CHAPTER - II

MATERIALS AND METHODS

CHAPTER - 2

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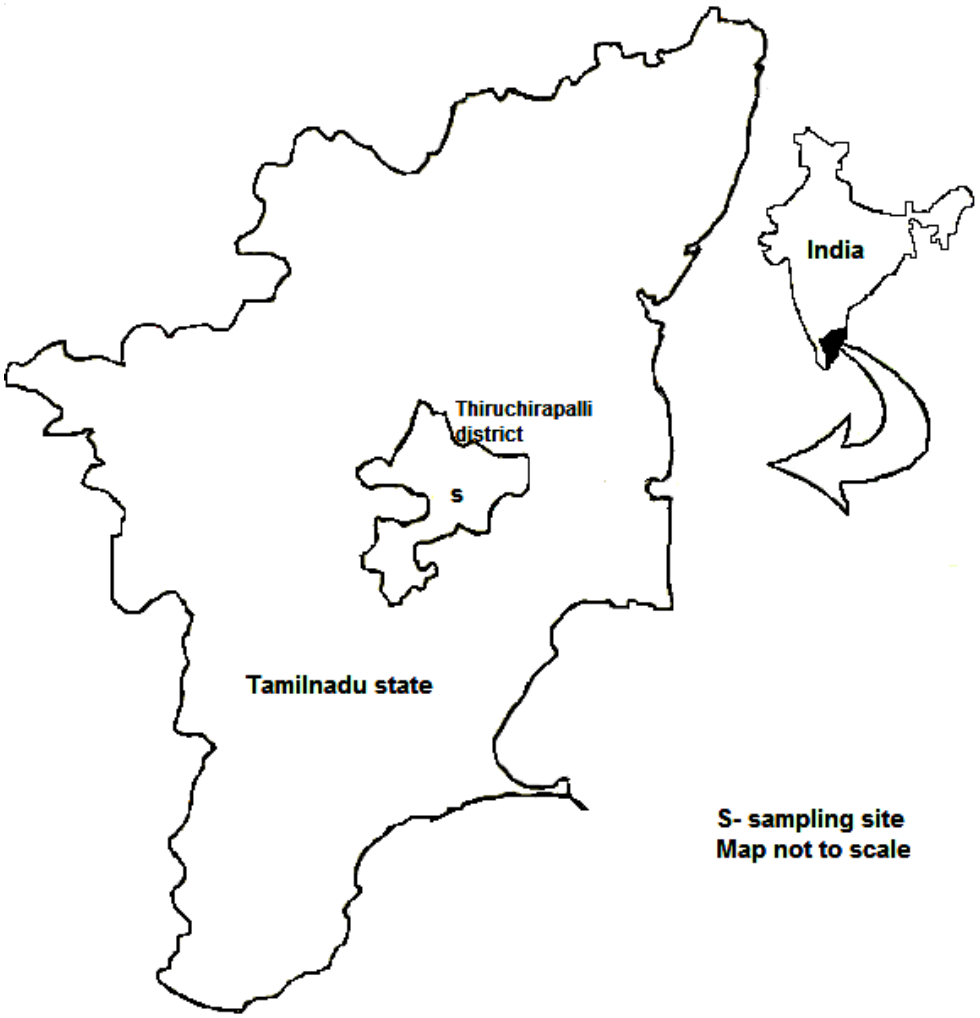
2.1. Study Area

The selected plant material collected from Rettamalai in Tiruchirappalli. Tamil Nadu. Rettamali is one of the sacred hills in Trichy. The Rettamalai hill is located in Karumandabam. The latitude and longitude of Rettamalai stands between 10.791222 & 78.659848. It was collected during February 2014 at 10.00 a.m.



Map of Thiruchirapalli district, Tamilnadu, showing sampling site

2.2. Herbarium preparation for selected plant specimen

The specimens (the specimen with leaves and flowers) were trimmed and immersed in denatured spirit and pressed in wooden press. The specimens were pressed and dried using a herbarium press and old newspapers. A concentrated solution 2% of mercuric chloride dissolved in ethanol was used for poisoning. Dried specimens were mounted on herbarium sheets of standard size using an adhesive.

2.3. Identification of Plant specimen

The plant was identified and authenticated (Accession number-BSI/SRC/5/23/2014-15/Tech/538) in Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu, India.

2.4. Taxonomical classification of *Barleria longiflora* L.f (Plate 1)

- **Kingdom - Plantae**
- **Phylum - Angiosperms**
- **Class - Dicotyledonous**
- **Order - Scrophulariales**
- **Family - Acanthaceae**
- **Genus - *Barleria***
- **Species - *longiflora* L.f**

2.5. Morphology

Barleria longiflora L.f is a unarmed shrub to 1.5(2.5) m. Leaves elliptic ovate, 3-6 × 2-5 cm, pubescent above, appressed-tomentose below, base truncate to rounded, margin ciliate, apex acute, lateral nerves ca, 6 pairs; petiole to 1.5 cm. flowers 3-5, in 1-sided racemes; bracts linear-lanceolate, 2 × 0.3 cm.

Calyx-lobes 4, scarious with age; outer 2 ovate, 2 × 1.5 cm, subacute; inner ones elliptic- ovate, 6 × 4 mm, apiculate. Corolla white, 2 cm across, salver-form; tube 5 cm long; lobes 5, ovate, 1cm, subacute. Stamens 2 to 1 cm; anthers 3 mm. ovary obovoid, 3 mm, 4-ovuled; style 2 cm. Capsule elliptic-oblong, 1.5 × 0.6 cm; Seeds appressed-hairy. Common. Peninsular India. Flowering: October-December.

2.6. Preliminary phytochemical analysis

Plants have been used as an substitute medicine to promote human health and endurance in many regions of the world since prehistoric times (Palanichamy *et al.*, 2018). Plants are the great sources of medicines, especially in traditional system of medicine, which are useful in the treatment of various diseases. (Pattar *et al.*, 2011). Plants are made up of secondary metabolites which are formed as products of primary metabolism and produced for defense against predators. Examples of such metabolites are tannins, flavonoids and alkaloids; they are known to be the brain behind the healing potentials of plants (Jeremiah Oshiomame Unuofin, Gloria Aderonke Otunola, 2017).

Secondary metabolites are generally not important for the growth and reproduction of organisms, but they play an important role in pharmaceutical field (Susanto *et al.*, 2017). Phytochemical tests were carried out to know about the qualitative existence of secondary metabolites in them (Thite *et al.*, 2013). Preliminary screening of phytochemicals is a valuable step, in the detection of the bioactive principles present in medicinal plants and subsequently may lead to drug discovery and development (Watal *et al.*, 2014).

Most of the research reports, acanthaceae family members has a novel and medicinal important phytochemical compounds in its (Nidhi and Kumar, 2013), (Yadav *et al.*, 2012), (Sharma *et al.*, 2014). Not only this, there was no previous work done in

this plant. Present study reveals the evaluation of phytochemical analysis in different solvents like petroleum ether, chloroform and ethanol extract of *Barleria longiflora* L.f.

2.6.1. Preparation and selection of plant extract

To remove the soil particles and adhered debris the leaf, stem and root were detached and washed clearly in running tap water. The plant part like leaf, stem and root were shade dried separately and ground well into powder and extracted by using hot continuous extraction technique in a soxhlet extractor using solvent like petroleum ether, chloroform and ethanol until the extracts were colorless in the siphon tube. The extracts were concentrated and dried under vacuum.

The three different solvents (Petroleum ether, chloroform and ethanol) were taken and preliminary phytochemical analysis was done by using the standard procedure (Velmurugan and Anand., 2016). To identify the presence of some secondary metabolites using the following tests.

2.6.1.1.Test for Alkaloids:

Dragendorff's test: 1 ml of plant extract were taken, 1 ml of Dradendorff's reagent was added and mixed well. A dark orange or orange red precipitates indicate the presences of alkaloids.

2.6.1.2.Test for Flavonoids:

Shinoda test: To 1ml of the extract, add 8 - 10 drops of concentrate HCl and a pinch of magnesium powder or filing. Boil for 10 to 15 minutes and cool. A red coloration indicates the presence of flavonoids.

2.6.1.3.Test for Steroids:

Libermann Burchard test: To 0.5 ml of the extract, add 2ml of acetic anhydride and 2ml of concentrate H2SO4 along the sides of the tube. The formation of green colour indicates the presence of steroids.

2.6.1.4.Test for Terpenoids:

Salkowski test: To 5ml of the extract, add 2ml of chloroform and 3ml of concentrated H2SO4. At the interface of the two liquids a yellow ring formed and turned reddish brown colour after two minutes.

2.6.1.5.Test for Tannins:

Modified Prussian blue test: To 1ml of the extract, add 1ml of 0.008M potassium ferricyanide and 1ml of 0.02M FeCl3 in 0.1 M HCl. Appearance of blue colour indicates the presence of tannins.

2.6.1.6. Test for Saponins:

Forth test: About 2g of the powdered sample is boiled with 20ml of distilled water in a water bath and filter. 10 ml of the filtrate is mixed with 5 ml of distilled water and shake vigorously for a stable persistent.

2.7. Phytochemical analysis by GCMS methods

The phytochemical analysis of the medicinal plants are also important and have commercial interest in both research institutes and pharmaceuticals companies for the manufacturing of the new drugs for treatment of various diseases. (Wyson *et al.*, 2016). Several phytochemical screening studies have been carried out in different part of the world by using GC-MS (Janakiraman *et al.*, 2012). These phytochemical compounds are considered biologically and pharmacologically important (Casuga *et al.*, 2016). In addition to this, the result of the GC-MS profile can be used as pharmacognostical tool

for the identification of plants (Janakiraman *et al.*, 2012). GC-MS analysis is the first step towards understanding the nature of active principles in the medicinal plant and this type of study will be helpful for further detailed study in line with the biochemical and phytochemicals (Oluwasegun Victor, 2015).

GC-MS analysis was carried out to detect the phytoconstituents present in the ethanolic extract of the leaves, stem and root of *Barleria longiflora* L.f. However, there has been no information available on phytochemical components and biological activity in the whole plant ethanol extract of *Barleria longiflora* L.f.

2.7.1. Ethanol Extraction

10 g of the plant material like leaf, stem and root were shade dried separately and grind well into powder and extracted by using hot continuous extraction technique in a soxhlet extractor using solvent ethanol until the extracts were colorless in the siphon tube. The extract were concentrated and dried under vacuum. Ethanolic extract of different parts of *B. longiflora* was subjected to GC-MS analysis. (Bhalodia and Shukla, 2011).

2.7.2. Analysis of the sample extract

Equipment Details:

- Column BR-5MS (5% Diphenyl / 95% Dimethyl poly siloxane), 30m x 0.25mm ID x 0.25µm df
- Equipment Scion 436-GC Bruker
- Carrier gas 1ml per min, Split50:1
- Detector TQ Quadrupole Mass Spectrometer
- Software MS Work Station 8
- Sample injected 2µl

Oven temperature Programme

- 80° C hold for 2 min
- Up to 160° C at the rate of 20 ° C/min-No hold
- Up to 285 ° C at the rate of 5° C / min- 5 min hold
- Injector temperature 280° C
- Total GC running time36 min

MS Programme

- Library used NIST Version-11
- Inlet line temperature 280° C
- Source temperature 250 ° C
- Electron energy 70 eV
- Mass scan (m/z) 50-500
- Solvent Delay 0- 3.5 min
- Total MS running time36 min

2.7.3. Identification of compounds

With the comparison of average peak area to the total areas the relative percentage amount of each component was calculated. The detection employed by using the National Institute of Standards and Technology (NIST) library ver.2.0 (2005). Based on Dr. Duke's phytochemical and ethnobotanical databases which are created by Dr. Jim Duke of the Agricultural Research Service/USDA the prediction of biological activity of compounds was. The GCMS Interpretation was conducted using the database of NIST library having more than 62,000 patterns. The unknown compound's spectrum was compared with known compound's spectrum stored in the NIST library ver. 2.0. The

name, molecular weight and molecular formula of components of the test materials were ascertained.

2.8. Antimicrobial activity

Medicinal plants are always recognized as rich source of antimicrobial agents and are widely used by different countries for medicinal purposes as they are powerful and potent sources of drugs (Gupta and Kumar, 2017). Infectious diseases are one of the major problems in developing as well as developed countries. Traditional medicinal plants are widely used to treat the microbial disease due to their rich source of antimicrobial activity (Hemalatha *et al.*, 2013). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases (Rojas *et al.*, 2003). Plants have a broad variety of antimicrobial agents which are extensively used as herbal drugs against different microbes. Antimicrobial susceptibility testing against pathogenic microorganisms is the most significant task of clinical microbiology laboratory. A variety of antibiotics (tetracycline, terramycin, ampicillin) has also been isolated from different medicinal plants (Ullah *et al.*, 2016). To the best of our knowledge, similar studies have not been carried out on the *Barleria longiflora* L.f.

2.8.1. Preparation and selection of plant extract

The collected plant material were shade dried, coarsely powdered and extracted by using hot continuous extraction technique in a soxhlet extractor using solvent ethanol until the extracts were colorless in the siphon tube. The extracts were concentrated and dried under vacuum (Bhalodia and Shukla, 2011).

2.8.2. Test microorganisms

The following bacteria and fungi were used for screening the antimicrobial activity. Gram Positive bacteria such as *Staphylococcus aureus* (NCIM 2079), *Bacillus subtilis* (NCIM 2063). Gram negative bacteria such as *Proteus vulgaris* (NCIM 2027), *Klebsiella aerogenes* (NCIM 2098) and fungi such as *Candida albicans* (NCIM 3102), *Aspergillus niger* (NCIM 1054) were utilized for the study. The test microorganisms were attained from National Chemical Laboratory Pune (NCL) in India and sustained by periodical sub culturing on nutrient agar and sabouraud dextrose agar medium for bacteria and fungi respectively.

2.8.3. Antibacterial and Antifungal activity

The antibacterial and antifungal activity analyses were carried out by disc diffusion technique (Madhumitha and Saral, 2011). The bacterial strains were inoculated into nutrient broth and incubated at 37 ^{0}c for 18 hours. The standardized inoculum about 0.1 ml was inoculated on Muller Hinton agar (Hi media) uniformly. The sterile disc (Watt man No. 2 of 6 mm diameter) was placed at equal interval on uniformly inoculated plate and a standard disc Ciprofloxacin 5 µg/disc was also placed by aseptic technique. The test sample about 100 µl was loaded to the sterile disc by using aseptic precautions. The plates were incubated at 37 ^{0} C for 24 hours.

The fungal strains were inoculated were brought to the active phase by sub culturing in Sabouraud Dextrose Broth and incubated at room temperature for 4 days. The standardized inoculum about 0.1 ml was inoculated on Sabouraud Dextrose Agar uniformly. The sterile disc (Watt man No. 2 of 6 mm diameter) was placed at equal interval on uniformly inoculated plate and a standard disc Nystatin 100 units/disc was also placed by aseptic technique. The test sample about 100 µl was loaded to the sterile disc by using aseptic precautions. The plates were incubated at room temperature for 2

to 4 days. At the end of incubation, inhibition zones formed around the disc. The diameter of the inhibition zones observed and its value noted (in mm).

2.8.4. Preparation of Muller Hinton Agar (plating medium)

This medium is recommended for the disc diffusion method of antimicrobial susceptibility testing of bacteria (BAUER *et al.*, 1966). When enriched with blood (chocolate), it can be used for Neisseria and Haemophilus species as well.

Beef extract	300 g
Peptone	17.5 g
Starch	1.5 g
Agar	17 g

1) Weigh and suspend the ingredients in 1000 ml of cold distilled water, heat to boiling.
2) Adjust PH to 7.4
3) Sterilize by autoclaving (121 ^{0} c for 10 minutes)
4) Cool to 50 ^{0} c before pouring. For preparing chocolate blood agar plates, read the procedure given earlier.

2.8.5. Preparation of Nutrient Broth (Sub culturing medium)

Nutrient broth is made from commercial meat extract. It is used to cultivate those bacteria which are not nutritionally fastidious.

Peptone	5 g
Beef extract	3 g
Sodium chloride	8 g
Distilled water	1000 ml

1) Weigh out all in the ingredients in an Erlenmeyer flask (2000 ml) or any other suitable container that can stand heating.
2) Dissolve by heating and constant stirring.
3) When cool adjust the pH to 7.4 to 7.6.
4) Distribute in tubes, bottles or flasks and sterilize by autoclaving at 121 ^{0} c for 15 minutes.

2.8.6. Preparation of Sabouraud Dextrose Agar (Plating medium)

This is the most useful selective medium for the culture of mycotic agents, particularly the filamentous moulds. With the addition of antibiotics (chloramphenicol or cycloheximide, or a combination of pencillin and streptomycin), growth of bacterial contaminants can be prevented.

Ingredients

Dextrose	40 g
Peptone	10 g
Agar	15 g
Distilled water	1000 ml

1) Dissolve agar in 1000 ml of distilled water by heating.
2) While hot , add peptone and dextrose
3) Boil gently until dissolved.
4) Adjust the PH 6.0.
5) Dispense into culture tubes (20-ml) with cotton plugs or caps.
6) Sterilize by autoclaving (121^{0} c for 15 minutes).
7) Cool the culture medium in slants, or when the temperature of the medium reaches 50^{0} c, pour in sterilized plates.

2.8.7. Preparation of Sabouraud Dextrose Broth (sub culturing medium)

Ingredients

Dextrose	40 g
Peptone	10 g
Distilled water	1000 ml

1) Dissolve the ingredients with gentle heating and stirring.
2) Dispense in 10 ml amounts in culture tubes.
3) Autoclave (121 ^{0} c for 10 minutes).

2.9. Anticancer activity

Cervical cancer is the second most common gynecological malignant tumor seriously harmful to the health of women, remains a leading cause of cancer-related death for women in developing countries (Zhang *et al.*, 2016). Cervical cancer kills over a quarter of a million women worldwide on an annual basis, with 80% of these deaths occurring in developing countries. (Pollack *et al.*, 2006). Having severe side-effects with conventional chemotherapy, alternate drugs and therapies are actively being investigated. There is a need for innovative drug discovery and design as existing cancer therapies are costly and not readily available. (Tiloke *et al.*, 2018). The large amount of scientific research has been reported on plants as a natural source of treatment agents for cervical cancer, it is currently scattered across various publications. (Wang *et al.*, 2013). Plant-based drugs with potent anticancer effects should add to the efforts to find a cheap drug with limited clinical side effects. Keeping this very purpose in mind, an attempt has been made in this review to explore the potential of plant extracts or constituents known to exhibit antitumorigenic activity or exert cytotoxic effect in human cervical carcinoma cells. (Kma, 2013). Plants are reservoirs for novel chemical entities and provide a promising line for research on cancer. Phytochemicals are selective in their functions and

acts specifically on tumor cells without affecting normal cells. Phytochemicals are considered suitable candidates for anticancer drug development due to their pleiotropic actions on target events with multiple manners. Many phytochemicals and their derived analogs have been identified as potential candidates for anticancer therapy. (Iqbal *et al.*, 2017). Plant derived agents are being used for the treatment of cancer. Several anticancer agents including taxol, vinblas-tine, vincristine, the camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world. (Shoeb, 2006b).

2.9.1. Cell Line

From National Centre for cell science which is in Pune the human cervical cancer cell line (HeLa) was attained and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were kept at 37^0C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passage weekly, and the culture medium was changed twice a week.

2.9.2. Cell Treatment Procedure

To make a single cell suspensions the monolayer cells were detached with trypsin-ethylenediaminetetra acetic acid (EDTA). And the viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well. And incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. With the serial concentrations of the test samples the cells were treated after 24 hrs. They were initially dissolved or dispersed in dimethyl sulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. For the result

of required final sample concentration, 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium. Following sample addition, the plates were incubated for an additional 48 h at 37^0C, 5% CO_2, 95% air and 100% relative humidity. The medium holding without samples were served as control. And triplicate was maintained for all concentrations.

2.9.3. MTT Assay

The compound MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the produced amount of formazan is directly proportional to the number of viable cells. After 48 h of keeping in incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined by the following formula: Percentage cell inhibition = 100 Abs (Sample) / Abs (Control) x 100 (Monks *et al.*, 1991).

2.9.4. Statistical Analysis

Nonlinear regression graph was plotted between % cell inhibition and log10 concentration and IC50 was determined using GraphPad Prism software.

2.10. DNA barcoding

Traditionally, taxonomic identification has relied upon morphological characters. In the last two decades, molecular tools based on DNA sequences of short standardized gene fragments, termed DNA barcodes. (Fišer Pečnikar and Buzan, 2014). DNA

barcoding is a widely used molecular-based system, which can identify biological specimens, and is used for the identification of both raw materials and processed food. (Galimberti *et al.*, 2013). DNA barcoding is currently gaining popularity due to its simplicity and high accuracy as compared to the complexity and subjective biases associated with morphology-based identification of taxa. The standard chloroplast DNA barcode for land plants recommended by the Consortium for the Barcode of Life (CBOL) plant working group needs to be evaluated for a wide range of plant species. (Bafeel *et al.*, 2012). The use of DNA barcodes, which are short gene sequences taken from a standardized portion of the genome and used to identify species, is entering a new phase of application as more and more investigations employ these genetic markers to address questions relating to the ecology and evolution of natural systems. (Kress *et al.*, 2014a).

2.10.1. Tissue sampling and storage

Young leaves of the respective species were collected in sterile Ziploc bags and stored at -20 °C until further use.

2.10.2. DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel)

About 100 mg of the tissue is homogenized using liquid nitrogen and the powdered tissue was transferred to a microcentrifuge tube. 400 µl of buffer PL1 was added and vortexed for 1 minute. 10 µl of RNase A solution was added and inverted to mix. For ten minutes at 65^{o}C the homogenate was incubated. Then the lysate was transferred to a nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid was collected and the filter was discarded. Four hundred and fifty microlitres of buffer PC was added and mixed well. The solution was transferred to a nucleospin plant II column, centrifuged for 1 minute and the flow through liquid was discarded. Four hundred microlitre buffer PW1 was added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid was discarded. Then 700 µl PW2 was

added, centrifuged at 11000 x g and flow through liquid was discarded. In order to dry the silica membrane 200 µl of PW2 was added and centrifuged at 11000 x g for 2 minutes. The column was transferred to a new 1.7 ml tube and 50 µl of buffer PE was added and incubated at 65^{o}C for 5 minutes. The column was then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4^{o}C (Osathanunkul *et al.*, 2016).

2.10.3. Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. At 75V electrophoresis was performed with 0.5X TBE as electrophoresis buffer until bromophenol dye front has migrated to the bottom of the gel. In a UV transilluminator (Genei) the gels were pictured and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 7).

2.10.4. PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	**Primer Name**	**Direction**	**Sequence (5’ → 3’)**
matK	390f	Forward	CGATCTATTCATTCAATATTTC
	1326r	Reverse	TCTAGCACACGAAAGTCGAAGT
*rbc*L	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724_rev	Reverse	GTAAAATCAAGTCCACCRCG

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

2.10.5. PCR amplification profile

matK

98 ºC	-	30 sec	
98 ºC	-	5 sec	} 40 cycles
50 ºC	-	10 sec	
72 ºC	-	15 sec	
72 ºC	-	60 sec	
4 ºC	-	∞	

rbcL

98 ºC	-	30 sec	
98 ºC	-	5 sec	} 40 cycles
60 ºC	-	10 sec	
72 ºC	-	15 sec	
72 ºC	-	60 sec	
4 ºC	-	∞	

2.10.6. Agarose Gel electrophoresis of PCR products

In 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide the PCR products were checked. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and

the image was captured under UV light using gel documentation system (Bio-Rad) (Figure 2).

2.10.7. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes namely, exonuclease I and shrimp alkaline phosphatase (SAP), in a specially formulated buffer for the removal of dNTPs and unwanted primers from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37^{o}C for 15 minutes followed by enzyme inactivation at 80^{o}C for 15 minutes.

2.10.8. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96^{o}C for 2 minutes followed by 30 cycles at 96^{o}C for 30 sec, 50^{o}C for 40 sec and 60^{o}C for 4 minutes for all the primers.

2.10.9. Post Sequencing PCR Clean up

The master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction was made and 12µl of master mix I was added to each reaction containing 10µl of reaction contents and are properly mixed.

Master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction was made. 52 µl of master mix II was added to each reaction. Contents are mixed by inverting. It was incubate at room temperature for 30 minutes. The content was spinned at 14,000 rpm for 30 minutes.

The supernatant was decanted and added 100 µl of 70% ethanol. It was spinned at 14,000 rpm for 20 minutes. The supernatant was decanted and washed 70% ethanol. Finally the supernatant was decanted and it was air dried.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

2.10.10. Sequences

Sequence for matK

CCTTTTTAGAGGACAATTTTTCACATTTAAATCTTCTATTAGATATACTAATACCCCACT
CTGTCCATGTGGAAATCTTGGTTCAAACTCTTCGCTGTTGCTTAAAAGATGCCTCTTCTT
TGCATTTATTACGATTTTTTCTCAACGAGAATTGTAATTGGAATACTCTTAGTAGTATTA
GGCCAAAGAAAGCTGCTTCCTCTTTTTCAAAAATAAATCAAAGATTATTCTTATTCTTA
TATAATTCTCATGTATGGGAATATGAATCCATTTTCTTATTTTTACGTAAGCAATCTTCT
CATTTCCGGTCAACATCTTCTGGAGTTTTTCTTGAACGAATCCATTTCTATGGAAAAATA
GAACGTCTTGTGAACGTCTTAGTTAAGGGTTTTCAGGTGAACCTATGGTTGGTGAAGGA
GCCTTGCATGTATTCTATTAGGTATCAAAGAAGATTCATCTTGGCTTCAAAAGGGATGT
CACTTTTCATGAATAAATGGAAATCTTACCTTGTCACTTTTTGGCAAGGGCATTTTTCGC
TGTGGTTTCATCCAAGAAGGATTTATAGAACCCAATTATCCAATCATTTCCTTGAATTTT
TGGGCTATCTTTCAAGCGTGAGGATCAAACCCTTGGTCGTACGGACCCAAATTCTGGAA

AATGCATTTCCAATCAATAATGCTATTAAGAAGTTTGATACCCTTATTCCAATTATTCCT
CTGATTGCATCGTTGGCTAAAGCGAAATTTTGTAACGTAATAGGGCATCCTATTAGTAA
GCCGGTTTGGGCTGATTTATCAGATTCTAATATTATTGACCGATTTGGGCGTATATGCA
GAAATCTTTCTCATTATCATAGCGGATCTTCTACAAAAAAGAGTTTGTATCGAATAAAA
TATAT

Sequence for rbcL

AAGTGTTGGATTCAAAGCGGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTGAAT
ACGAAACCAAGGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCCGGAGT
TCCGCCTGAAGAAGCAGGGGCAGCGGTAGCTGCCGAATCTTCCACTGGTACATGGACA
ACCGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGGCGATGCTACCACA
TCGAGCCCGTTGCTGGCGAAACAGATCAATATATCTGTTATGTAGCTTACCCTTTAGAC
CTTTTTGAAGAAGGTTCTGTTACCAACATGTTTACTTCCATTGTAGGAAATGTATTTGGA
TTCAAAGCCCTGCGTGCTCTACGTCTGGAAGATCTGCGAATCCCTACTGCTTATATTAA
AACTTTCCAAGGTCCGCCTCATGGGATCCAAAGTGAGAGAGATAAATTGAACAAATAT
GGTCGTCCTCTGCTGGGATGTACTATTAAACCTAAATTGGGGTTATCTGCTAAAAACTA
TGGTAGAGCATGTTATGAATGTCTTCGTGGTGGACTTGATTTTACGAAAGATGATGAGA
ACGTGAACTCCCAGCCATTTATGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCA
ATTTATAAATCACAGGCCGAAACAGGCGAAATCAAAGGGCATTACTTGAAT

2.10.11. Sequence Analysis

The sequence quality was checked using sequence scanner software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

PLATE 1

Habit of *Barleria longiflora* L.f

Flower of *Barleria longiflora* L.f