CHAPTER - III

RESULT AND DISCUSSION

CHAPTER - 3

RESULT AND DISCUSSION

3.1. Phytochemical Studies

The phytochemical screening of petroleum ether, chloroform and ethanol extracts of *B. longiflora* are presented in Table1. Medicinally active metabolites were present in *B. longiflora*. The leaf, stem and root were extracted with different solvent and the results showed the presence and absence of alkaloids, flavonoids, steroids, terpenoids, tannins and saponins in all the extracts. The natural products (like alkaloids, flavonoids, steroids, terpenoids, tannins and saponins) still play a very important role in medicine. Plant extract has a potential application as natural medicine and to treat diseases as well as the microbiological safety of the human health (Kaminidevi *et al.*, 2014).

Medicinal plants and the parts of medical plants denote a rich source of antibacterial agents. Plants are the source of many powerful and potent drugs and are used medicinally in different countries. Different traditional medicinal plants extracts have been tested. Several reports have shown the efficiency of traditional herbs against microorganisms (Velmurugan and Anand., 2016). Medicinal plants contain some natural products which perform definite physiological action on the human body and these bioactive substances include alkaloids, flavonoids, steroids, terpenoids, tannins and saponins (Thite *et al.*, 2013).

Owing to the significance in the above context, such preliminary phytochemical screening of plants is the need of the hour in order to discover and develop novel therapeutic agents with improved efficacy. Towards the biological activities of medicinal plants these secondary metabolites contribute significantly towards the biological

activities of medicinal plants. For example hypoglycemic, anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anti-carcinogenic, antimalarial, anticholinergic activities etc. (Watal *et al.*, 2014). Hence, phytochemical screening serves as the first early step in predicting the kinds of potential active compounds from plants (Santhiya *et al.*, 2016).

Alkaloids, as reported by Elekwa *et al.* in 2008, have been seen to interfere with cell division which makes them an important plant part to possibly be used as remedy in the treatment of cancer. Noble (1990) corroborated that alkaloids are widely used as cancer chemotherapeutic agent. Cardiac glycosides have been reported to be effective in congestive heart failure (Itoandon *et al.*, 2012). From the phytochemical screening it is observed that the petroleum ether and chloroform extracts gave a negative result with Dragendorff's which indicate the absence of alkaloids in both extracts but it is present in ethanolic extract of leaf. Whereas in stem petroleum ether showed negative result but chloroform and ethanolic extract showed the positive result. And also when the same test was done for the root of the *Barleria longiflora* L.f petroleum ether and chloroform gave negative result and ethanolic extract gave a positive result.

Flavonoids are hydroxylated phenolic substances which are known to be manufactured by plants in response to microbial infection. And they have been found to be antimicrobial components against wide array of microorganisms (Thite *et al.*, 2013). Flavonoids are referred to as bioflavonoid, are polyphenol antioxidants found naturally in plants. Flavonoids are also known as vitamin P or natural biological modifiers. (Joseph *et al.*, 2013). In the test of flavonoids petroleum ether showed the negative result which indicates the absence of flavonoids but chloroform and ethanolic extract showed the positive result which indicates the presence of flavonoids in the leaf sample. Whereas in the stem sample petroleum ether and ethanolic extract showed positive result and

chloroform showed negative result. For the root the result was obtained as we got for the leaf sample.

Steroids have been reported to have antibacterial properties and they are very important compounds especially due to their relationship with compounds such as sex hormones (Samejo *et al.*, 2013). When it comes for the test of steroids petroleum ether and ethanolic extract of leaf showed positive result with libermann burchard test by indicating the presence of steroids. But chloroform extract of leaf showed the absence of steroids. In the same test in the stem samples petroleum ether and chloroform showed the absence of the steroids whereas ethanolic extract showed positive result.

It is well known that terpenoids possess antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, antiinflammatory and immunomodulatory properties. In storing agriculture products terpenoids can be used as protective substances.(Watal *et al.*, 2014) The growth of many fungi, yeasts, bacteria and viruses was inhibited by tannins. tannins are attributed for analgesic and anti-inflammatory activities (Savithramma *et al.*, 2011).

In the salkowski test, petroleum ether gave negative result indicating the absence of terpenoids. Chloroform and ethanolic extract of leaf sample showed the presence of terpenoids. In the stem samples showed the previous mentioned results. But in the case of root sample petroleum ether and chloroform showed the negative result but ethanolic extract of root sample showed the positive result.

Tannins are polyphenolic compounds found in most plants and are generally thought to function as chemical defenses against pathogens and herbivores. In recent work points to a number of beneficial effects of tannins at moderate concentrations, including being a natural detergent to reduce bloat and a suitable substitute for synthetic

anthelmintics and having antimicrobial activity and antioxidant activity (Sung *et al.*, 2012). When the attempt was done to find the tannins presence petroleum ether and chloroform showed the absence of tannins with modified prussian blue test in leaf sample. But ethanolic extract showed the presence of tannins. In the case of stem petroleum ether showed the negative result whereas the chloroform and ethanolic extract showed the positive results.

Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning. However, they are also having beneficial pharmacological effects including anti-inflammatory, anti-parasitic and anti-viral properties (Qadir *et al.*, 2015). As a detergents, piscicides and molluscicides saponins have been extensively used. In addition to their industrial applications as foaming and surface active agents and also have beneficial health effects.(Savithramma *et al.*, 2011). Saponins are helpful in lowering cholesterol, as antioxidant and anti-inflammatory agents (Samejo *et al.*, 2013). The leaf extract of petroleum ether, chloroform and ethanolic extract showed the negative result with forth test which indicates the absence of saponins. The stem extract of petroleum ether and chloroform gave negative result of saponins but the ethanolic extract showed the positive result. And the saponins were found to be absent in petroleum ether and ethanolic extract of root sample and present in chloroform extract of root sample.

3.2. Gas chromatography and mass spectroscopy

The plant is endowed with various chemical components such as flavonoids, alkaloids, triterpenes, steroids, polysaccharides, tannins, saponins, proteins, amino acids, volatile oils, and free reducing sugars (Vidhya and Udayakumar, 2015). The phytoconstituents are the major important compounds which are responsible for the

medicinal properties of the shrubs. Hence medicinal plants could be a potential source for nutraceuticals. Phenols and flavonoids which are the substances of phytochemicals become the major important substances responsible for the medicinal value of the plants including antioxidant, anticancer, antimicrobial activities, etc. (Rajeswari and Krishnakumari, 2010).

The identification of the phytocompounds was carried out based on the retention time and molecular formula. The name of identified compounds in the different parts of *B. longiflora* with their retention time (RT), molecular formula (MF), molecular weight (MW) and peak area percentage were represented in Tables 2, 3 and 4.

The leaf extract of *B. longiflora* showed twenty three phytocomponds such as Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]- (10.05%), Caryophyllene (9.00%), E)-β-Famesene (1.13%), Humulene (2.24%), β-copaene (1.52%), α-Guaiene (1.10%), Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-(0.28%), Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-, [1R-(1α,3α,4β)]- (0.54%), γ-Elemene (0.67%), Caryophyllene oxide (0.41%), α-acorenol (0.83%), 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (1.81%), Tetradecanoic acid (0.28%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.20%), Phytol, acetate (0.34%), n-Hexadecanoic acid (5.64%), Phytol (6.97%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (36.13%), Squalene (2.84%), 2,6,10,14-Hexadecatetraenoic acid, 3,7,11,15-tetramethyl-, methyl ester, (E,E,E)- (0.86%), dl-α-Tocopherol (3.32%), p-tert-Octylresorcinol (9.21%), β-Sitosterol (3.60%). (Table 2).

The sixteen different phytocomponents were identified in stem extract of *B. longiflora* such as 2-Cyclohexen-1-one, 4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl-, [R-

[R*,R*-(E)]]- (0.90%), α-acorenol (0.52%), 2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol (2.71%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (9.03%), cis-9-Tetradecen-1-ol (1.59%), Phytol, acetate (3.09%), n-Hexadecanoic acid (8.36%), Hexadecanoic acid, ethyl ester (0.60%), Phytol (7.78%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (4.05%), Diisooctyl phthalate (1.98%), Lupeol (2.41%), Squalene (44.94%), 2,6,10,14-Hexadecatetraenoic acid, 3,7,11,15- tetramethyl-, methyl ester, (E,E,E)- (6.65%), Stigmasterol (4.79%), β-Sitosterol (0.59%). (Table 3).

Twenty five phytocompounds such as 5-Hydroxymethylfurfural (0.02%), Ethanone, 1-(2-hydroxy-5-methylphenyl)- (1.26%), Phenol, 4-ethyl-2-methoxy- (0.21%), Benzoic acid, 4- hydroxy-3-methoxy- (1.57%), Benzeneacetic acid, 4-hydroxy-3-methoxy-, methyl ester (0.22%), 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (3.20%), Tetradecanoic acid (0.82%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.14%), Phytol, acetate (1.29%), n- Hexadecanoic acid (14.18%), Phytol (0.81%), 9,12-Octadecadienoic acid (Z,Z)- (5.35%), Octadecanoic acid (0.08%), 17-Octadecynoic acid (0.42%), 1-Eicosanol (1.57%), Eicosanoic acid (0.28%), Z,E-2,13-Octadecadien-1-ol (0.24%), Diisooctyl phthalate (2.41%), Squalene (1.09%), Octadecane, 3-ethyl-5-(2-ethylbutyl)- (1.35%), Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate (0.34%), Cholestan-3-ol, 2-methylene-, (3β,5α)- (0.17%), Ergost-5-en-3-ol, (3β)- (4.14%), Stigmasterol (26.97%), β-Sitosterol (30.87%) were identified in the root extract of *B. longiflora* (Table 4).

The GC-MS analyses showed that the presence of Twenty three different phytocompounds in ethanolic extract of leaf of *B. longiflora*. The highest peak area of 36.13% for 9,12,15-Octadecatrienoic acid,(z,z,z)- was identified in leaf of *B.longiflora*. The ethanolic stem extract of *B. longiflora* showed that the presence of sixteen different phytocompounds. The highest peak area of 44.94% for Squalene. The root extract of

B.longiflora showed that the presence of Twenty five different bioactive compounds. The root of *B.longiflora* showed β-Sitosterol with the highest peak area of 30.87%.

In this study, the phytocompounds were identified in the ethanol extract of *B. longiflora* by GC-MS analysis and predicted their biological activities based on Dr. Duke‟ s Phytochemical and Ethnobotanical Databases created by Dr. Jim Duke of the Agricultural Research Service/USDA (Table 5, 6, 7).

3.3. Antimicrobial activity

The occurrence of antibacterial and antifungal substances in higher plants is well established (Singh and Jain, 2011). Plants have delivered a source of inspiration for novel drug compounds as plants derived medicines have made major contribution towards human health (Singh *et al.*, 2011). Phytomedicines can be used for the treatment of diseases as is done in case of Unani and Ayurvedic system of medicines of it can be the base for the development of a medicine, a natural blueprint for the development of a drug (Parekh and Chanda, 2007). Successive separation of bioactive compounds from plant material is chiefly dependent on the type of solvent used in the extraction procedure.

Barleria longiflora L.f stem on showed maximum zone of inhibition was against Gram positive bacteria *Staphylococcus aureus* (30mm) and Root on minimum against Gram negative bacteria *Bacillus subtilis* (8mm) (Table 8, Figure 4 & Plate 2). *Barleria longiflora* L.f root showed maximum antifungal activity towards *Candida albicans* (28mm) and leaf and stem showed minimum antifungal activity (26mm). The stem and leaf showed maximum antifungal activity towards *Aspergillus niger* (28mm) and minimum activity 25mm showed in root (Table 9, Figure 5 & Plate 2).

The results showed significant activity of ethanolic extract of *Barleria longiflora* L.f and suggesting its use as natural antimicrobial agent. The result of present study

indicated that ethanolic extract of *Barleria longiflora* L.f shows potent antibacterial and antifungal activity.

3.4. Anticancer activity

The results for cell growth inhibition by the extract against HeLa cell lines for various concentrations is shown in table 10. In the present study HeLa cells showed growth inhibition in a dose dependent manner when treated with *Barleria longiflora* L.f extract (Leaf, stem and root) at concentrations ranging from 12.5µg to 200µg. The percentage of dead cells for each concentration was found to be 0.24, 0.06, 3.06, 20.4, and 54.4. The 50% cytotoxic effect (IC_{50}) of *Barleria longiflora* L.f extract was found to be 184.3µg / ml (Table 10 and Figure 6 and Plate 3). The utility of cell lines acquired from tumors allows the investigation of tumor cells in a simplified and controlled environment (Donipati and Sreeramulu, 2015). There are specific advantages and disadvantages to exploit cancer cell lines over animal models. These then dictate the nature of the experiment that can be organized (Arya *et al.*, 2011). In the last few decades, studies with cell lines can serve as an initial screen for agents that might regulate drug resistance (Geetha and Santhy, 2013). *Barleria longiflora* L.f has been widely studied for its antioxidant activity. Now-a-days, after this antioxidant was found to offer protection against the occurrence of cancer activity (Geetha and Santhy, 2013). In the present study the HeLa cell lines are used as a model for studying cervical cancer. Several mechanisms of action were detected in HeLa cells. The IC_{50} of extract on cell line less than 100 µg / ml is categorized as a potential cytotoxic substance (Karthikeyan et al. 2017). In the present study, ethanol extract of *Barleria longiflora* L.f was found to be moderately cytotoxic towards human HeLa in MTT assay and the concentration required for 50% cell death was found to be 184.3 µg / ml. Hence present study shows the efficacy

of *Barleria longiflora* L.f for the antiproliferation of HeLa cells thus suggesting protection against cervical cancer.

3.5. DNA barcoding

The plant *Barleria longiflora* L.f belongs to the family Acanthaceae were collected from Rettamalai hill. Approximately 1000bp DNA was isolated during the quality check through agarose gel electrophoresis method. The gene amplification adopted in the present study yielded enough quantity of DNA for further sequence analysis of matK and rbcL. The matK gene had 898bp and the rbcL gene had 697bp and the same sequence had been deposited in the Gene Bank with the Accession Number KR861702.1 and KR861703.1. This study provided an opportunity to utilize matK and rbcL sequence for identification of this species in future. During the BLAST search no sequence matches for this gene could be identified from databases on plant. Hence it may be concluded that the matK and rbcL sequence of *B. longiflora* was a first record for Gene Bank.

The results of Neighbor-Joining method (NJ) analysis of 898bp fragment of the matK and 697bp fragment of the rbcL gene belonged to *B. longiflora* with the nine sequences obtained through BLAST showed different branch lengths in the Phenogram. Maximum identical sequences were not available for *B. longiflora* in this NJ analysis in both matK and rbcL gene. (Fig. 1 & 2).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and M, 1987). The optimal tree with the sum of branch length of matK = 22.53425277 and rbcL = 1069.42380238 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the

Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 70 positions in the final dataset in matK and 40 in rbcL. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

A detailed estimate of evolutionary divergence of rbcL and matK sequence of with their similar sequences through BLAST search is provided in Table 11 and 12. The results on the distance analysis indicated that the overall average for all species *B. longiflora* in matK was 5.905 and rbcL was 323.245. The maximum evolutionary distance observed between *Barleria longiflora* L.f and *Acanthus ebracteatus* was 11.165 in matK and 543.525 in rbcL (Table 11 and 12). Therefore, it is concluded that rbcL sequence of *B. longiflora* may be used for the identification of this species reported from any part of the world through BLAST analysis if the identical sequences are submitted to Gene Bank in future.