INTRODUCTION

1.1. Background

For thousands of years, medicinal plants played an important role in the treatment of diseases and health disorders (Van Wyk and Wink, 2004). World Health Organization (WHO), estimated that 80% of developing countries use medicinal plants for sustainable health and vitality (Trivedi, 2006). Low pricing, absence of side effects, alternate solutions for diseases and disorders, time tested remedies and preventive approaches etc. showed interests for herbal therapies among peoples (Malik et al., 2012).

Threat to medicinal plants are mainly caused by several manmade activities such as, over collection of whole plant, the rate of fruit and unripe fruit collections, mass collection of seeds, harvesting underground parts, poor fruit setting, low seed germination and most importantly habitat destruction of plants. These were the serious cause for their loss in wild population (Singh, 2004; Sher, 2011). Besides several drawbacks, (poor seed setting, decline in seed viability) in conventional propagation of many medicinal plants, a non-conventional and modern method of propagation is required to fulfil the demands of pharmaceutical companies.

1.2. Conservation of medicinal plants

Extensive utilization and increased demand of medicinal plants in different forms of formal markets increased the value of medicinal plants in global levels (Ncube et al., 2011). The raw materials for drug preparations were being traded from harvested plant materials (Mander and Mckenzie, 2005) and as a result
depletion of the population in wild occurred. Even though, conventional cultivation of medicinal plants is a logical conservation strategy, it has challenged to several drawbacks like climate and seasonal variations, water and land availability, slow growth rates, diseases and pests (Pierik, 1987; Arikat et al., 2004).

Micropropagation is a modern biotechnological tool for the production of pathogen free and identical clones for the conservation of selected species (Mousami et al., 2006). Regeneration of plants have been carried out by direct and indirect methods (somatic embryogenesis) (Abdin and Ilah, 2007). The commercial propagation technology is based on growing multiple cells, tissues and organs on defined solid or liquid medium under aseptic and controlled conditions (Rehman et al., 2003). Micropropagation is being achieved successfully from small stem cuttings, axillary buds and also from embryo cells in suspension cultures. Plant regeneration through tissue culture provides an attractive way for utilization of plant genetic resources (and its bioactive compounds) as well as conservation of desired species (Bhojwani and Dantu, 2013).

Micropropagation of plants allows an opening for ethno pharmacological exploitation of cells, tissues, organs or entire plants at their early stages of growth. Use of the modern technology in traditional medicine could offer several advantages by avoiding the collection of wild plant sources, and huge production of pharmacological compounds irrespective of the above-mentioned risk factors (Pierik, 1987; Arikat et al., 2004; Ncube et al., 2011). However, the success of this ideology depends upon the validation of micropropagated plants through pharmacological screening on their suitability for use in traditional medicine (Ncube et al., 2011).
1.3. Molecular analysis of plants

Plant growth regulators and in vitro culture conditions play an important role in increase of biological properties (Dakah et al., 2014). In vitro production of plant secondary metabolites through plant cell suspension cultures have been reported from various medicinal plants (Gantet and Memelink, 2002). Variations in secondary metabolites (phytochemicals) of plants in suspension cultures have influence on the changes in molecular or genetic level and vice-versa (Suman et al., 1999; Yaseen et al., 2009). DNA profiling techniques like RAPD, DNA microarrays etc. serve as suitable high throughput molecular tools for the simultaneous analysis of multiple genes and analysis of gene expression, that are necessary for providing details about regulatory mechanism, biochemical and cellular pathways (Yaseen et al., 2009). It is important to maintain the genetic integrity of in vitro regenerated plants irrespective of the mother plant to uphold the agronomic traits and horticultural necessities (Goel et al., 2009). Chances of somatic variations in the plant culture is influenced under several factors like species, genotypes of donor, type of explants, modification in culture medium, composition, physical culture conditions and period of repeated sub-culturing (Larkin and Srowcroft, 1981; Kishor and Devi, 2009) and the changes may be heritable (Jain, 2001). Sometimes, alteration in DNA methylation is found as a major cause of epigenetic modifications (Rai et al., 2011). Hence, it is valid to appraise whether the in vitro regenerants are genetically identical with mother plant or not.

Recently, molecular markers are playing a significant role in establishing the genetic variability and stability among different plant samples (Rai et al., 2010). Among many PCR based molecular markers, random amplified
polymorphic DNA (RAPD) is commonly considered for the molecular identification of genetic diversity. The RAPD technique has the advantage of being technically simple, quick to perform and requires only small amounts of DNA (Ceasar et al., 2010). Goto et al., (1998) explained the reasons for occurrence of genetic variations, mutations among the in vitro cultures, and it may depend upon factors such as source of explants, duration of regeneration, in vitro culturing and incubation methods. This technique is more appropriate to study the distribution of secondary metabolites (using molecular tools) and to identify their genomic level organization in plants (Kalpana et al., 2004).

1.4. Antimicrobial Activity of medicinal plants

Human diseases particularly those caused by dreadful microorganisms (like bacteria, fungi and virus) cause serious infections. Infectious diseases caused by the microorganisms are responsible for large scale morbidity and mortality worldwide (Ingale and Hivrale, 2010). For example, Staphylococcus aureus causes pyogenic inflammatory diseases, food poisoning, and toxic shock syndrome in humans. It is also known for its opportunistic infections or nosocomial infections. Salmonella typhi readily causes typhoid fever in humans and nearly 20 million peoples died due to this microbial infection (Kidgella et al., 2002). Proteus vulgaris causes urinary tract infections, kidney stone formation and obstruction of urinary tract (Almeida et al., 2013). Pseudomonas aeruginosa is an opportunistic pathogen that commonly infects patients with impaired immune responses (Sarlangue et al., 2006). Fungal pathogens like Aspergillus niger commonly causes otomycosis (fungal ear infections). Mucor species causes skin allergies (Bekada et al., 2008) and Candida albicans are the causal agent of opportunistic infections
in oral, genital and candida onychomycosis (an infection in nail plate) in humans (Ryan and Ray, 2004).

Even though treatment of disease causing microorganisms with modern drugs are rapid, they were having huge drawback as they were causing side effects or raised resistance of the bacterial pathogens against those modern drugs (Kumar et al., 2014). Various medicinal plants have been used in daily life to treat many diseases all over the world. Hence, plant based herbal remedies for treatment of several infections and need to control those pathogens without any adverse effects are required (Purohit and Mathur, 1999). Most of the drugs today obtained from natural sources or semi synthetic derivatives of natural products and used in the traditional systems of medicine.

According to WHO, medicinal plants would be the best source to obtain a variety of drugs (Santos et al., 1995), and approximately 20% of the known plants were subjected to biological tests and sustainable number of new antibiotics were introduced into the market (Sukanya et al., 2009; Bairu et al., 2010). Concurrently, the characterizations of antimicrobial compounds from medicinal plants have been very challenging to the researchers (Kuzel et al., 2009). Hence, a systemic screening of plants for the identification of antimicrobial compounds to act on microbial pathogens is needed. Recently, many researchers have focussed on the isolation of potential compounds form micropropagated medicinal plants in order to produce high quality and potential secondary metabolites responsible for the control of microorganisms (Taware et al., 2010). Dakah et al., (2014) reported that antimicrobial compounds from micropropagated plants and callus materials had more potential antimicrobial properties than wild plants.
1.5. Phytochemical nature of medicinal plants

Phytochemicals are the plant secondary metabolites produced during plant metabolisms, which are not involved in the primary metabolic function of the plants. Such metabolic compounds (alkaloids, flavonoids, tannins, phenolics, amino acids, steroids, terpenoids, glycosides etc.) are having higher antimicrobial and antioxidant properties by breaking protein synthesis or by blocking metabolic pathways (Westh, 2004). Presence of polyphenolic contents of plant extracts are reported to have antioxidant and anticancer properties (Duraipandiyen et al., 2006).

The accumulation of phytochemicals in the plant cell cultures have been studied for above three decades and the generated knowledge had helped in realization of using cell cultures for production of desired phytochemicals (Castello et al., 2002). The isolated phytochemicals were purified for their active biological properties and they were used in the pharmaceutical industries in large scale. Due to increased demand of secondary metabolites in the past few decades, lot of efforts have been put for micropropagation of plant cell, tissue and organ culture as alternate method of whole plant propagation and the large scale production of pharmacologically important plant secondary metabolites (Rao and Ravishankar, 2002; Amoo et al., 2012; Dakah et al., 2014).

1.6. Antioxidant and anticancer activity of medicinal plants

Oxidative stress is considered to be the leading cause for several diseases like cellular damage, diabetes, cancer, heart failure and sometimes it induces sudden death (Mattson and Cheng, 2006). Natural antioxidants play a key role in
health maintenance and prevention of the various chronic and degenerative diseases, such as diabetes, carcinogenesis, neurodegeneration and rheumatism, atherosclerosis, cardiac ischemia, cerebral ischemia, DNA damage and ageing (Uddin et al., 2008; Jayasri et al., 2009). Cancer is the second most deadly disease leading to high death rate. Medicinal plants with high antioxidant potential are considered to be anti-carcinogenesis due to presence of phenolic or polyphenol compounds (Liu, 2004).

Antioxidant research is an important one in food and medical field. Oxidative process is the important route in production of free radicals in food, drug and in some living systems which leads to several disorders in human body (Cook and Samman, 1996; Kumpulainen and Salonen, 1999). Free radicals are said to cause adverse effects in immune system of the human body, leading to excessive oxidation of cellular substrates (oxidative stress) and resulting in type II diabetes, neuro-degenerative disease, cardiovascular disease, cancer etc (Pourmorad et al., 2008). Currently, there is huge demand for natural antioxidants in food industry, for replacing the synthetic preservatives used to prevent fat rancidity or colour loss (Adam et al., 2008). In addition, antioxidants are an important group of medicinal compounds used in food additives for inhibiting detrimental changes of easily oxidizable nutrients (Tadhani et al., 2007).

Amongst the antioxidants, polyphenols (such as tannins, flavanones, isoflavones, anthocyanins, resveratrol and ellagic acid etc.) were used in the food and neutraceutical industries (Espin et al., 2007). Phenolic compounds exhibit a significant antioxidant (free-radical scavenging) activity and have been evaluated for their reactivity as electron donating agents. The resulting antioxidant derived
radicals are stable and has high reactivity with other radicals and metal chelation tendencies (Lee et al., 2001; Wojdylo et al., 2007). The antioxidants, which are now being used in markets, such as Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT), have been suspected for causing several diseases including liver damage and carcinogenesis. Therefore, there is an urgent need for development and utilization of more effective antioxidants, derived or obtained from the natural origin (Oktay et al., 2003). Natural antioxidants can protect the human body from free radicals and hold back the progress of rancidity in foods (Gulcin et al., 2002). Antioxidant properties have been studied in several plants for the development of natural antioxidant formulations in the fields of food, medicine and even in cosmetic industries (Miliauskasa et al., 2004; Sajeesh et al., 2011; Dakah et al., 2014).

1.6.1 Anticancer property of medicinal plants

Cancer is an abnormal growth and proliferation of cells and considered as one of the most life threatening diseases, which possess many health hazards around the world with 6 million mortalities every year (Pandey et al., 2012). Cancer is being caused by various factors including physical, chemical, genetic and environmental issues (pollution and radiation), use of tobacco, alcohol and smoking etc. It may be uncontrollable and incurable, and may occur at any age, in any part of the body (Umadevi et al., 2013). The most common types of cancers prevailed among men and women are lung, prostate, colorectal, stomach, breast and cervical cancer (WCR report, 2014).

Among them, breast cancer is one of the leading cancers globally with highest rate of cancer deaths in women. Tissue invasiveness and metastatic spread
of breast cancer cells are liable for most of the morbidity and mortality allied with the disease (Nukumi et al., 2007; Ali et al., 2010). Hence, there is an urgent need to diagnose at an early stage, though early detection of breast cancer have an advantage of hormone therapy, radiotherapy and surgery as remedy, there are huge risk of reoccurrences of malignancy or metastatic cancers (EBCTCG 2005; Ali et al., 2010; Jemal et al., 2011). MCF – 7 is a breast cancer cell line with characteristics of differentiated mammary epithelium, having the ability to process estradiol via cytoplasmic estrogen receptors and capability of forming domes (Lacroix and Leclercq 2004) is selected for present study.

The search for novel anticancer drugs from plant sources started in early 1950’s, the development of potential anticancer drugs such as vinca alkaloids, vincristine and vinblastine laid the strong foundation for development of several plant based drugs (Cai et al., 2004; Cragg and Newman, 2005). Previously, several medicinal plants were screened for the isolation of anticancer compounds (such as podophyllotoxin, vinorelbine, vindesine etc) and they were found successful in treatment of several cancer including Kaposi sarcoma, breast and lung cancers (Lee and Xiao, 2005). Earlier, Paclitaxel analogs have provided a major renewable natural source and were used mostly in breast cancer and Kaposi sarcoma, it was later found to be unproductive in several cases (Cragg and Newman, 2005). An imperative need existing among the treatments for breast cancer without damaging healthy cells and hence the identification of naturally occurring phytocompounds from medicinal plants to combat cancers has become highly sought after in recent decades due to their very low side effects (Sun et al., 2013).
1.7. Separation of bioactive compounds

Separation of pure compounds from plants for the pharmacological applications is a long term process and very tedious. It is necessary to identify an easier methods to separate a pure or single compound (bioactive) from the mixture of thousands of different molecules from the plant crude extracts (Peter, 2004). Various techniques are employed for the separation of the potential bioactive components in the crude extracts of medicinal plants, and they are systematically fractionated to screen their bioactivity. Those sub-fractionated compounds are analysed for their characteristic nature (Cannell, 1998). Chemical profiling of the extracts establish the characteristic chemical nature. Generally, the separation of bioactive compounds from the crude (organic solvents) extracts of medicinal plants is done by chromatographic techniques. TLC and Column chromatography are simple and cheapest method of detection of plant metabolites because of their little equipment, reproducibility and easy run methods (Battu and Reddy, 2009; Devi and Thanagam, 2010; Jothy et al., 2011).

High performance liquid chromatography (HPLC) is however considered as the sensitive and selective detection of even lower amounts of secondary metabolic compounds present in plant materials (Lyne et al., 1976). Hence, HPLC is used as a viable tool for qualitative determination of compounds. In addition, many analytical techniques such as, gas chromatography, column chromatography, and spectrophotometric methods with high resolving powers are also frequently used for quality control and standardization of the isolated compounds (WHO, 1998; Dai and Mumper, 2010; Siddiqui et al., 2011).
Liquid Chromatography coupled with Mass Spectrometry (LC/MS) is an extensive technique for detection of minute components of the plant extracts (He, 2000; Cai et al., 2002). It is known to produce abundant information for structural elucidation of the compounds when the chromatography is coupled with tandem mass spectrometry. Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds present in medicinal plants (Ye et al., 2007).

Gas Chromatography – Mass Spectrometry (GC – MS) is the combination of two analytical methods into a versatile technique for the identification of complex volatile materials. Gas chromatography (GC) effectively separates different constituents of the sample for subsequent analysis and identification by mass spectrometry (MS). Mass spectrometry is used to identify the nature of each component.

The non-chromatographic techniques like Fourier Transform – InfraRed spectroscopy (FT – IR) has proven to be a valuable tool for the characterization and identification of functional groups (chemical bonds) of compounds present in an unknown mixture of plants (Eberhardt et al., 2007; Hazra et al., 2007). FT – IR has been used to obtain the scattering, absorption and emission of diverse particles such as gas, liquid and solids. It also collects wide range of spectral data. Thus, it deliberates a significant advantage over other methods/ techniques used for the detection of narrow range of multiple wavelengths at a time. The spectrum of an unknown compound can be identified by comparison to a library of known compounds (Griffith et al., 2012).
1.8. Need for this study

Due to increased demand of herbal medicines, micropropagation technique used for mass propagation of medicinal plants are widely employed to produce large amounts of drugs and secondary metabolites and the obtained products can be used throughout the year without any disturbance to wild population (Sidhu, 2010). The medicinal potential of the micropropagated plants also need to be tested for their pharmacological applications and its utilization in commercial purposes. Simultaneously, their bio-efficacy has to be tested for confirming their potential for treatment of several diseases caused by pathogenic microorganisms. Hence, the present study is planned in such a way as to explore the biological (antimicrobial, antioxidant and anticancer) properties of wild and micropropagated medicinal plants belonging to Amaranthaceae family i.e. Aerva javanica (Perumpoolai) and Aerva lanata (Sirupoolai).
AIM AND OBJECTIVES

The present work is aimed at comparative analysis of antimicrobial, phytochemical, antioxidant and anticancer potential of wild and micropropagated medicinal plants (A. javanica and A. lanata).

OBJECTIVES

- Micropropagation of A. javanica and A. lanata using shoot-tip and nodal explants.
- RAPD analysis of micropropagated and field grown plants for confirmation of genetic similarity/variation and distribution of secondary metabolites.
- Antimicrobial properties of in vivo, in vitro leaf and callus extracts of plants and test their antibacterial activity of isolated fractions from potential extracts against target organisms.
- To analyse the phytoconstituents of different solvent extracts of plant materials.
- Perform the antioxidant and anticancer (methanol extract) properties of plant extracts.
- Separation and identification of bioactive compounds from the potential extracts using chromatography (preparative TLC and HPLC) and spectral analysis (UV-Spectroscopy, GC – MS, LC – MS and FT – IR).