6. DISCUSSION

Medicinal plants and their extracts are well known worldwide for alleviating ailments of humankind. However, only a small proportion of plant species has been thoroughly investigated for their medicinal properties and undoubtedly there are many novel biologically active compounds yet to be discovered (Frame et al., 1998; Heinrich and Gibbons, 2001). Compounds derived from natural products are still proving to be invaluable medicines for human application, in spite of the recent interest in drug discovery by molecular modeling, combinatorial chemistry, and other synthetic chemistry methods.

Medicinal plants are increasingly gaining acceptance probably due to the increasing inefficacy of modern drugs used against many infections such as typhoid fever, gonorrhea, tuberculosis etc. and the increase in resistance by several bacteria to various antibiotics (Van den Bogaard et al., 2000; Smolinski et al., 2003). Initially plant-based medicines were distributed in the form of crude drugs such as tinctures, teas, powders, and other herbal formulations and served as an archetype to develop more effective and less toxic medicine. With the advancement in organic chemistry, medicinal chemists have started preparing analogs from these drug prototypes to provide safer and more potent drugs instead of using it directly as drugs or drug prototypes to cure human disease.

The isolation of morphine from opium in 19th century has become the stepping stone isolation of drugs from plants (Butler, 2004). The reason behind targeting natural products in drug discovery is chemical diversity, the effects of
evolutionary pressure to create biologically active molecule and the structural similarity of protein targets across many species (Harvey, 2007).

The present study attempts to identify compounds from plant sources, which can serve as ideal candidates for drug development against tuberculosis (TB). Although TB is a curable disease, due to the increase in MDR and XDR-TB strains, the potencies of the currently available drugs are on the decline. WHO plans to eliminate tuberculosis by 2050, but the different resistant forms of tuberculosis pose the biggest challenge to achieve this objective. Newton et al. (2000) reviewed the antimycobacterial activity of natural products of which 123 were from the plant species. Although India is a country with rich biodiversity and traditional knowledge of medicine, it was noted that only limited work has been done for screening of natural products to test their antimycobacterial activity. The present study mainly focuses on identifying potent medicinal plants based on traditional usage and literature survey from in and around Virudhunagar district against clinical isolates of *M. tuberculosis*.

Virudhunagar district of Tamil Nadu, India comprises of very different vegetation belts like dry lands to thick forest. The latitude and longitude of Virudhunagar district is 9.5842°N and 77.9582°E respectively. Subbu and Prabha in 2009 studied the medicinal plant diversity of Virudhunagar District and they have reported 175 species belonging to 61 families which are being used by villagers for primary healthcare to cure various ailments. A similar study was conducted by Mutheeswaran et al. (2011) to document the medical plants used by traditional healers in the same district. Totally 227 species were identified as medicinal plants used for preparing 611 formulations for treating
36 illnesses including cough, fever, malaria etc. Based on these documentations, 28 medicinal plants were selected from in and around Virudhunagar district for this study.

The extraction of different phyto-constituents from a crude plant material depends primarily on the solvent selection. For the preliminary extraction of plant parts; chloroform, hexane, methylene chloride and methanol are commonly used. Chloroform and its impurities produce quaternary salts and other products with some compounds. As methanol and ethanol are polar and alcoholic solvents, they can efficiently penetrate cell membrane and extract high amounts of endocellular components. Chloroform and hexane due to their low polarity are usually employed as defatting solvents to extract extracellular contents, unwanted waxes, oils and large portion of impurities from the crude extracts making the separation further easier (Mukherjee, 2002). Hence in this study, hexane and methanol were used for preliminary extraction of selected medicinal plants.

The conventional soxhlet extraction has been used worldwide as the main extraction technique for a number of decades, surpassing the performance of other leaching alternatives. It is being used as an efficiency reference for the comparison of its new counterparts. By this method the most possible sample extraction can be achieved (Ahmad et al., 2010).

Ahmad et al. (2010) studied the optimization of soxhlet extraction and separation of chemical constituents in the medicinal plant of *Herba leonuri*. The study showed that methanol extraction produced higher yield compared to hexane when performed at three extraction times viz. 6h, 9h and 12h. The highest yield obtained with methanol was 14.18%; while that of n-hexane was
7.25%. For methanol extraction, the percentage yield increased up to 14.18% with increasing length of extraction period whereas for hexane extraction, the percentage yield was not consistent with increasing length of extraction period. This correlates with our finding that methanol extracted a larger percentage of the dry mass material than n-hexane. The highest yield obtained with methanol was 11.72% whereas in n-hexane; the yield was only 6.31%.

Development of new drugs to combat *M. tuberculosis* has been hampered by non-availability of a simple method for screening large number of compounds. The luciferase reporter phage assay has great promise in this regard, since the assay uses very small drug quantities and the results are obtained within 3 days. In comparison to BACTEC and Alamar blue broth dilution methods, the LRP assay has the great advantage of being simpler, faster, less laborious and amenable to high throughput screening and generates numerical results that can be helpful in interpretation (Sivaramkrishnan et al., 2013). In LRP assay dimethyl sulfoxide (DMSO) is used as solvent vehicle to dissolve the plant extracts and it helps in improving the substrate transportation into the cell also.

Moreover, screening methods using LJ medium or 7H11 agar require large amounts of crude extracts or purified compounds to be incorporated in the media. It also requires about 3 or more weeks of incubation to produce results. Even though molecular based detection are rapid and provide bedside solution to the problem, they have limitations in certain areas like cost, dedicated infrastructure, expertise and need for region specific re-standardization or evaluation. Shawer et al. (1997) screened 480 plant extracts using both luciferase and colorimetric broth dilution assays, where an
overall agreement of 99 percent between these two methods has been
described. Natural products from various natural sources like plants,
actinomycetes, fungi (Prabu Seenivasan et al., 2006; Ignacimuthu and
Shanmugam 2010; Radhakrishnan et al., 2010; Molly et al., 2012) and
derivatives from natural products (Sivakumar et al., 2007; Kumar et al., 2011)
were screened for antimycobacterial activity by LRP assay.

Muthuswamy et al., in 2013 screened methanol extracts of 32 medicinal
plants selected from Western Ghats of south India for antimycobacterial
activity using LRP assay and reported that the methanolic extracts of the Ruta
graevalens exhibited good antimycobacterial activity against all the tested
strains of M. tuberculosis H37Rv (76.60%), MDR, (87.25%) and drug sensitive
strain (94.32 %) at a concentration of 100µg/ml. Similarly Prabuseenivasan, in
2006 screened 21 plant essential oils against M. tuberculosis by LRP assay.
Among these plants, the essential oil of Cinnamomum zylonicum was
identified as potent antimycobacterial agent thus bringing out the efficiency of
LRP assay allowing large scale screening and supports the search for new
classes of anti-tuberculosis agents urgently needed to control this disease.

In the present study 13 out of 28 plants showed high with the cut off at
> 50% RLU Reduction) antimycobacterial activity against one or more strains
at 500 µg/ml concentration. Among these, promising activity (>80% RLU
reduction) was exhibited by the methanolic extract of Andrographis paniculata
against M. tuberculosis H37RV, drug sensitive, MDR clinical strains of M.
tuberculosis. Since A. paniculata showed maximum % reduction of RLU when
compared with other plants it was identified as a potent plant having anti-TB
activity and selected for further investigations.
A. paniculata has been effectively used in traditional Asian medicine for centuries as an immune booster and it is commonly known as “King of bitters”. In Ayurveda system A. paniculata is being used popularly for treatment of various liver disorders (Kapil et al., 1993). In traditional Chinese medicine it is an important “cold property” herb used to get rid of the body heat, fever and to dispel toxins from the body (Deng, 1978). It acts against common cold (Caceres et al., 1997), upper respiratory infections and act as anti-inflammatory. It is beneficial for asthma, bronchitis, jaundice, colic dysentery and as an antidote against poisons of snakes (Shen et al., 2002).

The active compounds from A. paniculata have been proved to exhibit various bioactivities including anti-inflammatory (Chiou et al., 2000; Shen et al., 2002; Levita et al., 2010), anti-cancer, anti-tumour (Satyanarayana et al., 2004; Zhou et al., 2010), hepato-protective against various inducers (Handa and Sharma, 1990; Visen et al., 1993), immunomodulator (Wang et al., 2010), anti-oxidant (Sheeja et al., 2006; Akowuah et al., 2008), anti-diabetic (Zhang, et al, 2009 anti-microbial (Shen et al., 2006), and anti-viral (Calabrese et al., 2000).

The antimicrobial potencies of A. paniculata against both bacterial (Stapylocococcus aureus, Klebsiella pneumonia, Bacillus subtilis and Escherichia coli) and fungal species (Aspergillus niger and Aspergillus flavus). The result showed that the methanol extracts of the plant inhibited the growth of majority of the tested isolates (Divya et al., 2011). Zhang and Tan (2000) studied the ethanolic extract of whole plant it was found to be having antihyperglycaemic property and reduce the oxidative stress in diabetic rats. Gabrielian et al. (2002) has reported that A. paniculata extract showed positive
effects in treating acute upper respiratory tract infections and relieving the inflammatory symptoms of sinusitis.

A number of researchers have reviewed and demonstrated that *A. paniculata* and its compounds exhibit various biological activities. Plant components usually have multiple beneficial effects, often acting beyond a symptomatic treatment of disease. For example, Yoxen (1983) studied *Hydrastis canadensis* (organe root) exhibit antimicrobial activity and also increases blood supply to the spleen to release mediating compounds. Although *A. paniculata* exhibits various biological activities the scientific basis for the use of *A. paniculata* intreating clinical isolates of *M. tuberculosis* is still unclear. Recently, Tawde *et al.*, in 2012 reported that ethanolic extract of *A. paniculata* showed a potent activity against *M. tuberculosis* at a MIC concentration of 2.5 mg/ml using agar diffusion method.

Biochemical assays are very important aspects in pharmacognostic evaluation of medicinal plants (Harborne, 1973; Choudhury *et al.*, 2009). Preliminary qualitative tests are useful in the detection of bioactive principles and consequently may lead to drug discovery and development (Mallikharjuna *et al.*, 2007). Copp (2003) studied plant-derived antimycobacterial products and reported that it covered a large proportion of phytochemicals such as lipids/fatty acids, simple aromatics, phenolics and acetogenic quinones, peptides, alkaloids, terpenes (monoterpenoids, diterpenes, sesquiterpenes, sesterterpenes) and steroids. In the present findings the preliminary phytochemical study of active methanolic extract of *A. paniculata* indicates that the major chemical constituents are alkaloids, flavonoids, terpenoids, phenols, saponins and glycosides. Similar observations were reported by Siripong *et al.*
(1992) that *A. paniculata* extract contains three major groups of phytochemical compounds, namely, terpenes, flavonoids and stigmasterols.

The bottleneck in natural products chemistry depends on separation and purification of target compounds from complex mixtures and their structure elucidation. Chromatography is one of the most useful means of separating mixtures of compounds that includes TLC, LC, GC and HPLC (Vogler and Setzer, 2006). The bioassay guided fractionation procedure used to identify bioactive natural products is often perceived as rate limiting and resource intensive (Butler, 2004). In the present work, TLC system was used followed by fractionation using column chromatography to deduce the components of the extract. Among Six fractions, fraction four (FR IV) was found to be active against *M. tuberculosis*. Purity of the active compound was confirmed with HPTLC. Syed *et al.* (2013) justified the use of HPTLC in checking the purity of the isolated compound and it also offers a better resolution and estimation of active constituents with reasonable accuracy in a shorter time. Similar studies were done by Chen *et al.* to isolate Fourteen diterpenoids from the 85% ethanol extract of *A. paniculata* by the silica gel, Sephadex LH-20, ODS column chromatography and HPLC, and their structures were identified by the spectral analyses and chemical evidences (Chen *et al.* 2006).

Modern spectroscopic methods have largely revolutionized compound identification and tremendously accelerated the pace at which the compounds are isolated. The identified active compound (FR-IV) was found to be Andrographolide by means of different spectral analysis (NMR, IR, and UV).
Andrographolide is a colourless, bitter bicyclic diterpenoid constituent of A. paniculata.

Andrographolide contains an α-alkyldene γ- butyrolactone moiety, two olefin bonds at C-8(C-17) and C-12(C-13), and three hydroxyls at C-3, C-19 and C-14 (Levita et al., 2010). Sule et al. (2012) investigated the antifungal activity of the whole plant extracts and isolation of active principles from A. paniculata. Structures of compounds were elucidated through spectroscopic techniques and comparisons were made with previously reported data for similar compounds. Bioassay guided extraction from dichloro methanol and methanol showed 3-O-β-d-glucosyl-14-deoxyandrographiside, 14-deoxyandrographolide, and 14-deoxy-11,12-didehydroandrographolide as antifungal compounds. The lowest minimum inhibitory concentration (MIC) of 50 µg/mL and minimum fungicidal concentration (MFC) of 50 µg/mL was exerted by 14-deoxyandrographolide on Microsporum canis.

Chao and Lin (2010) reviewed the biological activities of andrographolide analogues present in A. paniculata. The andrographolide analogues, 14-deoxy-11, 12-didehydroandrographolide is immunostimulatory, anti-infective and anti-atherosclerotic; neoandrographolide is anti-inflammatory, anti-infective and anti-hepatotoxic; 14-deoxyandrographolide is immunomodulatory and anti-atherosclerotic; andrograpanin is both anti-inflammatory and anti-infective; 14-deoxy-14,15-dehydroandrographolide is anti-inflammatory and isoandrographolide, 3,19-isopropylideneandrographolide and 14-acetylandrographolide are tumor suppressive.
Rajagopal et al. (2003) studied the mode of action of andrographolide against human cancer and immune cells in vitro. Cancer cells are directly targeted by the compound by arresting the GO/G1 phase of cell cycle by inhibiting the protein P$_{27}$ and by decreasing the expression of CDK. It also boosts the immune system by enhancing the tumor necrosis factor-alpha (TNF-$\alpha$) production and CD marker expression. Hidalgo et al., (2013) has standardized the A. paniculata extract (30% andrographolide) in clinical trials and showed effectiveness for symptom relief and reduction in serological parameters in patients with Rheumatoid Arthritis. In anti-HIV activity, andrographolide prevents transmission of the virus to other cells and stop the progress of the disease by modifying cellular signal transduction.

In the present study MIC value of the active compound for M. tuberculosis, Andrographolide was determined by LRP assay. The MIC of the compound Andrographolide ranged between 100 to 200$\mu$g/ml for all tested clinical isolates by the LRP method. The absolute concentration method is the widely used Drug susceptibility testing (DST) method especially in resource-limited settings for the diagnosis of drug-resistant TB. Based on this method MIC of active compound andrographolide was found to be between 200 to 400$\mu$g/ml. This finding is corroborated with the findings of Lee and Heifets, 1987. They have studied the MIC of 5 antituberculosis drugs viz. isoniazid, rifampin, streptomycin, ethionamide, and ethambutol by adopting radiometric (BACTEC) broth method and by the agar plate proportion method against seventeen M. tuberculosis strains isolated from patients before treatment. The MIC values of 4 drugs, except streptomycin, were 2 to 4 times lower in 7H12 broth than in 7H11 agar. The broth-determined MIC was at least 2 to 4 times lower than the achievable serum concentrations. The broth determined MIC
are probably much closer to the true MIC values than those determined in agar plates because of the lower degree of absorption and degradation in the liquid medium. In the case of natural compounds, prolonged incubation may reduce the efficacy of the drug.

The MIC values of a few compounds such as allicin isolated from garlic oil (Delaha and Garagusi, 1985), Hypargenin F, from the roots of *Salvia hypargeia* (Ulubelen *et al.*, 1988), triterpenes from *Borrichia frutescens* (Cantrell *et al.*, 1996) were found to be higher than that of Andrographolide for *M. tuberculosis*. In contrast, compounds such as ambroxol, a semi-synthetic derivative of vasicine from the Indian shrub *Adhatoda vasica* (Grange and Snell, 1996) and alkaloids isolated from *Galipea officinalis* (Houghton *et al.*, 1999) etc. exhibited better activity than Andrographolide with lower MIC values.

In order to ensure that the plant based compounds and their derivatives are safe for human consumption, cytotoxicity screening is very essential. Cytotoxicity screening provides an important preliminary data in selecting plant compounds with less side effects for future work. It enables the characterization of the intrinsic toxicity of the plant and the effect of acute over dose (Padmaja *et al.*, 2002). In this study the active compound andrographolide was subjected to MTT assay to check the toxicity level against Vero cell lines. Almost 90% of cell viability was observed in concentration up to 250µg/ml. The LC_{50} value of the active compound was 500µg/ml where there was 50% cell death exhibiting cytotoxic activity. Prakash and Manavalan (2011) studied the acute toxicity of Andrographolide and clearly
demonstrated that andrographolide treated animals were devoid of any toxic sign and indicated that it is safe up to the dose of 2000 mg.kg-1 body weight.

Compared to antimicrobial compounds from microorganisms, plant species have not yet been studied well for their potential as source of antibacterial agents. However some plant extracts and their compounds have been reported to have potent antitycobacterial properties (Newton et al., 2000, Gautam et al., 2007). Bromhexine is a semi-synthetic derivative of the alkaloid vasicine, which is found in *Adhatoda vasica*, an Indian shrub that has long been used as mucolytics. This compound exerts inhibitory effect against *M. tuberculosis* in vitro (Grange and Snell, 1996). The crystalline cinnamic acid which is the oxidised form of cinnamaldehyde is antitubercular and is reported to have been used as injection in phthisis (Nadkarni, 1976).

Andrographolide has been reported to have several targets in human involved in various biological activities such as cancer, inflammatory, diabetes, immune modulators etc. Some of the targets of andrographolide in human are cAMP-dependant protein kinase, Human Abl kinase, PI3 kinase, epidermal growth factor receptor etc. (Sharmila et al., 2013). Andrographolide is reported to interfere with the binding of Nuclear factor kappaB (NFKB) thereby inhibits the expression of COX-2 (Raghavan et al., 2012).

Andrographolide were docked to selected mycobacterial `drug targets from MTBSD and from a recently reported work (Chung et al., 2013). While analysing the interaction of andrographolide with these targets, it was found that Isocitrate dehydrogenase (ICDH) and Aminoglycoside 2'-N-acetyltransferase (AAC) proteins interact with andrographolide with 60.98 and 68.01 GOLD score respectively. AAC protein had been already reported as a
target for kanamycin and its crystal structure complex with kanamycin is available in PDB database. Comparing the docked interaction of andrographolide and crystal structure of kanamycin, it is understood that interactions are found to be very similar with the key residues.

Isocitrate dehydrogenase-1 (ICDH) protein of MTB is 409 amino acid long having isocitrate/isopropylmalate dehydrogenase domain and Isocitrate / isopropylmalate dehydrogenases signature (NYDGDVqSDtvAqgy.GSLGL). ICDHcatalyzes the oxidative decarboxylation of isocitrate using NAD (P)^+ as a co-substrate to form α-ketoglutarate (αKG), CO_2 and NADPH. ICDH protein is located in the branch point between TCA cycle and glycosylate shunt in organisms having glycosylate shunt which is absent in humans. In several organisms, utilization of this shunt is very important for their virulence and persistence (Dunn et al., 2009). In the latent phase of M. tuberculosis, the glycosylate shunt bypasses the two decarboxylative steps of the TCA cycle, allowing organisms to survive under nutrient limiting conditions (McKinney et al., 2000). Thus, ICDH makes an important target for M. tuberculosis and can be used for the development of inhibitors against the glycosylate shunt and thus can act against persistent M. tuberculosis. Exploration of new drug targets for persistent M. tuberculosis is especially important because this phase of the life cycle is non-replicating.

AAC catalyzes the coenzyme A (CoA) - dependent acetylation of the 2' hydroxyl or amino group of a broad spectrum of aminoglycosides. It confers resistance to amino glycosides like kanamycin. The exact physiological function of AAC is not known (Vetting et al., 2002).
With the observations of *in silico* results, both ICDH and AAC are predicted to be targets of andrographolide in *M. tuberculosis*. As andrographolide is having multiple targets in human, several derivatives can be developed by substituting with other side groups in order to make it very specific to *M. tuberculosis*.

These studies indicate a possible role of andrographolide in the therapy of tuberculosis, although further studies are required to determine whether this compound could act synergistically with existing antituberculosis drugs.