloral hydrate solution, warmed up and mounted in glycerin solution. The slide was observed under compound microscope. The characters were drawn with the help of camera

lucida using 40X objective lens and 10X eyepiece lens. For scaling, standard stage micrometer having 100 divisions with 0.01 mm distance between each division, was traced under the same magnification. This was measured with scale and average was taken of such three observations.

3.2.3 Genomic DNA isolation, purification and analysis

Genomic DNA isolation of high quality is the first step for the molecular techniques. DNA isolation from the leaves of Shankhpushpi plants and the stem bark samples of *Terminalia* species was done. General CTAB method was followed for the herbs whereas optimizations were needed for the stem bark. These methods were described in the section below.

3.2.3.1 DNA isolation from leaves of *C. microphyllus* and *E. alsinoides*

Leaves of *C. microphyllus* and *E. alsinoides* were used for DNA isolation. The protocol is based on the CTAB method described by (Doyle and Doyle 1990) with the minor modifications.

(A) Preparation of CTAB extraction buffer

The extraction/homogenization buffer include 2% CTAB (w/v), 1.4 M NaCl, 2 g PVP (w/v), 100 mM Tris-Cl (pH 8) and 20 mM EDTA (pH8). Buffer was prepared by mixing all these components and sterilized by autoclaving. As, β-mercaptoethanol and ascorbic acid are the heat labile components, so added just before the DNA isolation. β-mercaptoethanol at the concentration of 0.6 % and 5 mM of ascorbic acid were used.

(B) Procedure of DNA isolation

Homogenization buffer (2 ml) was preheated at 65º C for 30 minutes. Leaves (50-100 mg) were homogenized in a mortar and pestle with preheated extraction buffer along with 12 µl β-mercaptoethanol and 10 µl of 1 M ascorbic acid. Homogenate was transferred to a microfuge tube and incubate at 65º C for ½-1 hours with intermittent shaking. Equal volume of chloroform : isoamyl alcohol (24 : 1 v/v) was added and contents were mixed by inverting the tube up and down for 5 minutes. Homogenate was subsequently centrifuged at 6000 rpm for 10 minutes at 24º C. The upper aqueous layer was collected in a new microcentrifuge tube; isopropyl alchohol was added and kept for 20 minutes at 4º C. Subsequently, the tube were centrifuged at 8000 rpm

for precipitation of DNA pellet. Pellet was washed two times with 70% ethanol, dried and dissolved in 1XTE buffer containing RNAse. DNA solution was incubated at 37° C for half an hour and again repurify with absolute ethanol which was further washed with 70% ethanol and finally dissolved in 1XTE buffer.

3.2.3.2 DNA isolation from stem bark of *Terminalia* species

DNA isolation from stem bark tissue of *Terminalia* species was found very challenging. In comparison of leaves of Shankhpushpi plants, it was found more tough tissue to be crushed. Bark of *T. arjuna* along with other species is reported to be highly rich in polyphenolics, flavanones and tannins which is characteristic of this genus (Khatoon et al. 2008). Lots of efforts were made to obtain spectrophometrically pure DNA form the stem bark samples of *Terminalia* species. The protocol developed for the DNA isolation was described below.

In order to develop a protocol many previously reported methods were tried. At first DNA isolation methods by Doyle and Doyle and by Dellaporta et al. were tried with or without modifications (Dellaporta et al. 1983; Doyle and Doyle 1990). In the method by Doyle and Doyle, CTAB was used in the extraction buffer whereas SDS was included in the method developed by Dellaporta (1983). Based on the results CTAB buffer was preferred and all the modifications were integrated into CTAB method based on the reports by (Khanuja et al. 1999; Manning 1990; Porebski et al. 1997). The method is described in the following sections.

(A) Tissue preparation

Stem bark samples were surface sterilized with 0.1% $HgCl_2$ for 2 minutes, to remove microbial DNA contamination. Sterilized tissue samples were soaked in water overnight at 37° C prior to crushing for making the crushing easy

(B) DNA extraction method

For DNA extraction from the stem bark samples of *Terminalia* species, sterilized and soaked tissue was homogenized in the preheated modified extraction buffer. The modified extraction buffer had higher concentration of CTAB (3%) and NaCl (2M) along with the other components same as mentioned in the section **3.2.3.1(A).** Incubation time with isoamyl alcohol and

chloroform was increased upto 15 minutes helped to improve DNA quality. DNA precipitation was done with ethanol in place of isopropanol. The remaining steps were followed as same in **section 3.2.3.1(B).** DNA, thus obtained was brown in color in cases of *T. bellirica* and *T. chebula*. A pinch of charcoal was added in the DNA solutions (Krizman et al. 2006). Charcoal was removed by centrifugation and subjected to ethanol precipitation. This depigmented DNA with other samples was dissolved in 1XTris-EDTA buffer (pH 8) and subsequently subjected to quality and yield checking using UV/Vis spectrophotometric analysis.

3.2.3.3 Qualitative and Quantitative assessment of isolated genomic DNA

(A) Spectrophotometer Analysis

Extracted DNA from each sample was subjected for evaluation of quality and measurement of quantity. Absorbance was taken at three different wavelengths falls under the UV region of the light spectrum. Absorbance at 230 nm showed the presence of polysaccharides and organic solvents. Absorbance at 260 nm and 280 nm displayed the presence of nucleic acids and protein respectively. Appropriate amount of DNA was diluted with MiliQ. Equal volume of 1xTE was taken in place of DNA to prepare blank. Absorbance were taken for each sample and ratios of optical densities on different wavelengths were calculated as 260/230, 260/280 to check the presence of any contamination. Quantity of DNA was calculated using following formula (Sambrook and Russell 2001).

DNA Concentration (µg/ml) = 50 X OD on 260 nm X Dilution Factor

(B) Agarose Gel Electrophoresis

Agarose gel (1%) was prepared in 0.5X TAE buffer for genomic DNA analysis. One gram of agarose was weighed and mixed in 100 ml of 0.5XTAE buffer. The mixture was heated until the complete dissolution of agarose particles. Ethidium bromide was mixed when the gel mixture cooled down to approximately 45°C. Comb was inserted and gel mixture was poured into the gel cast tray and left it for solidification. After 30 to 45 minutes comb was removed and gel was kept into the buffer tank filled with 0.5X TAE running buffer. DNA samples were mixed with bromophenol blue tracking dye and added into the wells. Gel was run on 80 volts for 30 to 60

minutes. DNA was observed under the UV light and documented with the help of gel documentation systems.

(C) PCR amplification

Amenability of DNA for downstream processes like restriction digestion and PCR is another way of quality assessment. DNA isolated of each sample was subjected for amplification of ITS locus region. Qualitative assessment of DNA from the leaves of *C. microphyllus* and *E. alsinoides* was revealed, its sufficient purity for PCR and positive results were obtained. Results of qualitative assessment and PCR amplification of DNA from stem bark samples were not found consistent in spite of tremendous optimization in DNA isolation procedure. Therefore, some of the optimizations were also done during PCR amplification for the robust results which were mentioned in the next section.

3.2.4 PCR amplification of ITS locus

ITS locus of each species was amplified and subsequently sequenced. Similarity of the sequence with the gene database proved the authenticity of the samples. In some of the samples whole ITS region was amplified and in some of them ITS1 and ITS2 were amplified individually. PCR was optimized for each part of the ITS locus. Heterogeneous amplification of these loci in the stem bark samples of *Terminalia* species was overcome by using PCR facilitators. These facilitators were reported to inhibit the effect of various PCR inhibitors in the DNA solution.

3.2.4.1 Selection of universal primer pairs for ITS region

Various universally applied primers were reported for the amplification of ITS locus. These primers were developed based on the conserved flanking regions of ITS region. The primers developed by White et al. (1990) were used in this study. Sequences of these primers were given in the **Table 3.1** and their attachment sites were mentioned in **Figure 3.1.**

Table 3.1 Universal Primers for ITS amplification

Name of Primer	Sequence (5' to 3')
ITS 1	TCCGTAGGTGAACCTGCGG
ITS2	GCTGCGTTCTTCATCGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC
ITS 5	GGAAGTAAAAGTCGTAACAAGG

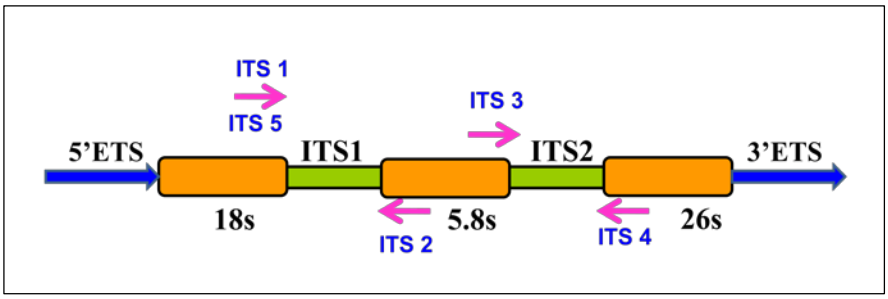

Figure 3.1 Attachment sites of different universal primers for ITS region.

3.2.4.2 Evaluation of PCR enhancers

Isolated DNA was further subjected for PCR amplification with reference to complete or partial ITS region. In contrast to *C. microphyllus* and *E. alsinoides*, in case of *Terminalia* species, amplification of ITS region was observed inconsistent. Unpredictability in PCR success was empowered using different PCR facilitators. The reaction optimized for PCR amplification of ITS locus was supplemented with different compounds with varying concentrations to find best PCR additive. In the amplification reactions different PCR enhancers namely DMSO, PEG, Tween 20, Formamide and Bovine Serum Albumin (BSA) were tested on different concentrations.

3.2.4.3 Amplification reaction of ITS region

Extracted DNA was amplified for complete ITS region using universal primers ITS5 and ITS4 **(Table 3.1)**. For the amplification in total 25 µl of PCR mixture containing 1X Taq buffer, 2.5 mM of $MgCl_2$, 200 µM of dNTP each, 10 pm of forward and reverse primer, 1U of Taq polymerase and 50 ng-60 ng of isolated DNA of each species. Based on the results of evaluation of PCR inhibitors as described in **section 3.2.4.2**, the reaction was supplemented with 0.5 µg/µl

bovine serum albumin (BSA) and 5% DMSO was added. The reaction was carried out with initial denaturation at 94º C for 10 minutes and then 30 cycles of denaturation at 94ºC for 1 minute, annealing at 52º-55º C for 45 sec and extension on 72º C for 1 minute and finally extended for 10 minutes at 72º C.

3.2.4.4 Amplification of ITS 1 and ITS 2

In some of the species problem was observed in sequencing of complete ITS region so ITS1 and ITS2 amplify individually and subjected to sequencing discretely. For amplification of ITS1 region primer pair ITS5 and ITS2 were used whereas ITS2 locus was amplified with primer pair ITS3 and ITS4 **(Table 3.1).** PCR conditions were followed as same for the amplification of complete ITS region in **section 3.2.4.3.**

3.2.4.5 Agarose gel analysis for PCR products

The amplified products were run on 1.5 % agarose gel in 0.5X TAE buffer and visualized in U.V light and gel documentation was done. Gel was prepared as in **section 3.2.2.3 (A).**

3.2.5 Bidirectional sequencing of ITS amplicons

All the amplicons generated related to ITS locus were subjected to bidirectional cycle sequencing. It was done by following the chemistry developed by Applied Bio system. Sequencing was performed at Xcelris Genomics Labs at Ahmedabad and Dr. C.G. Joshi Lab at College of Veterinary Science & Animal Husbandry, Anand with the help of concerned technical person. Cycle sequencing is based on the chain termination method developed by Sanger et al (Sanger et al. 1977). In this method deoxynucleotides were mixed with special type of nucleotides named as dideoxynucleotides (ddNTP's), during the DNA extension process. Dideoxynucleotides are devoid of 3' OH group which is responsible for the formation of phosphodiester bond between the growing strand and the coming nucleotide. Addition of ddNTP's does inhibit the reaction and a number of bands differing in size are accumulated which are further separated by gel or capillary electrophoresis platforms. Dideoxynucleotides are fluorescently labeled with different dyes which give different coloured signal upon addition of different ddNTP's in the growing chain. Sequencing was performed from both the sides as 5' to

3’ and vice a versa known as bidirectional sequencing. Methods followed during whole procedure of sequencing are given below.

3.2.5.1 Purification of PCR amplicons

PCR products are required purification before sequencing to remove excess of remaining nucleotides, salt or primer dimers in the sample. Purification was performed following three methods as per the requirements.

(A) ExoSap purification

The PCR samples were treated with 1 U of Shrimp Alkaline Phosphatase and 10 U of Exonuclease. This mixture was incubated at 37º C for 15 minutes subsequently at 80º C for another 15 minutes. This step helped in removing extra dNTPs, single stranded DNA or the primer dimers.

(B) Ethanol purification

The samples processed at College of Veterinary Science & Animal Husbandry were precipitated with absolute alcohol, washed with 70% ethanol dried and dissolved in 1XTE buffer.

(C) Gel elution

The highly problematic samples having intense background smear were purified by extracting the amplicons from the agarose gel. For gel elution Qiaquick gel elution kit from Qiagen was used and manufacturer’s protocol with slight modifications was followed. In the summarize form, band was excise with the help of sharp blade and weighed. Three volume of QG buffer (provided in the kit) was added and heated at 50º C until the gel was dissolved. Equal volume of isopropanol was added and the mixture was applied to the silica columns supplied with the kit. Centrifugation was done and filtrate was removed. Column was washed again with 500 µl QG buffer twice, centrifuge and then incubated with PE buffer for 10 minutes. Centrifugation was done to remove PE buffer. Column was loaded with elution buffer, kept in dry bath for 10 minutes at 50º C and elute was collected.

3.2.5.2 Cycle sequencing

Purified samples were subjected to sequencing. Bidirectional cycle sequencing method have three steps; first amplification of amplicons using reaction mixture having DNA polymerase and fluorescently labeled ddNTPs (BigDye® terminator reaction mix by ABI), second step is purification and denaturation of amplified products and at the last denatured samples are run in capillary electrophoresis station.

(A) Reamplification of purified samples

In order to complete the first step of sequencing, reactions were kept in 96 well plates. Each well contained total 10-12 µl reaction mixture having 20-30 ng PCR sample, forward or reverse primer (each 10 pm concentration), DMSO, 1X sequencing buffer and reaction mixture.(BigDye terminator cycle sequencing kit, Applied Biosystem). The primers for sequencing were used as same for the PCR amplification of ITS locus (**Table 3.1, Figure 3.1**). All the samples were amplified in thermal cycler using PCR program as denaturation at 96 º C for 10 seconds, primer annealing at 50-56º C for 5 seconds and extension at 60º C for 4 minutes. This was repeated for 25 cycles.

(B) Purification and denaturation

Samples were precipitated with ethanol and EDTA solution. Plate was centrifuged to precipitate the pellet which was subsequently washed with 70% ethanol, dried and dissolved in formamide. A short spin was given for complete mixing and then denaturation was done first by keeping the plate on 95ºC for 3 minutes and then immediately transferred at 4ºC.

(C) Separation of bands

Plate was loaded into 96 capillary sequencer platform. Samples were inserted into capillaries through electrokinetic injection and started to separate according to size. Signals were generated which were converted into electropherogram with the Sequence analysis software. Electropherogram files with extension of .ABI can be opened in ChromasLite software.

3.2.5.3 Quality analysis and editing of sequences

The next step of sequencing is the identification of the base (base calling) and characterization of their quality. Various softwares are available which have integrated algorithm which assigned the quality values (Q) for each base on the basis of various parameters related to peak shape and their resolution (Bonfield and Staden 1995; Ewing and Green 1998). It can be defined as the property logarithmically related to the probability (P) of error in base calling. Mathematically it is represented as $\mathbf{Q = -10 \log_{10} (P).}$ For example if a base has been assigned 30 quality score so the chances of incorrect base calling is 1 in 1000. Different companies also developed algorithms to calculate Phred like quality scores. Sequence analysis software was used to measure quality score of each base and the complete raw reads. Trace files were added and processed for the analysis. This software assigned the quality values of each base which was used further to determine the clear range for trimming the low quality bases from the sequence with the same tool. The reads have QV > 20 were considered for further analysis (Tripathi et al. 2013).

3.2.5.4 Assembly of sequences

The processed raw reads were assembled to make consensus sequences. Sequence assembly was done using Codon code aligner (Codon Code, Dedham, MA, USA).). It is an easy-to-use DNA and RNA sequence assembler, aligner, and editor. This software automatically assigned the quality score for each base, align the forward and reverse raw reads and select the base having higher quality score to make consensus. The trace file related to a plant species was imported using importing option in the main menu and a new project was created. Reads were selected and assemble option was clicked in the contig menu. The project window automatically was showing the assembled sequence which were saved for further analysis.

3.2.5.5 Verification and *in silico* analysis of the sequences using BLAST

BLAST or basic local algorithm searching tool is the bioinformatic program freely provided by NCBI. This tool was used for confirmation of all the sequences generated for ITS region. Algorithm for BLAST, searches the whole nucleotide database for similarity between the query sequences and other nucleotide strings present in it (subject sequence). Assembled sequences of each species were uploaded separately with default settings and click on blast button. Query

sequences were analyzed similarity scores and e- values were calculated. Expect value or e- value stands for finding the similarity in the entire nucleotide database by chance. This value should be more towards zero showing high similarity between the query sequence and the subject. On the basis of matches between the sequences generated in this project and present in the database for a particular species, it can be concluded that the collected samples were from authentic plant species. BLAST results also verified the absence of any mixing of DNA from other organism like fungus and bacteria (Hollingsworth 2011). Thus purity and authenticity of the sequences were validated. All the ITS sequences belong to six species in this project, were submitted to NCBI through their BankIt tool and accessions numbers were obtained.

3.2.5.6 Alignment, sequence characterization and tree based species identification

Complete ITS region has three components ITS1, 5.8S rRNA gene and ITS2. Sequence boundaries of each component were determined by comparing the consensus sequence of each species with the reference sequence in the NCBI database. ITS sequences of all the species with their corresponding adulterants were aligned using Clustal W tool integrated in phylogenetic software MEGA (Tamura et al, 2011). Aligned sequences were used to determine their length, GC content, aligned length and to measure number conserved, variable sites and parsimony informative sites between them. Aligned sequences were use to analyzed classification of species through phylogenetic tree. Neighbour joining trees were prepared to check the effectiveness of ITS region in species assignment using MEGA software.

3.2.6 Development of species specific primers

The aligned sequences were used to find variable nucleotides among the authentic species and the adulterants to develop specific primers to differentiate them at genomic level. Identification of variable sites for primer designing was done manually. Each variable site/ nucleotides were analyzed *in silico* to check its suitability for primer development. Two different online softwares were used for primer analyses OligoCalc (Kibbe 2007) and Primer3 (Rozen and Skaletsky 2000). OligoCalc provide convenient web interface for calculating physical properties of DNA/RNA stretch as it calculate the length of primer, GC content, give opportunity to check the formation of any hairpin loop as well as self complementarities and BLAST for the sequence entered can be

performed . OligoCalc calculate melting temperatures on the basis of three different methods- Basic Tm, Nearest Neighbour method and Tm based on salt adjustment. Primer 3 also calculate the melting temperature, GC content, thermodynamic stability of most stable hairpin loops, 3' inter and intra self-complementary region between forward and reverse primers. This software also measures the approximate amplicon size. Designing of reverse primer needed the reverse complementation of the 3' DNA template sequence so for this purpose on line tool Reverse comp was used (http://www.bioinformatics.org/sms/rev_comp.html). Among the pool of all possible primers, some primers were selected and taken further for experimental validation. Primers were synthesized by Sigma Aldrich, USA and Xcelris Labs Ahmedabad. During the primer development it was tried to develop the species specific primers having similar physical properties. Another consideration is that amplicon size should be differing in size so that bands were visually identified simply running on the gel. Some more factors were considered in selection of primers which have been discussed in the **Chapter 4**: **Primer sequences are mentioned in the tables 4.16 and 4.17.**

3.2.7 Development of species specific PCR assays

Bioinformatically verified primers were taken further to develop species specific PCR identification assays. Experimental validation and PCR assay development was done following the three parameters – (1) Amplifiability of the primers, (2) Cross reactivity of the primers and (3) Sensitivity of the reactions. Amplifiability of the primers describes its bonding with the original template DNA and should give the same amplicon size as determined in *in silico* analysis. Cross reactivity refers to check if the primers used for the original species are giving any amplification in the adulterant species or the vice versa. Sensitivity of the reaction can define as the minimum requirement of the template DNA required to obtain amplicon concentration at the detectable level. First the singleplex PCR assays were developed and verified according these three criteria and subsequently, multiplex PCR assays were developed aiming the identification of original species and their adulterants when present in mixtures. Each multiplex assay was validated. No template control (no DNA) was included in each reaction to ascertain the absence of any false positive results.

3.2.7.1 Development of assays for *C. microphyllus* and *E. alsinoides*

The final reactions were given as follows:

(A) Identification of *C. microphyllus*

The primer pair was checked on a range of temperatures to identify the best suitable annealing temperature. The highest annealing temperature was preferred. Each component of the PCR was optimized and final reaction conditions are given in the **Table 3.2.** Specificity (cross reactivity) of the primers and the reaction was evaluated by amplifying *E. alsinoides* DNA with the primer of *C. microphyllus*. PCR assay was tested for its sensitivity. Different dilutions of DNA template were made (2 ng, 5 ng, 10 ng, 25 ng, 50 ng, 75 ng and 100 ng) to test the limit of amplification and detection on agarose gel. The final reaction was used to amplify DNA of the same species collected from the different locations for validation.

Table 3.2 PCR reaction for identification of *C. microphyllus*

Reactants Concentration	Cycling Parameters
1X Taq buffer, 1.50 mM $MgCl_2$, 200 µm dNTP, FP (CM 200) 10 pm, RP (ITS4) 10 pm, Taq Polymerase 1U, BSA 0.5µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 60°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)

(B) Identification of *E. alsinoides* with the selected specific primer pairs

Three different primer pairs were selected to develop PCR assays for identification of the *E. alsinoides.* All the final reaction conditions are given in **Table 3.3**. Each primer pair was checked for its amplifiability, cross reactivity and the sensitivity. Cross reactivity of the primers and the reaction was evaluated by amplifying *C. microphyllus* DNA with the *E. alsinoides* specific primers. PCR assay was tested for its sensitivity applying different dilutions of DNA template as in section **3.2.7.1 (A).** Each component of the PCR were optimized to obtained the desired results. The final reaction was used to amplify DNA of the same species collected from the different locations for validation.

Table 3.3 PCR reaction for identification of *E. aslinoides*

Reactants Concentration	Cycling Parameters
1X Taq buffer, 1.20 mM $MgCl_2$, 200 µm dNTP, FP (EA 596) 2 pm, RP (ITS4) 2 pm, Taq Polymerase 1U, BSA 0.5 µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 62°C for 20 sec, 72°C 1 minute, Final extension 72°C (5 minutes)
1X Taq buffer, 1.50 mM $MgCl_2$, 200µm dNTP, FP (EA 596) 5 pm, RP (EAR519) 5 pm, Taq Polymerase 1U, BSA 0.5 µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 68°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)
1X Taq buffer, 1.50 mM $MgCl_2$, 200 µm dNTP, FP (ITS3) 2 pm, RP (EAR519) 2 pm, Taq Polymerase 1U, BSA 0.5 µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 64°C for 20 sec, 72°C 1 minute, Final extension 72°C (5 minutes)

(C) Multiplex PCR assay to identify the admixtures of CM and EA with the primers CM200/EA596/ITS4

A multiplex assay was optimized to detect presence of the species in the mixtures by combining the selected primers verified with singleplexes. Final reaction conditions are given in the **Table 3.4.** The specificity of multiplex assay was also verified. This was done by putting two controls i.e. in control-1 DNA of *C. microphyllus* was mixed with both the primers and in control-2 DNA of *E. alsinoides* was added in place of *C. microphyllus*. Optimizations were done to suppress cross reactivity. Sensitivity of the method was also tested by mixing the DNA templates of both the species in equal ratios from minimum 2 ng to maximum 100 ng as in **section 3.2.71 (A).** The developed multiplex PCR method was further validated by amplifying DNA isolated from the simulated blended material to mimic the mixing in market. Dried and powdered material of plants species were mixed in various ratios. DNA was isolated from each mixture and subjected to amplification with developed multiplex PCR assay. PCR products were analyzed using agarose gel electrophoresis.

Table 3.4 Multiplex PCR reaction for identification of *C. microphyllus* and *E. aslinoides* in mixture

Reactants Concentration	Cycling Parameters
1X Taq buffer, 2.50 mM $MgCl_2$, 200 µm dNTP, FP (CM 200) 20 pm, FP (EA 596) 2 pm, RP (ITS4) 10 pm, Taq Polymerase 1U, BSA 0.5 µg/µl	94°C (10 min), 35 cycles of 94 °C for 1 minute, 60°C for 20 sec, 72°C 1 minute, Final extension 72°C (5 minutes)

3.2.7.2 Development of PCR assays for Terminalia species

Similar to *C. microphyllus* and *E. alsinoides* individual reactions were developed for identification *T. arjuna* bark and its adulterants. No consistent polymorphic site was determined between ITS sequence of *T. arjuna* and *T. tomentosa*, so PCR assay were developed only for *T. bellirica* and *T. chebula* along with *T. arjuna*. Bioinformatically validated primers were taken forward for development of assays. Amplifiability, cross reactivity and sensitivity was also checked for the primers.

(A) Identification of *T. arjuna* with identified specific primers

Two different primer sets were experimentally tested for *T. arjuna*. The optimum annealing temperature of each primer set was identified by amplifying the *T. arjuna* DNA on different temperatures. Each PCR component was optimized. The other three species were also amplified following the same reaction conditions to check the cross reactivity of the primers and the assay **(Table 3.5)**. Sensitivity of the each assay was tested using a range of DNA concentrations as in **section 3.2.7.1 (A).**

Table 3.5 PCR reaction for identification of *T. arjuna*

Reactants Concentration	Cycling Parameters
1X Taq buffer, 2.50 mM $MgCl_2$, 200 µm dNTP, FP (TAF517) 10 and RP (ITS4) 10 pm each , Taq Polymerase 1U, BSA 0.5µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 60°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)
1X Taq buffer, 2.50 mM $MgCl_2$, 200 µm dNTP, FP (TAF517) and RP 20 (TAR 452) pm each , Taq Polymerase 1U, BSA 0.5µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 60°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)

(B) Identification of *T. bellirica*

At the first step optimum annealing temperature was identified and a PCR assay was developed at 60°C for specific identification of *T. bellirica* bark. Cross reactivity was inhibited with suitable optimizations. Sensitivity assessment was done **(Table 3.6).**

Table 3.6 PCR reaction for identification of *T. bellirica*

Reactants Concentration	Cycling Parameters
1X Taq buffer, 2.50 mM $MgCl_2$, 200 µm dNTP, FP and RP 5 pm each, Taq Polymerase 1U, BSA 0.5µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 60°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)

(C) Identification of *T. chebula*

Two individual primer combinations was check experimentally for identification of *T. chebula*. Similar to other PCR assays, optimum annealing temperatures were identified, each PCR component was optimized in order to check cross reactivity and sensitivity testing was performed. Final reactions are given in the **Table 3.7.**

Table 3.7 PCR reaction for identification of *T. chebula*

Reactants Concentration	Cycling Parameters
1X Taq buffer, 2.50 mM $MgCl_2$, 200µm dNTP, FP (TCF686) and RP (ITS 4) 10 pm each , Taq Polymerase 1U, BSA 0.5µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 58°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)
1X Taq buffer, 2.00 mM $MgCl_2$, 200 µm dNTP, FP (TAF244) and RP (ITS4) 5pm each, Taq Polymerase 1U, BSA 0.5µg/µl	94°C (10 min), 30 cycles of 94 °C for 1 minute, 60°C for 45 sec, 72°C 1 minute, Final extension 72°C (5 minutes)

(D) Multiplex assay for the simultaneous detection of *T. arjuna, T. bellirica* and *T. chebula* using primers TAF517/TBF150/TCF244 and ITS4

A multiplex PCR method was developed combining primers selected and tested in species specific singleplex reactions **(Table 3.8).** Appropriate controls were used to prove the specificity of the primers as well as the assay. Total six controls were put to check the cross reactivity of the primers. The first three control tube was contained all the primers and DNA of any one species in a single tubes. In the second control tube set of three tubes had primers of all the three species

and DNA of combining any two species as *T. arjuna* with *T. bellirica* in one tube and with *T. chebula* in second tube. The third tube had DNA of *T. bellirica* with *T. chebula*. In the seventh reaction all the primers were mixed with DNA of each species combined which was considered as test tube. Sensitivity was checked by combining DNA of all the three species and mixing them in equal ratio. In this case validation of the assay was performed by mixing DNA of each species in varying ratios.

Table 3.8 Multiplex PCR reaction for identification of *Terminalia* species in mixture

Reactants Concentration	**Cycling Parameters**
0.75X Taq buffer, 2.50 mM $MgCl_2$, 400 µm dNTP, 20 pm TA517, 1.5 pm TBF150, 10 pm TCF244, 15 pm ITS4, Taq Polymerase 2U, BSA 0.5 µg/µl, 5% DMSO	94°C (10 min), 35 cycles of 94 °C for 1 minute, 50°C for 45 sec, 65°C 1 minute, Final extension 65°C (5 minutes)

3.2.8 Agarose gel electrophoresis for PCR products analysis

PCR amplicons generated in each reaction were analyzed on 1.8 to 2 % agarose gel electrophoresis. 2% gel was prepared as in **section 3.2.2.3 (A)** and ran in 0.5X TAE buffer. Gel was visualized in UV and documentation was done. In each gel size of the amplicons was determined using 50 bp DNA gene ruler.