CHAPTER I
INTRODUCTION

1.1. MEDICINAL PLANTS: A SOURCE FOR DRUG DISCOVERY

Ever since the prehistoric era, nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been derived from natural sources, many of these isolations were based on the uses of the agents in traditional medicine. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and/or reduced toxicity.

The World Health Organization (WHO) estimated that 80% of the earth’s inhabitants rely on traditional medicines for their primary health care need, and most of this therapy involves the use of plant extracts or their active compounds. Secondary plant metabolites have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals in plants. It is well-known that plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases.

Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glucosides.

Phytochemicals such as vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals that have antimicrobial and antioxidant activity (Madhuri and Pandey, 2009).

Free radicals induce Oxidative damage to lipids, proteins and nucleic acids, which eventually cause atherosclerosis, ageing, cancer, diabetes, inflammation, AIDS and several degenerative diseases in humans are well documented (Halliwell et al., 1994; Maxwell et al., 1997). Antioxidants are compounds that show reducing activity.
Table 1.1 Bioactive Phytochemicals in Medicinal Plants

<table>
<thead>
<tr>
<th>Classification</th>
<th>Main groups of compounds</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSA (Non-starch polysaccharides.)</td>
<td>Cellulose, hemicellulose, gums, mucilages, pectins, lignins</td>
<td>Water holding capacity, delay in nutrient absorption, binding toxins and bile acids</td>
</tr>
<tr>
<td>Antibacterial &amp; Antifungal</td>
<td>Terpenoids, alkaloids, phenolics</td>
<td>Inhibitors of micro-organisms, reduce the risk of fungal infection</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Polyphenolic compounds, flavonoids, carotenoids, tocopherols, ascorbic acid</td>
<td>Oxygen free radical quenching, inhibition of lipid peroxidation</td>
</tr>
<tr>
<td>Anticancer</td>
<td>Carotenoids, polyphenols, curcumine, flavonoids</td>
<td>Inhibitors of tumor, inhibited development of lung cancer, anti-metastatic activity</td>
</tr>
<tr>
<td>Detoxifying Agents</td>
<td>Reductive acids, tocopherols, phenols, indoles, aromatic isothiocyanates, coumarins, flavones, carotenoids, retinoids, cyanates, phytosterols</td>
<td>Inhibitors of procarcinogen activation, inducers of drug binding of carcinogens, inhibitors of tumourogenesis</td>
</tr>
<tr>
<td>Other</td>
<td>Alkaloids, terpenoids, volatile flavor compounds, biogenic amines</td>
<td>Neuropharmacological agents, anti-oxidants, cancer chemoprevention</td>
</tr>
</tbody>
</table>

(Saxena et al. 2013)

They protect the components of cells and biomolecules from oxidation by scavenging or donating an electron / hydrogen atom to free radicals / reactive oxygen species (ROS) such as superoxide, hydroxyl, and perox radicals. Antioxidants play many vital functions in a cell and have many beneficial effects when present in foods. They are effective in prevention of degenerative illnesses, such as different types of cancers, cardiovascular and neurological diseases, cataracts, and oxidative stress disfunctions (Stahelin et al., 1989; Riemersma et al., 1991; Ames et al., 1993). Ultimately Antioxidants reduce risk of cancer.

The specific function of many phytochemicals is still unclear; however, a considerable number of studies have shown that they are involved in the interaction of plants/pests/diseases. Antimicrobial screening of plant extracts and phytochemicals, represents a starting point for antimicrobial drug discovery. (Shakeri et al., 2012).

### 1.1.1. Anti-cancer drug discovery

Cancer is one of the most dreaded diseases of the 20th century and spreading further with continuance into the 21st century. Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into the world of medicine. Cancer is formed through a process known as
carcinogenesis. Carcinogenesis is a multistage process by which a normal cell is transformed into a cancerous cell. Transformation involves initiation, typically from DNA damaging agents; promotion, during which cell proliferation is increased; and progression, involving additional genetic alterations. Chemoprevention strategies target each of these steps including anti-initiation strategies (e.g., DNA repair, detoxification, free radical scavenging and carcinogen metabolism) and anti-promotion/anti-progression strategies (e.g., free radical scavenging, proliferation suppression, differentiation induction, immunity enhancement, inflammation reduction, increase in apoptosis, altered gene expression and decrease in angiogenesis).

Recently, a greater emphasis has been given towards the search on complementary and alternative medicine that deals with cancer management. Several studies have been conducted on herbs under a multitude of ethnobotanical grounds. Data have been collected on about 3000 plants, those of which possess anticancer properties and have subsequently been used as potent anticancer drugs (Balachandran and Govindarajan, 2004).

Drug discovery from medicinal plants has played an important role in the treatment of cancer. Indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer. The majority of available anticancer drugs are natural products or derived from natural products (Balunas and Kinghorn, 2005).

A recently approved plant-derived anticancer drug in EU is paclitaxel, more commonly known by its trademark name, Taxol. Taxol, a complete terpene-based molecule is derived from the Pacific yew (Taxus brevifolia). Extracts of Pacific yew were found to stop the growth of several mouse tumours. The plant was traditional used for lung ailments and not specifically for cancers or tumours. Should the traditional usage been strictly followed this could have overshadowed the usage of paclitaxel for treatment of tumours. Paclitaxol is now being used to treat lung cancers that do not respond to other therapies. Thus, although a particular plant species is ethnobotanically used for a particular condition that does not rule out other usage towards different conditions. Ethnobotanical usage can only serve as a lead to screen the plant species for biological activity.

For prevention of cancer the first FDA approved chemopreventive agent was tamoxifen, for reducing the risk of breast cancer. This agent was found to reduce the breast cancer incidence by 50% in women at high risk. With tamoxifen, there is an increased risk of serious side effects such as uterine cancer, blood clots, ocular disturbances, hypercalcemia, and stroke. This clearly indicates the need for agents, which are safe and
Introduction

efficacious in preventing cancer. Diet derived natural products will be potential candidates for this purpose (Anand et al., 2008).

Several classes of anticancer drugs have been developed and many of them are of from natural origin. Natural products have been the mainstay of cancer chemotherapy for the past 30 years (Mann, 2002). It is well established that plants have been a useful source of clinically relevant antitumor compounds (Cragg et al., 1994). Plants have long history of use in the treatment of cancer.

1.2 Taxonomy of selected medicinal plants

1.2.1 Dandaliyo thor (Pencil cactus)

Kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked): Rosids

Order: Malpighiales

Family: Euphorbiaceae

Genus: Euphorbia

Species: E. tirucalli

Euphorbia Tirucalli plant:

Habit: A much branched and ever green

Leaves: small and slender, up to 12 x 1.5 mm, rarely seen, as they fall very early..

Inflorescence: One-sided racemes or corymbs.

Flowers: yellow, inconspicuous, and carried in clusters at the apex of the short branches

Fruit: tripartite capsules (divided into three parts), about 12 mm in diameter, longitudinally very slightly lobed, short-stalked (8 mm), pale green, with a pink tinge and conspicuously pubescent (clothed with soft hairs).

Seeds: oval, about 4 x 3 mm, glabrous, smooth and dark brown with a white line around the small white caruncle (fleshy wart near the hilum of the seed). This spineless species contains large quantities of latex which is freely exuded by the twigs and branchlets at the slightest injury.

Flowering and Fruiting time: September – December
1.2.2 **Katakiyo (Cadaba fruticosa)**

**Kingdom:** Plantae

*(unranked):* Angiosperms

*(unranked):* Eudicots

*(unranked):* Rosids

**Order:** Brassicales

**Family:** Capparaceae

**Genus:** Cadaba

**Species:** *C. fruticosa*

**Habit:** A straggling much branched shrub.

**Stem:** Erect, branched, cylindrical and solid, with smooth stems.

**Leaves:** Simple, entire, elliptic-oblong, obtuse, mucronate, base rounded.

**Inflorescence:** One-sided racemes or corymbs.

**Flowers:** Greenish-white, bracteate, sepals 4, unequal, ovate, acute, petals spatulate with narrow claws, disk funnel-shaped, stamens 4, excreted half-way on the gynophore, ovary 1 celled, raised on the gynophore, style absent.

**Fruit:** Dehiscent, cylindric, and slightly moniliform.

**Seeds:** Kidney-shaped, immersed in orange-red arillate pulp.

**Flowering and Fruiting time:** November - March.

---

1.2.3 **Koyali Grass.**

**Kingdom:** Plantae

*(unranked):* Angiosperms

*(unranked):* Monocots

*(unranked):* Commelinids

**Order:** Poales

\Family: Poaceae

**Genus:** Panicum

**Species:** *P. maximus*

**Habit:** A long-lived (i.e. perennial) grass with short underground stems (i.e. rhizomes) forming tufted clumps and aboveground stems that are usually upright (i.e. erect) in nature. Guinea grass (*Panicum maximum var. maximum*) grows up to 3 m tall, but is usually about 2 m in height.
Stems and Leaves: The stems may be branched and vary from being hairless (i.e. glabrous) to quite hairy (i.e. pilose). The leaves consist of a sheath, which encloses the stem, and a spreading leaf blade. These long and narrow leaves are very large (15-100 cm long and 5-35 mm wide) with entire margins and pointed tips (i.e. acuminate apices).

Flowers and Fruit: The seed-heads (i.e. inflorescences) are loosely branched (i.e. open panicles) and 12-60 cm long. Their lowest branches are arranged in a cluster (i.e. whorl), while the branches further up the seed-head are variously arranged. The flower spikelets are small (3-4.5 mm long) and oval (i.e. elliptic) or oblong in shape. They are generally green in colour, but occasionally may be purplish or reddish in colour. These flower spikelets are hairless and have only one fertile floret. They are shed from the seed-head entire when mature.

1.2.4 Hanuman phal (Annona Muricata)

Kingdom: Plantae

Clade: Angiosperms

Clade: Magnoliids

Order: Magnoliidae

Family: Annonaceae

Genus: Annona

Species: A. muricata

Habit: Evergreen tree.

Leaves: oblong to oval, 8 centimetres (3.1 in) to 16 centimetres (6.3 in) long and 3 centimetres (1.2 in) to 7 centimetres (2.8 in) wide. glossy dark green with no hairs above, and paler and minutely hairy to no hairs below. The leaf stalks are 4 millimetres (0.16 in) to 13 millimetres (0.51 in) long and without hairs.

Flowers: Flower stalks (peduncles) are 2 millimetres (0.079 in) to 5 millimetres (0.20 in) long and woody. They appear opposite from the leaves or as an extra from near the leaf stalk, each with one or two flowers, occasionally a third. Stalks for the individual flowers (pedicels) are stout and woody, minutely hairy to hairless and 15 millimetres (0.59 in) to 20 millimetres (0.79 in) with small bractlets nearer to the base which are densely hairy.

Fruit: The fruits are dark green and prickly. They are ovoid and can be up to 30 centimetres (12 in) long, with a moderately firm texture. Their flesh is juicy, acid, whitish and aromatic.

Flowering and Fruiting time: April- July
1.3. Strategies used in the determination of bioactive compounds in medicinal plants

Medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and are still widely used and have considerable importance in international trade. Plant preparations have a very special characteristic that distinguishes them from chemical drugs: a single plant may contain a great number of bioactive phytocompounds and a combination of plants even more. This complexity is one of the most important challenges to phytoscientists attempting to identify a single bioactive phytocompound or chemical group in the enormous universe that comprises a single crude extract.

Current strategies for choosing candidate plant species for isolation of bioactive components are based on ethnobotany, chemical ecology and plant anatomy.

1.3.1. Extraction of phytochemicals

Structural diversity of the phytochemicals affects physicochemical behaviour such as solubility and partitioning making optimization of the recovery system difficult in all but the simplest cases. Extraction method and solvent choice are generally critical as well as extraction time and temperature (Asima et al., 2003; Caldwell et al., 2005; Hinneburg and Neubert, 2005). No single solvent will provide optimum recovery of all phytochemicals or even a limited range of phytochemicals they exhibit considerable diversity in terms of acidity as well as polarity ranging from hydrophobic and hydrophilic in character. The range of physicochemical behaviors should be considered when determining sample handling strategies (Bidlack et al., 2000).

Once the material has been dried to constant weight, it is ground up to smaller particles and extracted usually using a gradient solvent extraction. Numerous extraction techniques are available which include: Cold extraction, Hot percolation and Soxhlet extraction.

1.3.2. Bio extracts screening

Bioassay is a very crucial stage in assessing the pharmacological actions of plant extracts and their ethno medical uses. The isolation of new bioactive compounds from plants can be directed by bioassays. Alternatively, new uses of compounds can be identified when known compounds are tested in new bioassays. The availability of specific in vitro bioassays has facilitated the screening of numerous bioactivities of natural products. Screening has uncovered new pharmaceuticals and structure-activity relationships which has provided leads for designs of new drugs (Baker et al., 1995).
There are many types of pharmacological screens that are specific for bacteria, fungi, protozoa, intestinal worms, viruses, etc. The efficacy of compounds against health problems such as cancer and inflammation is often probed while the effect on physiological and anatomical systems such as reproduction, digestion, etc. There are two kinds of bioassays which are quantal (all or none response) assay and Graded (proportionate increase in the observed response following an increase in the concentration or dose) assays (Panuganti, 2015).

The method widely employed in the evaluation of antioxidant activity is the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. DPPH radical is used as an indicator in testing hydrogen-donating capacity and thus antioxidant activity (Dorman et al., 2003).

For researching and developing new antimicrobial agents from various sources (I. E., medicinal plants) to combat microbial resistsants, Several bioassays such as disk-diffusion, well diffusion and broth or agar dilution are well known and commonly used (Balouiri et. al., 2016).

The MTT/MTS in vitro cell proliferation assay is one of the most widely used assays for evaluating preliminary anticancer activity of both synthetic derivatives and natural products and natural product extracts. The highly reliable, colorimetric based assay is readily performed on a wide range of cell lines. In the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is bioreduced by dehydrogenase inside living cells to form a coloured formazan dye, while in the MTS assay, a similar bioconversion takes places utilising 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent (phenazine ethosulfate) PES.

1.3.3. Purification of natural products by High-Throughput Screening (HTS)

The purification of natural products remains a challenging, lengthy and a tedious task (Mahler and Thomason, 2005). Spectroscopic methods coupled with good separation techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. Sound strategies have helped in the isolation and characterization of many bioactive molecules (Rios et al., 1991). Fractionation and separation of samples obtained from nature remain time-consuming, tedious and extremely expensive even though the assays for testing these samples have become faster and more
cost-effective thanks to advanced high-throughput screening (HTS) processes (Bhandari et al., 2011).

High-throughput screening, often abbreviated as HTS, is a method of scientific experimentation especially relevant to the fields of biology and chemistry. Using robotics, data processing/control software, liquid handling devices, and sensitive detectors, high-throughput screening allows a researcher to quickly conduct millions of chemical, genetic, or pharmacological tests. Through this process one can rapidly separate, identify active compounds, antibodies, or genes that modulate a particular biomolecular pathway. The results of these experiments provide starting points for drug design and for understanding the interaction or role of a particular biochemical process in biology.

Today, sepbox is the standard technology used for separating compounds from natural resources. Fractionations and separation of samples obtained from nature remain very difficult. The unique sepbox concept allows processing sample automatically and will make up to 30 times faster than by using a conventional process. The sepbox concept is based on a patented combination of high-performance liquid chromatography (HPLC) and solid-phase extraction (SPE) that provides a universal platform suitable for processing large sample numbers. This facility is commonly called as sepbox or the separation box. The facility is being used for the fractionation of plant extracts or pre-fractionated extracts and combinatorial reaction products. Using two-dimensional separations the recovery rate for both polar and non-polar substances is usually above 90% per cent. By Using an automated and highly reproducible process one extract can be completely separated per day. The pure individual components are soluble in suitable solvents and can be collected in microtiter plates or vials (Bhandari et al., 2011).

1.3.4. Structural characterization of purified natural products

Once the biological evaluation has been performed and the separation of the natural product has been achieved, the chemist will attempt the structural characterization of the compounds (Pieters and Vlietinck, 2005). Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR), Infra Red (IR) and UV-Visible, Mass Spectrometry (MS) and X-Ray analyses (Balunas and Kinghorn, 2005; Lindsey et al., 2006). Liquid chromatography time-of-flight mass spectrometry (LC–TOF-MS) analysis provides an expansive technique for identifying many known and unknown analysts. LC–TOF-MS was able to resolve many isobaric compounds by accurate mass correlation within 15 ppm mass units and a narrow retention time interval of less than 10 s
of separation (Guale et al., 2013). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an efficient tool for large biomolecule analysis.

1.4 Practical utility of research problem:
Herbal medicines are an essential and growing part of the international pharmacopeia. Knowledge of their medicinal properties is growing as a result of research and testing, which will make them an increasingly safe alternative or a preferred option to allopathic medicine. In this current era of diseases, cancer is prompt as a major diseases of current scenario. Allopathic medicines for cancer are widely used now a days with having huge side effects to human anatomy. As a remedy for this herbal extracts from selected medicinal plants which target specific cancer cell line have huge potential to inhibit cancer growth without any side effects. So to isolate and identify anticancer active compounds from medicinal plants will reveal important study to combat cancer.

1.5 Objectives:-
1. To identify and collect anticancer medicinal plants
2. To extract phytochemicals (secondary metabolites) from above plants through different solvents.
3. To study physicochemical properties of above medicinal plants.
4. To evaluate in vitro biological activities of above medicinal plants extracts.
5. To separate secondary metabolites from above medicinal plants extracts.
6. To identify the phytochemicals (secondary metabolites) isolated from above medicinal plants extract.
7. Proteomic study of cancer inhibitory proteins of cancer cell line.
Plate 1.1 The appearance of *Euphorbia Tirucalli* (Dandaliyo thor) (A-C), *Cadaba Fruticosa* (Katakiyo) (D-F).
Plate 1.2 The appearance of *Panicum Maximus Jacq.* (Koili grass) (A-C), *Annona Muricata* (Hanuman Phal) (D-F).