CHAPTER 1

INTRODUCTION

Several plant products are known for their therapeutic properties. The use of plant products in treatment of numerous diseases is common in India. Plant extracts as medicines have been reported to assist treating numerous ailments such as blood pressure, cancer, anxiety, depression and so on. They have also assisted in helping people feel happier from within. Other abnormalities such as hay fever, irritable bowel syndrome, and menstrual problems are also extensively treated with plant extracts. In recent time, natural-product-based drug discovery has been increasing. As per a report by the World Health Organization on several Asian and African countries, an 80% of residents depend on traditional medicine for their regular principal health care (WHO 2008).

The use of plant extracts as therapeutic agents is familiar from ancient Indian history in the name of ayurveda, which has been suitably accepted again in recent days. The recovery period after treating with plant extracts is tremendously slow, not only because crude extracts hold the drug at low magnitude, but also the release of drug is exceedingly sluggish. The invasion of India by the Europeans led to the introduction of synthetic medicines. Speedy revival, in case of synthetic medicines might have resulted in confined use of ayurvedic medicine. The source for synthetic active ingredient medicine being the same, production is getting different and the same ayurvedic extracts are produced synthetically for use in treating diseases.

In the early 1950’s, the thought process of scientifically exploring natural organisms as the basis of supportive anti-cancer substances, was started (Gordan and David 2005). Of late, scientists are of the opinion that "the use of natural products has been the single most successful strategy in the discovery of novel medicines"
(Martin Tulp and Lars Bohlin 2002). Plants find it necessary to protect themselves from attack by micro-organisms by producing several chemicals. They specially generate several anti-fungal compounds that are lethal to fungi. Since human cells and fungal cells are biochemically comparable, the chemical compounds intended for protecting plant from fungus, comprise an inhibitory effect on human cells, including human cancer cells (Cardenas et al. 1999).

Plant extracts used as medicines are known to have fewer side effects, compared to synthetic medicines. From an individual plant, each individual part which includes the leaf, bark, fruit, seed and root, either is regularly used in treating diverse symptoms in isolation or may be used as a medicine in combination. Plants play a decisive role as a source for potential anti-cancer compounds (Mullauer et al. 2010) and extracts from the parts or plant parts as such are consumed as medicines in different forms. Likewise, plant extracts as well as few plant products, emphasize remedial feature against several therapeutic symptoms. As per many ancient medical literature reports, surgery was performed by physicians, but they also recommended the use of several natural, especially plant, products to cure the disease. Large-scale anticancer drug discovery and screening programs those sponsored by the National Cancer Institute (NCI) have played a decisive role in the improvement of anticancer natural compounds (Nobili et al. 2009).

Plant derived compounds with anti-tumor activity comprise camptothecin derivatives, topotecan and irinotecan, etoposide derived from epipodophyllotoxin along with paclitaxel, and vinca plants which exhibits potential anti-tumor properties amidst several important anti-cancer plants extracts (Gordan and David 2005). There are at least 86 alkaloids extracted from vinca genus. Among these, vincristine, vindesine and vinflunine extracted from Catharanthus roseus (formerly known as Vinca reosea) are used to treat leukemia, lymphomas and childhood cancers, as well as several other types of cancer including some non-cancerous conditions (Jonathan Roepkea et al. 2010). Vinorelbine, one of the plant extracts from Catharanthus roseus, has been reported to show activity against breast cancer protein transferase. Gonzalez-Angulo et al. (2007) in their review have emphasized that breast cancer
could be the most common cancer and the second leading cause of cancer death among women.

In silico modeling helps in identifying plant originated ligands, through data base search. Further, ligand is docked to the protein of interest which helps in calculating the total energies depending on the shape of the molecule as well as electrostatics. In silico modeling is a multidisciplinary process that integrates mathematical models with experimental (in vitro and in vivo) and clinical data (Sandeep Sanga et al. 2007 & Abhinav Grover et al. 2010). Drug discovery being an extensive and expensive process, widespread and scheduled application of screening methods, bring down expenditure and time in drug discovery. The docked ligand is visualized for best docked output. Geometry optimization of the ligand facilitates improvising the molecule for better docking energy or by increasing the common binding sites. In silico trials were exploited for exposing the anti-cancer properties of several herbal ligands.

Traditionally, in vivo and in vitro pharmacology models were used in predicting ability of molecules to be used as medicine and in therapeutics. These models were helpful in forecasting assumptions and further were helpful in advancement of science for innovations. Both in vitro and in vivo studies have a specific model for a particular disease. Generally, in vitro studies help to choose doses for the molecule through cytotoxicity determination. The cells are exposed to different concentrations of molecule to verify the cytotoxicity. Based on this cytotoxicity, doses for the animal models are decided. Each prospective drug is experimented with in vivo pharmacology models to determine their capability to control the diseases. Mice in vivo models are common and they are available as inflammation and immunology, ocular, oncology, respiratory and cardiovascular/metabolic disease and so on, as per diverse ailments. In case of cancer, different models are available for each type and nude mice of different types are available as models. Added advantages of animal models of cancer in mice are that, they facilitate in finding out the etiology and therefore, better management of malignancy. Besides this, a route of administration can be easily demonstrated in the animal models. Similarly, these studies help in each and every aspect of studying the
diseases. Thereby, the animal models are a resource of immense potential for cancer medicine (Frese and Tuveson 2007).

Further, animal models necessitate examining and manipulating complex disease process through easy handling of these animals which might be impossible to execute in patients. Though animals are handled humanely and experiments are conducted with as few animals as possible, the results from these experiments can more or less directly transmit to human beings. A good number of results in animal studies are expedited and directly passed on to similar cases in human beings. In the recent past, a number of studies have been conducted using animal models to understand the pathogenesis and response to treatment of many of the cancer and other diseases (Vandamme 2014).

Cancer is one of the diseases diffusing prominently and primarily becoming the reason for death among the human population. Hippocrates, the father of medicine, employed the word ‘cancer’ which originated from the Greek word ‘Karkinos’ to describe tumors. Treating cancer in the present world is entirely different from those early days when cancer was detected. The identification of cancer was just through literary description in the latter (Alanna Skuse 2015). Understanding the progress in symptoms and treatment was prolonged, and was based on progress in pathological anatomy (Faguet 2015). According to the World Health Organization (WHO), cancer is one of the principle causes of fatality with approximately 14 million new cases annually, and as high as 8.2 million cancer associated deaths only in the year 2012 (World cancer report, 2014). Breast cancer is one of the most prevalent types of cancer. Breast cancer tumor is classified as benign tumors and malignant tumors (WHO 2003). The benign tumors are generally harmless and the malignant tumors are cancerous. Also, breast cancer is classified into categories (histopathological type, the grade of the tumor, the stage of the tumor, and the expression of proteins and genes) as per different schemes criteria and serving a different purpose.

According to information published by Health and Human Services Agency for Healthcare Research and Quality, while tamoxifen (a drug presently used
for treating breast cancer) significantly reduces breast cancer, it has side effects. For example, lower-limb lymphedema was correlated with the use of tamoxifen owing to the blood clots and deep vein thrombosis caused by its inappropriate use (US department of health and human services 2002). Tamoxifen-treated breast cancer patients show evidence of reduced cognition, considered as a major side effect (Paganini-Hill and Clark 2000). Similarly, doxorubicin another drug for breast cancer has side effects such as alopecia, cardiotoxicity and so on (Jonsson et al. 1991).

Extracts from *Catharanthus roseus*, are known to contain quite a few medicinal properties (Culine et al. 1999); vinorelbine one of the compound from extract, well known for its anti-cancer properties (EMC Medicines 2009) is currently synthesized, which is expensive. The compound is yet to be explored for therapeutic purposes in its natural form. The use of an extracted compound in its natural form has the advantage that, it may be safer because of lesser side effects coupled with less cost of production. In this context, an effort is made in the current exploration to study vinorelbine, a natural plant extract from *Catharanthus roseus* for its anti-breast cancer properties through *in silico* and *in vivo* approaches.

Through the evidences it was demonstrated that the synthetic medicines are significantly expensive and they are not in the reach of widest range of world’s unprivileged population. On the contrary, the plant based medicines extracted or gathered straight from the natural world are inexpensive or available at no cost (DaSilva et al. 2002).

The main objective of the present investigation is virtual screening of different herbal based ligands exhibiting anti-tumor properties, specifically against breast cancer through docking exercises in an *in silico* model. Further, based on virtual screening, one of the plant extracts is extracted, purified and verified in the breast cancer animal model. In this context, an attempt is made in the present investigation to study vinorelbine, a natural plant extract from *Catharanthus roseus* for its anti-breast cancer properties using *in vivo* approaches.

1.1 Aim: Identification and Characterization of Anti-Tumor Compound from Leaves of *Catharanthus roseus* (L).

The experiemnt was designed with virtual screening of different herbal based ligands exhibiting anti-tumor properties, specifically against breast cancer in
an *in silico* model. One of the plant extract is extracted and purified. This purified product, after successful identification is verified in the breast cancer animal model using *in vivo* approaches.

1.1.1 Objectives

- Virtual screening of anti-tumor compounds and its derivatives.
- Synthesis of the potential anti-tumor derivative.
- Animal model studies to test the efficacy of the potential anti-tumor derivative.
CHAPTER 2

REVIEW OF LITERATURE

2.1 Plant Extract as Medicines

In the food chain, plants are crucial and are at the start of the chain as primary producers. Further, they also provide us with the necessary raw materials for food and shelter. In addition, pharmaceutical properties of each part of several plants make the plants crucial for livelihood of human population. For centuries, the use of herbal medicines to treat various disorders is detailed. Moreover, these medicines facilitate the betterment of the individual and get rid various ailments. Employing plants as pharmaceutical agents by human beings has been practiced from the traditional era. These practices are not limited to rural areas of third world, but also in developed countries where contemporary medicines are principally consumed and are generally agreed to (Kamboj 2000). The use of herbal medicines in the western world is gradually on the rise with approximately 40 % of the population reporting use of herbs in therapeutic use (Bent and Ko 2004).

As per the data released by World Health Organization (WHO), among 21,000 plants that are intended for curative purposes around the world, roughly 2,500 species are of Indian origin and 150 are commercially viable. This establishes India as the largest producer of medicinal herbs and hence is referred to as the botanical garden of the world (Seth and Sharma 2004). Further, it estimates that 80 % of populations in developing countries depend exclusively on traditional medicine for their primary health care needs and 85 % people in underdeveloped countries use plants or their extracts as active substances in health care systems (Shome et al. 1996 & Sheldon et al. 1998). Indian Materia Medica includes about 2,000 drugs of natural origin, a majority of which are derived from our traditional system; 400 of these are
of mineral origin and the rest are of plant origin. Ayurveda is based on the natural products of nearly 2,000 cultivated and wild plant species. The written records of Ayurveda like Charaka Samhita, Shushruta Samhita and others contain more than 8,000 herbal remedies. There are literally millions of plants, combinations, traditions and household remedies to treat a variety of diseases and boost health (Pearce and Moran 1994, Subrat et al. 2002). The Himalayan region in India itself comprises of more than 1,748 plant species (1,685 angiosperms, 12 gymnosperms and 51 pteridophytes) consisting therapeutic importance (Samant et al. 1998 & Samant and Paul 2003). For more than 1,000 years, a number of medicinal plants are conventionally used in herbal preparations in the Indian health care system, in the name of rasayana (Scartezzini and Speroni 2000).

Awareness of medicinal properties of plants is based on keen assessment and is habitually transferred through subsequent generations by word of mouth, rather than in written form. Ethnomedical plant-use data in many varieties was seriously made use of in the development of formulations and pharmacopoeias, give a major focus in global health care, as well as contributing considerably to the drug development programme (Graham et al. 2000). Plants employed in traditional medicine encompass a wide range of therapeutical products that are used to treat chronic as well as infectious diseases (Duraipandiyan et al. 2006). Several reviews show that parts of plants are important source of medicine and herbal medicine has been in vogue for centuries to treat various ailments. Low toxicity of herbal medicines and the general belief that they contain a few side effects make them a preferred substitute that may decrease the incidence of quite a few diseases (Kaefer and Milner 2008 & Singh et al. 2010). Anti-viral, anti-bacterial and anti-cancer biological properties have been reported by numerous natural compounds (Newman and Cragg 2007). Several reviews show that parts of plant are important source of medicine and herbal medicine is in vogue for centuries for treating various ailments. Plant parts such as root, bark, petioles, leaves, fruits, and seeds of herbs *Toona sinensis*, (Luo et al. 2000; Cho et al. 2003a & Cho et al. 2003b) have been widely used to treat several diseases including enteritis, dysentery, itchiness, abdomen tumor, diabetes and hypertension (Edmonds and Staniforth 1998; Chang et al. 2002; Chang et al. 2006 & Fan et al. 2007). *Acorus calamus* (sweet flag) has numerous
traditional and ethno-medicinal applications. Rhizome of sweet flag, has been used in diverse medicinal systems such as ayurveda, unani, Chinese medicine and siddha, for treating a variety of pains like nervous disorders, appetite loss, bronchitis, chest pain, colic, cramps, diarrhea, digestive disorders, flatulence, gas, indigestion, rheumatism, sedative, cough, fever, bronchitis, inflammation, depression, tumors, hemorrhoids, skin diseases, numbness, general debility and vascular disorders (Sandeep Rajput et al. 2014).

L-Dopa, a non-protein amino acid from leguminous plants *Vicia faba*, is comprised of a therapeutically rich secondary metabolite used for treating Parkinson’s disease (Simmonds and Grayer 1999). These are readily available for usage and a few of them are present in easily extractable forms. Sinapic acid, a small naturally occurring carboxylic acid, present in edible plants is a potent antioxidant (Nenadis et al. 2007). Various parts like leaves, flowers and stems of perennial shrubs like rosemary, include several medicinal properties that are utilized in treating inflammation, circulatory disorders, rheumatism, muscular affections, ulcers, diabetes etc. These parts are reported to contain anti-nociceptive, antioxidant, antidiabetic, anticolitic, antifungal, antimicrobial, antiulcer, antidepressant, antibacterial, hepatoprotective, neuroprotective, anticancer properties (Sandeep Shandil and Souravh Bais 2014). Manisha Madhok et al. (2007), have recently reviewed the detailed use of several herbal drugs and plants in the treatment of diabetes, occurring in the Indian sub-continent.

Further, herbal medicines are reported to assist in relaxation and they can also deal with anxiety, depression and other abnormalities such as hay fever, irritable bowel syndrome, menstrual problems etc. Some vegetables, fruit, and medicinal herbs, are known to possess a variety of anti-oxidant effects and other biological activities. The presence of phenolic compounds in these plant materials is the origin of their anti-oxidant activity, which is mainly due to their redox properties and their capacity to block the production of reactive oxygen species. Many of spice-derived potent anti-oxidant compounds have drawn the attention of biologists and clinicians since they might help in protecting the human body against oxidative stress and inflammatory processes (Rubio et al. 2013).
2.2 The Role of Plant Extracts in Cancer Treatment

Natural products play significant role in cancer treatment therapy, with considerable numbers of anticancer agents used in the clinic being either natural or products derived from natural sources such as plants, animals and microorganisms. In addition, conventional use of plant based medicine has been a main source for the discovery of novel anti-cancer agents. As per the latest review, most of the new and approved anticancer drugs are either natural products or their derivates (Kinghorn et al. 2009a & Kinghorn et al. 2009b). Further, Cragg and Newman (2000), in their review, exposed that nearly 60 % of the drugs now in clinical trials for the multiplicity of cancers are either plant based herbal products or their derivates. These include pharmacophores, either obtained from active natural compounds or from modified natural products connected to targeting systems.

Advanced to this research, National Cancer Institute is involved in widespread drug discovery and screening programmes (Jean-Decoster et al., 1999). Drug discovery and screening programmes have encouraged the development of natural anticancer compounds and have taken significant steps towards increasing, natural-product-based drug discovery (Nobili et al. 2009). Precise biological activities of these compounds may be because of their extremely complex molecular structures joined with different chiral centres in relation to small molecules (Olaf van Tellingen et al. 1993). Most significant development in recent times is that plants and their constituents or extracts contain several clinically proven properties against the dreaded disease, cancer. Most of the anticancer compounds are in the form of phenolic acids and these compounds play a major role in antioxidants as agents of chemoprevention (Arpita Basu et al. 2010).

Natural phenolic compounds isolated from medicinal herbs and dietary plants play a significant role in cancer hindrance and treatment. These compounds include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and so on. In addition to low toxicity of the herbal medicines, Kaefer and Milner (2011), have also reported that various bio-activities of phenolic compounds are responsible for their chemo-preventive properties such as anti-oxidant, anti-carcinogenic, or anti-mutagenic and anti-inflammatory effects. In
addition to this, phenolics also contribute in encouraging the apoptosis by (i) arresting cell cycle, (ii) controlling carcinogen metabolism and ontogenesis expression, (iii) slowing down the DNA binding and cell adhesion, migration, proliferation or differentiation and (iv) obstructing signaling pathways (Huang et al. 2010). Total spirostanol saponins (TSSP) glycosides extracted from the rhizome of Paris polyphylla, is a new type of contractile agonist for the uterus. Their synergism may be responsible for the therapeutic effect of TSSP on abnormal uterine bleeding (Yu et al. 2010).

In the recent past, several experiments have been performed demonstrating the anti-tumor properties of several herbal plant extracts. An isomer of podophyllotoxin, epipodophyllotoxin, isolated from the roots of Podophyllum has anti-tumor properties (Stahelin 1973). Lyophilized powder of centrifuged crude water extract from Toona sinensis leaves (TSL-CE) performs an antioxidant activity that inhibits proliferation of human promyelocytic leukemia cells and induces apoptosis (Hseu et al. 2008). Withafarine (Withania somnifera), four different anti-proliferative phytocompounds (indole-3-carboxylaldehyde, weidelolactone, luteolin and apigenin from Wedelia chinensis), nimbolide (Azadirachta indica) and diosgenin from Dioscorea bulbifera have been identified as potential ligands exhibiting anti-tumor properties (Lin et al. 2007; Raju and Bird 2007; Harish Kumar et al. 2009 & Singh et al. 2010). Mayola et al. 2011, were successful in demonstrating antitumorigenic activity of withaferine against various cancer cells. In another study conducted by Koduru et al. 2010, withaferine inhibited notch-1 signaling and down regulates pro-survival pathways, such as Akt / NF-kappa B / Bcl-2 in different cancer cell lines and also, is active against breast cancer (Singh et al. 2010).

Lin et al. (2007), have reported phytocompounds such as indole-3-carboxylaldehyde, weidelolactone, luteolin and apigenin from Wedelia chinensis exclusively restrained the growth of androgen receptor dependent prostate cancer (PCa) cells and as a combination they also synergistically contained the growth in AR-dependent PCa cells. In addition cytotoxic effects of nimbolide on human breast cancer cells were subjected to treatment and reported that treatment with nimbolide hindered the growth of MCF-7 and MDA-MB-231 cell lines.
(Elumalai et al. 2012). Further, intrinsic and extrinsic apoptotic signaling molecules expression were associated with increased levels of pro-apoptotic proteins with reduced levels of the anti-apoptotic proteins. Diosgenin in an in vitro study demonstrated its effect against gastric cancer. It is a potent candidate for decreasing the ability of invasion and survival in cobalt chloride treated BGC-823 cells; when combined with HIF-1α specific short hairpin RNA (shRNA), diosgenin can inhibit BGC-823 cells more effectively. Anti-invasion role of diosgenin may be related to E-cadherin, integrinα5 and integrinβ6. These results suggest that diosgenin may be a useful compound in controlling gastric cancer cells in hypoxia condition, especially when combined with down-regulated HIF-1α (Zhu-Jun Mao et al. 2012).

Sulphur compounds present in or generated from garlic are effective anticancer agents (Ariga and Seki 2006). A mixture of homoharringtonine, isolated from Cephalotaxus harringtonia was successfully employed for the management of acute myelogenous leukemia and chronic myelogenous leukemia (Cragg and Newman 2005 & Kantarjian et al. 1996). Diosgenin from Dioscorea bulbifera have been identified as potential ligands exhibiting anti-tumor properties (Raju and Bird 2007). Nimboide, from Azadhiractha indica, has attracted worldwide prominence in recent years, owing to its wide range of medicinal properties. Neem has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine. Neem has a vast array of biologically active compounds that are chemically diverse and structurally complex (Subapriya and Nagini 2005). More than 140 compounds have been isolated from different parts of the neem tree. The leaves, flowers, seeds, fruits, roots and the bark, have been used traditionally for the treatment of inflammation, infections, fever, skin diseases and dental disorders. Medicinal utilities have been described especially for the neem leaf. Its constituents have been demonstrated to show evidence of immunomodulatory, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties. In addition nimboide from the neem plant is composed of anti-cancer properties (Harish Kumar et al. 2009).
2.3 The Role of Plant Extracts in the Treatment of Breast Cancer

Gonzalez-Angulo et al. (2007), in their review have emphasized that breast cancer could be the most common cancer and the second leading cause of cancer death among women population. This is sufficiently supported by the studies carried out by International Agency for Research on Cancer (IARC) and World Health Organization (WHO 2012).

Depending on clinical and molecular features of the tumor, a treatment of high efficacy and low toxicity, was selected with appropriate validation studies performed to stay away from confusing effects (McGuire 1991). In a majority of breast cancer samples, Hormone Receptors (HR), including the Estrogen Receptor (ER) and/or Progesterone Receptor (PR) were over expressed (Kocic et al. 2010 & Cossetti et al. 2015), whereas over expression of human epidermal growth factor receptor 2 (HER2) takes place only in 20–30% of cases (Mitri et al. 2012). Further it was confirmed that the most common breast cancer subtype is HR+/HER2− and postmenopausal women in particular, are more likely to have HR+/HER2− tumors, as HR over expression increases with age (Cadoo et al. 2013).

Currently, there are three main groups of medications used for adjuvant breast cancer treatment. The first being hormone-blocking agents, where anastrozole, a potent non-steroidal aromatase inhibitor is used (Ting Bao and Michelle 2011). The second medication type is chemotherapy, used from the second to the fourth stages, and is mainly favorable in estrogen receptor-negative (ER−) diseases. A couple of medicines used are Docetaxel (www.cancer.gov. 2015), Methotrexate (Rossi 2013) and Fluorouracil (5-FU) (Brayfield 2013). Employing a single or a combination of more drugs (docetaxel/doxorubicin/cyclophosphamide) is also a common technique in chemotherapy (von Minckwitz 2008). Another type of treatment for breast cancer is the application of monoclonal antibodies. The use of trastuzumab, a monoclonal antibody to HER2, has showed an improvement of up to about 87 % in patients suffering from breast cancer (Jahanzeb 2008). The National Comprehensive Cancer Network treatment guidelines for HR+/HER2− mBC recommend the use of
endocrine therapy, particularly a non-steroidal Aromatase Inhibitor (AI), as first-line treatment in postmenopausal women (Swallow et al. 2014).

Some breast cancers require estrogen to continue growing. They can be identified by the presence of Estrogen Receptors (ER+) and progesterone receptors (PR+) on their surface. These ER+ cancers can be treated with drugs that either block the receptors, e.g. tamoxifen, or alternatively block the production of estrogen with an aromatase inhibitor, e.g. anastrozole or letrozole (Harold Burstein et al. 2014). X-ray diffraction studies of the ligand binding domain of human estrogen receptor revealed that residues: try$^{383}$, leu$^{384}$, 387,391,428,525, met$^{388}$, 421, arg$^{394}$, phe$^{404}$, glu$^{419}$, ile$^{424}$, gly$^{420}$, 521 and his$^{524}$ were interacting with 4 hydroxy tamoxifen (Shiau et al. 1998). According to the report issued by the Health and Human Services' Agency for Healthcare Research and Quality, tamoxifen and additionally closely related medicines used for controlling breast cancer, while appreciably controlling breast cancer in women, also enhances the possibility of side effects. For instance, lower-limb lymphedema has been correlated to continuous utilization of tamoxifen due to the formation of blood clots and deep vein thrombosis which are caused by its inappropriate use (Tamoxifen, Wikipedia). Breast cancer patients treated with tamoxifen, confirmed evidence of reduced cognition (Paganini-Hill and Clark 2000).

Not only tamoxifen but also other molecules used in chemotherapy are often accompanied by serious side effects of treatment, with a severe impact on the patients’ health (Stockler et al. 2000 & Bergh et al. 2001). Adriamycin (Doxorubicin), another breast cancer drug, also confirmed the presence of side effects such as alopecia, cardiotoxicity and so on. This was observed when 76 patients with evaluable advanced breast cancer were treated with a weekly dose of Adriamycin as first line chemotherapy in a phase II study (Jonsson et al. 1991). In another experiment, a high dose of doxorubicin and cyclophosphamide were given to eighteen patients for 2 weeks in three cycles. The therapeutic outcome was exceptional. However, side effects like mild to moderate palmar and plantar inflammation were observed. The experiment also exhibited that survival gain is very limited and continuation of the medication could not be considered due to severe toxicity (Ferguson et al. 1993).
To offer more effective and less toxic treatments, selecting appropriate therapies requires careful consideration of the patient’s response to therapy, followed by clinical and molecular characteristics of the tumor under investigation (June and Hansjörg 1970). In an experiment Bonadonna and Valagussa (1981), observed that the clear dose-response effect of CMF (cyclophosphamide, methotrexate, and fluorouracil) was useful only when given in a full or nearly full dose (greater than or equal to 85 per cent of the planned dose). Systemic treatment of breast cancer includes cytotoxic, hormonal and immunotherapeutic agents. These medications are used in the adjuvant, neoadjuvant and metastatic settings. In general, systemic agents are active at the beginning of therapy in 90 % of primary breast cancers and 50 % during the metastases stage (Gonzalez-Angulo et al. 2007). Several times the unnoticed cancer tumor progresses to an extent that, the possibility of irreparable damage is high. At this point resistance to therapy is not only common but expected.

Based on the studies carried out by Joyce Nirmala et al. (2011), it is clear that vinca alkaloids are active against leukemia’s, lymphomas, advanced testicular cancer, breast cancer and lung cancer. Vinca plants are known to exhibit potential anti-tumor properties, within a number of plant-derived compounds which have been important sources of many clinically useful, anti-cancer agents (Gordan and David 2005). There are at least 86 alkaloids extracted from vinca genus (Manfred Hesse 2002) and chemotherapy agents such as vincristine, vindesine and vinflunine extracted from Vinca rosea are employed to control leukemia, lymphomas, childhood cancers, and additional kinds of cancer. They are also used to treat few non-cancerous circumstances.

2.4 Extraction, Purification and Characterization

Organic solvents are generally used to extract various natural derivatives from plants, which are used either singly or as a mixture. For instance, the elimination of surface waxes as well as additional hydrophobic surface chemicals from plant tissues is accomplished by dipping vinca leaves in chloroform (Samuels et al. 2008). In another experiment, vinblastine in sulfate form and vinca alkaloid as dimethyl sulfonate salt, were isolated using acetonitrile as a solvent (Olaf van Tellingen et al. 1993). Several extracts from Acorus calamus using various solvents
are employed against diverse symptoms such as antiepileptic (Jatinder et al. 2012), anti-inflammatory (Lad et al. 2010), anti-diarrheal potential (Shoba and Thomas 2001) etc.

The ethanolic extract of *Acorus calamus* exhibited an *in vitro* anti-cellular property (Malhrotra et al. 1962). Its methanolic extract from leaves is active against the lymphocytic leukemia cell line and several human cancer cell lines (Palani et al. 2010). Using combination of solvent blends, methanol-acetonitrile (75:25 v/v), Barthe et al. (2002) was victorious in extrication 11 alkaloids from vinca plants. Using aqueous acidic 0.1 M solution of hydrochloric acid, catharanthine and vindoline embonates were extracted from dried leaves of *Catharanthus roseus* which acted as the starting material for a semi-synthesis of the anti-cancer bisindole (Verma et al. 2007).

The Soxhlet apparatus was used for extracting secondary extracts from the young leaves of *Avicennia marina* by making use of diverse solvents (Soxhlet 1879). A compound with limited solubility in solvents needs to be extracted with a Soxhlet extraction and it cannot be used for thermolabile compounds (Nikhal et al. 2010). Several researchers have, considered sonification, heating under reflux or Soxhlet extraction for extracting bioactive compounds from plants (Moore et al. 1982; Huie 2002; Zygmunt and Namiesnik 2003 & Sasidharan et al. 2011).

Thin Layer Chromatography (TLC) is a chromatographic technique used to separate non-volatile mixtures (Harry Lewis and Christopher Moody 1989). Separation is achieved because of a different and definite rate of analyte movement on the TLC plate. These findings suggested that the plant root could be a potential source of natural antioxidants which is vital as a therapeutic agent and in preventing oxidative stress related degenerative diseases (Vogel 1989). Five individual solvents namely hexane, ethyl acetate, methanol, acetone and chloroform, were used for extracting the root of *Aerva lanata*, separately. These isolated phytochemical constituents may emerge to function as a source of useful plant based drugs. These five constituents were successfully purified using TLC (Guji et al. 2013). Antibacterial metabolites were screened of red clover phenolic compounds and were effectively separated using the TLC method. The studies confirmed that the
metabolites inhibited Hyper Ammonia-producing Bacteria (HAB) native to the bovine rumen (Kagan and Flythe 2014). The secondary metabolites of the young leaves of *Avicennia marina* were obtained by sequential Soxhlet extracts with petroleum ether, chloroform, ethyl acetate, ethanol and water as solvents. The extracts exhibited different degrees of growth inhibition against the tested bacterial strains. Components of young leaf extracts were separated by TLC (Abeysinghe Pushpa and Weeraddana Chaminda De 2011). Column chromatography is also employed as one of the techniques for purifying phytoextracts. Two phytoextracts present in the seeds of *Minusops elengi* were recognized using ethyl acetate and were purified by column chromatography (Hazra et al. 2007).

The IR spectroscopic method is commonly employed while identifying the unknown chemicals useful in the identification of plant extracts. It uses the spectral method for the identification of molecules involving absorption spectroscopy. Leaf fragments of three plants of different genera, namely, Ranunculus (Ranunculaceae), Acantholimon (Plumbaginaceae), and Astragalus (Leguminoseae) were collected. By making use of Fourier transform infrared (FT-IR), quick and precise molecular characterization and identification of these fragments was performed. Compositional and structural differences in macromolecules identified using FT-IR, successfully helped to characterize all three different plants accurately (Gorgulu et al. 2007). In an experiment conducted to analyse leaf extracts from petroleum ether, chloroform, ethyl acetate and methanol from leaves of medicinal plants Ashok kumar and Ramaswamy, (2014), successfully used the FT-IR spectroscopy method. Employing GC-MS and FT-IR, bioactive compounds of *Solanum torvum* leaves were estimated. While, chemical compositions were examined using Perkin-Elmer Gas Chromatography-Mass Spectrometry, the presence of alcohol, alkanes, aromatic carboxylic acid, halogen compound, alkyl halide were confirmed in FTIR analysis (Nithyadevi and Sivakumar 2015). In another experiment, flavonoids were extracted from leaves of various plant samples by ultrasonication and maceration. These flavonoids were analysed by employing Infrared (IR) spectroscopy combined with chemometrics (Lestyo Wulandari et al. 2016).
A Nuclear Magnetic Resonance (NMR) spectrum provides the leading information regarding the structure of a compound. In the NMR Spectroscopic method, a compound is placed in a strong magnetic field which affects the spin of atomic nuclei. A radio wave passes through the compound and reorients atomic nuclei. By turning off the wave, the nuclei release a pulse of energy that provides data on molecular structure of compound. The provided data is transformed into an image through computer techniques. Identification and characterization of active constituents, sesquiterpenes, and other metabolites extracted from arnica with the supercritical CO(2), was performed by combining literature data and information obtained by 2D-NMR experiments (Bilia et al. 2002). *Zehneria scabra* is an important climber which belongs to the family Cucurbitaceae and consists of bioactive chemical compounds, with medicinal values. These compounds were identified by Nuclear Magnetic Resonance (NMR) spectral analysis. Proton nuclear magnetic resonance spectra of the root sample were recorded and the chemical shift values of the various signals were identified by the presence of gypenoside. This bioactive compound is confirmed as the valuable medicinal compound likely to cure vast array of disease (Anand et al. 2011). Using the Soxhlet extraction, a leaf extract of *Euphorbia neriifolia* was collected, using ethanol. This extract was subjected to thin layer chromatography and high performance thin layer chromatography. Further, characterization was done through IR, $^1$H NMR and MS. The extract was isolated as flavonoids (Veena Sharma and Pracheta Janmeda 2014).

The *in vitro* cytotoxic activity of several plant extracts have been utilized for the purpose of selecting extracts exhibiting probable therapeutic properties (Soundararajan and Sreenivasan 2012; Hanisa et al. 2014; Ali et al. 2014 & Shruti Bandopadhyaya et al. 2015). The *in vitro* cytotoxicity of *Centella asiatica*, *Curcuma longa* and *Strobilanthes crispus* extracts were performed using three kidney cell lines acquired from an African green monkey, a baby hamster and a rabbit using MTT reduction assay. The results showed *Centella asiatica* was showing very small toxicity to all these experimented cell lines followed by *Strobilanthes crispus* and *Curcuma longa* (Hanisa et al. 2014). Out of the total of 14 wild angiosperms accumulateed from Saudi Arabia, cytotoxic activity displayed by the extract of
*Lavandula dentata* demonstrated hopeful potential as an anticancer agent having good anti-proliferative as well as apoptotic activity (Ali et al. 2014).

Gallic acid, a naturally occurring plant phenol was found to show cytotoxicity against a few cancer cells such as primary cultured rat hepatocytes and macrophages, fibroblasts and endothelial cells. It was showing cytotoxicity to these cells with IC\(_{50}\): 4.8-13.2 μg/mL (Inoue et al. 1995 & Inoue et al. 2000). Other related studies have revealed that intracellular Reactive Oxygen Species (ROS) induced by gallic acid helps in bringing out an early signal in apoptosis and particularly, H\(_2\)O\(_2\), which is derived from extracellularly generated superoxide anion may increase intracellular Ca\(^{2+}\) levels or cooperate with intracellular Ca\(^{2+}\), thus resulting in apoptosis induction (Inoue et al. 2000). Also, gallic acid, a bioactive compound extract of *Toona sinensis*, has been reported to have various effects on cultured cell lines, including an anti-proliferative activity in cancer cells (Yi-Chen Chia et al. 2010).

There are multiple experiments that show vinca based alkaloids extracted from *Catharanthus roseus* and their synthetic derivatives exhibit good anti-tumor properties (Culine et al. 1999; Jean-Decoster et al. 1999; Sevilcan Tuna et al. 2009; Ahmed and Jamil 2011 & Maryam Moudi et al. 2013). In a recent experiment, toxicity of Vinorelbine to the cells was tested along with additional neoplastic medicines (Geftinib, Cisplatin, 5-FU and Gemcitabine) by use of cervical cancer cell lines. From this study, cisplatin was found to be the most toxic drug (LC\(_{50}\) = 13μM), while vinorelbine was the least toxic (LC\(_{50}\) = 48 μM) (Ahmed and Jamil 2011).

The *In vitro* cytotoxic activity of various plant extracts has been used for the purpose of selecting extracts exhibiting potential therapeutic properties (Shruti Bandopadhyaya et al. 2015). Several studies showed vinca based alkaloids extracted from *Catharanthus roseus* (periwinkle) and their synthetic derivatives displayed good anti-tumor properties (Culine et al. 1999 & Maryam Moudi et al. 2013). Dose-dependent efficacy was established for cetuximab using Non-small cell lung cancer (NSCLC) cell lines. Cetuximab was dosed at half-maximal efficacy with chemotherapy used at maximum tolerated dose resulting in antitumor activity in NSCLC models expressing mutated epidermal growth factor receptor. It was also
observed that combination treatments increased the efficacy of cetuximab (Philipp Steiner et al. 2007).

Animal models of cancer using mice present supplementary alternatives of experiments to conclude the reasons of and treatments for malignancy, thus representing a resource of immense potential for cancer medicine. Further, these models support the investigators in scrutinizing and manipulating a complex disease process in a approach impossible to execute in patients. In the recent past, a number of studies have been conducted using animal models to understand the pathogenesis and response to treat cancer and other diseases (Valerie et al. 2004; Joann 2004; Beverly 2006; Frese and Tuveson 2007 & Barbara Szymanska et al. 2014).

Based on trials conducted on xenografted mice, causes and treatment for malignancy were successfully established. Plant extracts are tested initially for their cytotoxicity under in vitro condition followed by in vivo compatible tumor models in order to know its effectiveness against cancer (Gui-Zhen Han et al. 2005; Goetsch et al. 2005; Alami et al. 2007 & Hirokomi Ikeda et al. 2011). For instance, a combination treatment of vinorelbine with fulvestrant and other cytotoxic agents (doxorubicin, paclitaxel, docetaxel and 5-fluorouracil) has a synergistic effect in estrogen receptor-positive breast cancer. Outcome of the study is based on in vitro as well as in vivo investigations (Hiroko Ikeda et al. 2011). The influence of crude fucoidan, a sulfated polysaccharide, derived from brown algae was studies on mouse breast cancer in vitro and in vivo and results demonstrated inhibition of mouse breast cancer growth in both in vitro and in vivo. Further, it was suggested that fucoidan might be a prospective remedial means for breast cancer (Xue et al. 2012). By using the in vitro and further by nude mice models, a recombinant humanized anti-insulin-like growth factor receptor type I antibody enhanced the antitumor activity of vinorelbine and anti-epidermal growth factor receptor therapy against human cancer xenografts (Goetsch et al. 2005).

Several experiments in Catharanthus roseus (formerly Vinca rosea) have revealed that the different alkaloids present in its extracts are showing numerable therapeutic uses. Based on the studies carried out by Joyce Nirmala et al. (2011), it is clear that these extracts, commonly known as vinca alkaloids, are active against
leukemia’s, lymphomas, advanced testicular cancer, breast cancer and lung cancer. Few vinca alkaloids, vincristine, vindesine, vinflunine, vinblastine and vinorelbine extracted from vinca are familiar for extensive pharmaceutical applications. Vinorelbine, one of the alkaloid, is active against breast cancer protein transferase. Despite, vinorelbine is well known for its anti-cancer properties (Sevilcan Tuna et al. 2009 & Ahmed and Jamil 2011), the compound has not been investigated for therapeutic purposes, in its natural form. Instead, the molecule is synthesized which is expensive. In addition, the compound extracted in its natural form may be safer as well as cost effective.

2.5 In Silico Studies

In silico modeling is a multidisciplinary process that integrates mathematical models with experimental (in vitro and in vivo) and clinical data (Sandeep Sanga et al. 2007 & Abhinav Grover et al. 2010). In silico screening methods are routinely and extensively used to reduce the cost and time of drug discovery. In a study conducted by Enireddy Vamsidhar et al. (2010), 266 compounds from 7 plant sources revealed antihypertensive agents demonstrating their biological activity. The study also facilitated the identification of compounds against hypertension, based on screening, docking and consensus scoring techniques. In silico trials were used to reveal anti-cancer properties in many of the herbal ligands. Abhinav Grover et al. (2010) were successful in carrying out computational studies with the aim of exploring the proteasome inhibition capability of the herbal ligand, withaferin. Docking this compound onto the structures of bovine and human proteasomes validate this ligand’s capability to inhibit activity of mammalian 20S proteasomes by blocking the nucleophilic function of N-terminal Thr1. Thymoquinone, an active ingredient of Nigella sativa, which demonstrates anti-oxidant, anti-inflammatory and anti-tumor properties through mechanisms that are not fully understood. Molecular docking analysis revealed that TQ formed interactions with seven polar residues and six non-polar residues within the ligand-binding pocket of peroxisome proliferator-activated receptor gamma (PPAR-γ or PPARG) that are reported to be critical for its activity (Chern Chiuh Woo et al. 2011). An in silico study was carried out to verify the binding interactions of the
phenanthroindolizidine alkaloid, a phytochemical isolated from natural sources, with a COX-2 receptor. This showed that the alkaloids as well as the derivatives, possess a greater binding affinity towards the COX-2 enzyme and thus inhibits it are over expression. This binding affinity supports the anti-inflammatory as well as the anticancer activity of the phenanthroindolizidine alkaloid towards COX-2 enzyme (Anita Mandhare et al. 2015).

In the in silico docking study conducted using the GLIDE module (Glide, version 6.1, Schrödinger), an antiangiogenic compound present in the *Sophora interrupta* root extract, piceatannol, showed excellent interaction with vascular endothelial growth factor receptor 2 (VEGFR 2). The high docking score of piceatannol may be due to the presence of an extra hydroxyl group which is in good orientation with acidic part of the binding pocket. Further it was concluded that, piceatannol can be used as nutritional and pharmacological applications to treat a variety of cancer models (Pardhasaradhi Mathi et al. 2015). Lignads, zeaxanthin, translutein, quercetin 3-glucoside 7-rhamnoside and isorhamnetin is present in two plant species, *Hippophae salicifolia* and *Hippophae rhamnoides*, when docked against Ras protein using Autodock 4.0. A study revealed that, while, the binding energies for these were low and were different for these four ligands, all the compounds occupied the similar active site of Ras protein (Talambedu Usha et al. 2014). Five different compounds got from an ethanol extract of *Anisochilus carnosus* were docked with Bcl2 protein using the Auto dock tool and the results demonstrated that Imidazole, 2-amino5-[2carboxy] vinyl, had the least docking energy, indicating its superior anticancer property (Meenakshi 2012). E2F3, a tumor marker encodes a transcription factor that is important for cell cycle regulation and DNA replication, which helps in the development of various types of human cancer. The crystal structure of E2F3 was developed by an X-ray crystal structure. Further in docking studies, it was established that the ligand Vinblastine, an antitumor alkaloid isolated from *Vinca rosea*, resulted in better binding energy against E2F3 (Sinosh Skariyachan et al. 2010).

Based on these observations, it is possible to conclude that there is an urgent need to screen for different herbal based ligands exhibiting anti-tumor
properties, specifically against breast cancer, using computational strategy. The outcome of this can be useful to extract the same from *Catharanthus roseus* for its anti-breast cancer properties using *in vivo* approaches.
CHAPTER 3

MATERIALS AND METHODS

The information related to herbal ligands was obtained from extensive literature search; some of them showing a wide range of anti-tumor properties. Using this data as the basis, followed by a survey by the of PDB databank (www.rcsb.org), cancer related target proteins were segregated into transferases, isomerases or oxidoreductases. Herbal ligands specific against transferases were considered since these proteins were present in larger proportion, whose mol2 files were subsequently downloaded from ZINC database (www.zinc.docking.org) (Table 3.1) for the docking exercise.

Table 3.1 mol2 Files of Anti-Tumor Herbal Ligands Specific against Transferase Downloaded from ZINC Database

<table>
<thead>
<tr>
<th>Herbal Ligand</th>
<th>Alt. Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinorelbine</td>
<td>Daizdzin 2</td>
</tr>
<tr>
<td>Daizdzin</td>
<td>Diosgenin</td>
</tr>
<tr>
<td>Chalnone</td>
<td>Brassinin</td>
</tr>
<tr>
<td>Betulinic acid</td>
<td>Xanthohumol</td>
</tr>
<tr>
<td>Wdalactone</td>
<td>Withaferine</td>
</tr>
<tr>
<td>Theophyline-pheoforbide</td>
<td>Sinapic acid</td>
</tr>
<tr>
<td>Silymarin</td>
<td>Quercetin_1</td>
</tr>
<tr>
<td>Pheophorbide</td>
<td>Osthole</td>
</tr>
<tr>
<td>Nimbolide</td>
<td>Mellissic acid</td>
</tr>
<tr>
<td>Geniposide</td>
<td>Gallic acid</td>
</tr>
</tbody>
</table>
Plant extracts exhibiting anti-tumor properties against several cancer types is presented in Table 3.2.

### Table 3.2 Summary of Plant Extracts Showing Anti-Tumor Properties

<table>
<thead>
<tr>
<th>Name of the ligand</th>
<th>Plant source</th>
<th>Active against cancer type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melissic acid</td>
<td><em>Wedelia chinensis</em> (Pitabhrrnga)</td>
<td>Prostate cancer (putative)</td>
<td>Kaefer and Milner 2011</td>
</tr>
<tr>
<td>Withaferine</td>
<td><em>Withania somnifera Linn</em> (Ashwagandha)</td>
<td>Breast Cancer,</td>
<td>Singh et al. 2010</td>
</tr>
<tr>
<td>Nimbolide</td>
<td><em>Azadirachta indica</em> (Neem)</td>
<td>Choriocarcinoma (putative), colon cancer</td>
<td>Elumalai et al. 2012</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td><em>Vinc a rosea Linn.</em> <em>(Periwinkle)</em></td>
<td>Breast Cancer, Lung Cancer, Ovarian Cancer, non-small cell lung cancer</td>
<td>Jina Yu et al. 2004</td>
</tr>
<tr>
<td>Diosgenin</td>
<td><em>Dioscorea bulbifera</em> (Dukkar kand)</td>
<td>Colon cancer, breast cancer (putative)</td>
<td>Li-Xin Sun et al. 2006</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td><em>Humulus yunnanensis</em> <em>(Hop plant)</em></td>
<td>Lung Cancer (putative),</td>
<td>Yi-Chen Chia et al. 2010</td>
</tr>
<tr>
<td>Chalcone</td>
<td><em>Glycyrrhiza glabra</em> <em>(Yashti-madhuka)</em></td>
<td>Breast, Prostate and colon carcinoma</td>
<td>Koneni et al. 2010</td>
</tr>
<tr>
<td>Geniposide</td>
<td><em>Gardenia jasminoides</em> <em>(Gandharaj,)</em></td>
<td>Glioma</td>
<td>Koo et al. 2004</td>
</tr>
<tr>
<td>Osthole</td>
<td><em>Cnidium monnieri</em> <em>(She chuang zi)</em></td>
<td>Lung and Various cancers</td>
<td>Chou et al. 2007</td>
</tr>
<tr>
<td>wedalolactone</td>
<td><em>Eclipta alba (L.)</em> <em>(False daisy)</em></td>
<td>Various cancers</td>
<td>Mithun and Shashidhara 2008</td>
</tr>
<tr>
<td>pheoforbide</td>
<td><em>Carpinus betulus</em> <em>(Common hornbeam)</em></td>
<td>Various cancers</td>
<td>Cieckiewicz et al. 2012</td>
</tr>
<tr>
<td>Xanthohumol</td>
<td><em>Humulus yunnanensis</em> <em>(Hop plant)</em></td>
<td>hepatocellular carcinoma (putative), leukemia (putative)</td>
<td>Clarissa Gerhauser et al. 2002</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td><em>Various plants</em></td>
<td>Breast cancer</td>
<td>Janakiraman et al. 2014</td>
</tr>
<tr>
<td>quercetiin_1</td>
<td>Different plant source</td>
<td>Various cancer</td>
<td>Densie Webb 2008</td>
</tr>
<tr>
<td>daidzin</td>
<td>Leguminous plants</td>
<td>Various cancer</td>
<td>Kaufman et al. 1997</td>
</tr>
<tr>
<td>Betulinic acid</td>
<td><em>Ziziphus mauritiana</em> <em>(Madhuraphala)</em></td>
<td>Several cancers</td>
<td>Mullauer et al. 2010</td>
</tr>
</tbody>
</table>
3.1 Docking Studies

The 3D structures of all the chosen herbal ligands were docked onto the crystal structures of two kinase (kinases belong to larger family of phosphotransferases) complexes 1KDT (cytidine monophosphate kinase) (Bertrand et al. 2002) and 1J1B (human tau-protein kinase) (Aoki et al. 2004) deposited in the protein data bank. Cytidine monophosphate kinase 1KDT from E. coli, is a good phosphate acceptor and has been chosen to observe the phosphorylation reaction (Bertrand et al. 2002). The phosphorylation is related to the cause of metabolic disorders. Protein 1J1B, originated from human brain cells, is responsible for Alzheimer’s disease and chosen because of its virulent nature to human beings (Ishiguro et al. 1993). Similarly, for comparison, the selected ligand (vinorelbine) was docked onto the crystal structure of kinase 3ERT. This kinase is associated with tamoxifen, a drug presently used in the market for breast cancer treatment. Docking was carried out using HEX software (Ritchie 2003). Docked conformations and interaction energies were recorded at the end of the docking exercise. During the dock operation, total energies were calculated based on shape as well as electrostatics using a default grid spacing of 0.6 Å. Deep View package (Guex and Peitsch 1997) was used for later visualizing the best docked output. The interacting residues within 5 Å for each ligand were identified.

Geometry optimization as well as vibration frequency of the herbal ligand having the lowest calculated interaction energy was carried out using a Gaussian package (Raymundo Hernandez-Esparza et al. 2014) installed on SGI Altix UV10 supercomputing machine. To optimize the structure, Hartreefock theory with the basis set at “3-21g” was considered. Standard orientation of the optimized structure generated was visualized using the ARGUS lab package and the structure was saved in PDB format. A geometrically optimized structure was once again docked onto the same kinase receptor protein(s) using HEX software as explained earlier. The docked output was later validated by comparing the calculated interaction energy with the crystal structure of protein complexed with anti-tumor drug / ligand molecule deposited in the protein data bank.
Extraction and purification of vinorelbine from *Catharanthus roseus* was undertaken. Followed by this, extract was tested in animal models to prove the efficacy.

### 3.2 Plant

Details of plant used for extraction is provided in Table 3.3.

**Table 3.3 Details of Plant *Catharanthus roseus***

<table>
<thead>
<tr>
<th>Kingdom (unranked)</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Angiosperms</td>
</tr>
<tr>
<td>Order (unranked)</td>
<td>Eudicots</td>
</tr>
<tr>
<td></td>
<td>Asterids</td>
</tr>
<tr>
<td>Family (unranked)</td>
<td>Gentianales</td>
</tr>
<tr>
<td>Genus (unranked)</td>
<td>Apocynaceae</td>
</tr>
<tr>
<td>Species C. roseus</td>
<td>Catharanthus</td>
</tr>
<tr>
<td></td>
<td>C. roseus</td>
</tr>
</tbody>
</table>
3.2.1 Plant leaf Powder

Plants of *Catharanthus roseus* (formerly known as *Vinca rosea*) were collected from department of Horticulture Lalbagh, Bengaluru (Fig 3.1). The plant was identified and authenticated in National Ayurveda Dietetics Research Institute, Bengaluru. It was identified and was authenticated (authentication/S.M.P.U./N.A.D.R.R.I./BNG/2015-16/1200) as *Catharanthus roseus* (L.) G. Don, by the institute (refer Appendix 1).

The leaves from approximately 2,000 plants of *Catharanthus roseus* were separated and dried in a hot air oven at 40-50 ºC for overnight. The leaves were then powdered using a mixer. The drying process was conducted in Syngene International Limited Bengaluru.

3.3 Extraction

The leaf powder was subjected to extraction. Approximately one hundred gram dry leaf powder of *Catharanthus roseus* mixed with methanol was kept on shaker for 3 hours. Five flasks of were prepared similarly. Mixture was filtered using
Whatman filter paper. The resulting filtrate on filter paper was dried and introduced into the thimble. Further, it was extracted by Soxhlet extractor using methanol at 70-80 °C (Soxhlet 1879). The pure methanol extract retained in round bottom flask, was stored in refrigerated condition for 3 days.

Dried extract was placed in acidified distilled water (pH 2, with acetic acid, Merck Specialties) mixed and heated to about 50 °C for 24 hrs, leaving behind alkaloid rich residue. Strong alkali (NaOH, of 11 pH, approximately) was added to the residue drop wise. Warm non-polar organic solvent, hexane, was added to this and was kept at 37 °C with constant stirring for 20 minutes. Once the alkaloid gets migrated to hexane, it was kept for 24 hours to form a thick emulsion. Further, sodium hydroxide solution was included to break the emulsion formed and solvent layer was subsequently separated from the solution. The solvent was finally evaporated to yield alkaloids free from base (Elisha Solowey et al. 2014). The extraction procedure was carried out in Durga Femto Technologies and Research, Chamarajpet, Bengaluru.

3.4 Thin Layer Chromatography (TLC)

Silica coated plates were air dried at room temperature for one hour and dried at 80 °C in an oven for 30 minutes. Three alkaloid samples got from the extraction were spotted onto the TLC plate. The TLC plates were completely immersed inside the solvent mixture, chloroform and methanol (15:1), which was allowed to evaporate completely. Thin Layer Chromatography (TLC) was performed for differentiating the alkaloids (Meena and Vidya 2008).

Solid samples were dissolved methylene chloride, and the solution then was placed onto a single salt plate. The solvent was evaporated, leaving a thin film of the original material on the plate (Eberhardt et al. 2007). The TLC procedure was carried out in Durga Femto Technologies and Research, Chamarajpet, Bengaluru.

3.5 Column Chromatography

The silica gel was loaded into the column tube and methanol solvent was poured into it to run the silica gel. Once one round of methanol was run the methanol extract was introduced into the column. After around 2 hours compounds got
separated and were collected into a test tube. The column chromatography procedure was carried out in Durga Femto Technologies and Research, Chamarajpet, Bengaluru

3.6 Infrared Spectroscopy (IR)

The IR spectrum was recorded on Nicolet 6700 instrument using a universal ATR sampling unit. Neat dried sample was placed in ATR sampling unit and an IR spectrum was recorded. The IR procedure was conducted in Syngene International Limited, Bengaluru.

3.7 NMR Spectroscopy

NMR experiments were performed on a Bruker 400 MHz Instrument using 5 mm QNP probe at room temperature. 5 mg of the compound dissolved in 0.7 mL of DMSO-d6. The data were processed by Topspin software (Bruker). The NMR procedure was conducted in Indian Institute of Sciences, Bengaluru.

3.8 Animal Experiments

The compound vinorelbine was extracted from the leaves of Catharanthus roseus plant, separated through TLC and was purified using column chromatography. This was identified through NMR analyses and was further taken to verify its effect on xenografted nude mice.

3.8.1 Test System

The experiment was carried out using female mice (Mus musculus), strain Hsd: Athymic Nude-Foxn1nu (source; Harlan) of 5-6 weeks. Body weight of the animals varied between 20 and 22 g.

In vivo experiment was carried out per CPCSEA approved Institute of Animal Ethics committee (IAEC) protocol number SYNGENE/IAEC/537/08-2014.

3.8.2 Study design

Eight female mice each were tested in two groups, a control and a treatment group. The animals were xenografted with breast cancer cell line MDAMB231. Each animal was carrying 15x10^6 cells in right abdominal flank, subcutaneously. At study initiation tumor volume was approximately, 150 mm^3. The
treatment group animals were injected with vinorelbine at the rate 30 mg/kg body weight through intra-peritoneal route once weekly, for 4 weeks. Concurrent control group was not treated. Mice from each group were housed in separate 1285LN: type II long cage for mice with overall dimensions W x D x H: 396 (width) x 215 (depth) x 172 (height) mm, with floor area of 542 cm² with stainless steel top grills having facilities for holding pellet food and drinking water in bottle with stainless steel sipper tube. The corn cobb was used as bedding material (refer Appendix 5 for corn cobb report for its constituents, valid for an year). Each batch of bedding material was analysed in-house for excess bacteria (total bacterial count), fungi (total fungal counts) and pathogens (such as *Staphylococcus aureus*, *Escherichia coli* or *Salmonella*). The cages used for the study were sterilised along with bedding material in it.

3.8.3 Observations

The animals were routinely verified for their health by means of clinical signs. They were also observed for mortality and morbidity, daily once. The feed and water availability for the animals was checked daily. The cages where animals were housed was observed for the wetness of the bedding material and changed at least twice a week.

3.8.3.1 Cage Side General Observations

The All clinical signs for all the animals were observed, once daily, throughout the experimental period. Also, morbidity or mortality, of animals was observed twice daily.

3.9 Feed and Water

While the treatment is going on, the animals were provided with feed and water, *ad libitum* (refer Appendix 6 and Appendix 7 for report of feed and water, respectively for their constituents, valid for an year).

Each batch of feed was analysed in-house for presence of excess bacteria (total bacterial count), fungi (total fungal counts) and pathogens (such as *Pseudomonas aeruginosa*, *Escherichia coli* or *Salmonella*).
Water used for animal feeding was analysed every month for excess bacteria (total bacterial count) and pathogens (such as *Pseudomonas aeruginosa*, *Escherichia coli* or *Salmonella*).

### 3.10 Tumor Volume and Body Weight

The tumor volume of the animals was measured once in three days and also, the animal body weight (Barbara Szymanska et al. 2012).

Tumor volume (TV) was calculated as follows:

\[
TV (\text{mm}^3) = \frac{\text{Length of tumor} \times \text{width of tumor}^2}{2}
\]

Tumor growth inhibition (TGI) was calculated (Carlsson et al. 1983), as follows:

\[
\text{TGI} \% = \frac{\text{TV of treatment (mm}^3\text{)} - \text{TV of treatment (mm}^3\text{)}}{\text{TV of control (mm}^3\text{)}} \times 100
\]

### 3.10.1 Necropsy

Mice from both the groups were euthanized using CO₂ asphyxiation on day 30. The animals were transferred to the CO₂ chamber humanely and were confirmed that they were euthanised before they were taken out from the chamber.

### 3.10.2 Statistical analysis

Statistical analysis was carried out with the help of expert consultant using Student’s T test. The comparisons were determined at P<0.05 significant level (Blair Clifford and Higgins James 1980).

The animal experiment was conducted in Syngene International Limited Bengaluru per approved Protocol SYNGENE/IAEC/537/08-2014. The protocol for conducting animal experiment was approved by Syngene Animal Ethics Committee.
3.11 Comparison of Estimated Cost of Cultivation and Extraction of Vinorelbine from Catharanthus roseus with Synthetic Vinorelbine

Projected cost of cultivating *Catharanthus roseus* for the purpose of extracting vinorelbine is estimated. Cost of producing vinorelbine through synthetic approach (quotation Gila laboratories, GLPL/SI/343/16-17) is compared with cost of vinorelbine through plant extraction.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Docking studies

Docking studies were carried out to screen different herbal based ligands exhibiting anti-tumor properties, specifically against breast cancer through docking exercises.

Table 4.1. Docking Energy of Ligands with Protein

<table>
<thead>
<tr>
<th>Ligand from herbal source</th>
<th>Docking energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein 1J1B</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>-362.07</td>
</tr>
<tr>
<td>withaferine</td>
<td>-292.07</td>
</tr>
<tr>
<td>Nimbolide</td>
<td>-282.38</td>
</tr>
<tr>
<td>Melissic acid</td>
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</tr>
<tr>
<td>Diosgenin</td>
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<tr>
<td>Betulinic acid</td>
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<td>Chalcone</td>
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</tr>
<tr>
<td>wedalolactone</td>
<td>-228.05</td>
</tr>
<tr>
<td>Osthole</td>
<td>-239.75</td>
</tr>
<tr>
<td>quercetiin_1</td>
<td>-241.65</td>
</tr>
<tr>
<td>daidzin</td>
<td>-259.89</td>
</tr>
<tr>
<td>Sinapinic acid</td>
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</tr>
<tr>
<td>Xanthohumol</td>
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<tr>
<td>Gallic Acid</td>
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<tr>
<td>daidzin-2</td>
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<tr>
<td>theophylline- pheoforbide</td>
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</tr>
<tr>
<td>brassinin</td>
<td>NA</td>
</tr>
<tr>
<td>Tamoxifen (known marketed drug)</td>
<td><strong>-257.67</strong></td>
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</table>
Among the 18 anti-tumor herbal ligands that were selected for docking, vinorelbine – unrefined (ur) got docked with the lowest calculated interaction energy onto the kinase proteins (1J1B and 1KDT). While, the molecule got docked with interaction energy of -362.07 kcal mol\(^{-1}\) onto 1J1B, it got docked onto 1KDT with interaction energy of -297.60 kcal mol\(^{-1}\) (Table 4.1, Fig 4.1a and 4.2a).

Fig 4.1a. Docking of vinorelbine (colored magenta; -362.07 kcal mol\(^{-1}\)) and tamoxifen (colored red; -257.67 kcal mol\(^{-1}\)) onto the crystal structure of kinase protein (PDB ID: 1J1B)

The analysis of the binding site of vinorelbine – ur and tamoxifen with 1J1B revealed both the ligands got docked at different locations. While ile\(^{62}\), phe\(^{67}\), val\(^{70,135}\), arg\(^{141}\), lys\(^{85,183}\), gln\(^{185}\), asn\(^{64,186}\), leu\(^{188}\), cys\(^{199}\), gly\(^{63,65, 68, 202}\), ser\(^{66,203}\) and asp\(^{181,200,764}\) of 1J1B interacted with vinorelbine-ur, gly\(^{259,565}\), glu\(^{268}\), val\(^{267}\), gln\(^{265}\), pro\(^{268}\), tyr\(^{221}\), arg\(^{220,720}\), ser\(^{261,719}\), asp\(^{260,264,681,700}\), asn\(^{564,686}\), lys\(^{271,585,683}\) and phe\(^{567}\) of 1J1B made contact with tamoxifen (Fig 4.1b). The visualization of 1KDT protein docked with vinorelbine – ur and tamoxifen through Deep View package revealed that both the ligands appears to occupy the same binding site of the protein and
thereby share few common residues which include: asp\textsuperscript{10}, lys\textsuperscript{11}, tyr\textsuperscript{12}, arg\textsuperscript{20} and gln\textsuperscript{49} (Fig 4.2b).

Fig 4.1b. Active site residues of the crystal structure of kinase protein (PDB ID: 1J1B) interacting with vinorelbine (colored magenta) and tamoxifen (colored red) within a distance of 5Å
Fig 4.2a. Docking of vinorelbine (colored magenta; -297.6 kcal mol⁻¹) and tamoxifen (colored red; -220.7 kcal mol⁻¹) onto the crystal structure of kinase protein (PDB ID: 1KDT)

Since vinorelbine used for docking purpose is not a refined structure, it was subjected to geometry optimization using the GAUSSIAN package. The structure which got optimized at the end of 10 cycles recorded energy of -1585.89 atomic units (Fig 4.3). In addition to this, maximum force, RMS force, maximum displacement and RMS displacement computed by the package were less than the set threshold values. This suggested that the molecule has converged to global minimum energy.
Fig 4.2b. Active site residues of the crystal structure of kinase protein (PDB ID: 1KDT) interacting with vinorelbine (colored magenta) and tamoxifen (colored red) within a distance of 5Å
Note:

<table>
<thead>
<tr>
<th>Item</th>
<th>Value</th>
<th>Threshold</th>
<th>Converged?</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.000021</td>
<td>0.000450</td>
<td>YES</td>
</tr>
<tr>
<td>RMS Force</td>
<td>0.000004</td>
<td>0.000300</td>
<td>YES</td>
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<tr>
<td>Maximum Displacement</td>
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</tr>
<tr>
<td>RMS Displacement</td>
<td>0.000237</td>
<td>0.001200</td>
<td>YES</td>
</tr>
</tbody>
</table>

**Fig 4.3. 3D structure of vinorelbine geometrically optimized using GAUSSIAN software package [E (RHF) = -9.9x10^5 kcal mol^-1 after 10 cycles]**

There was no difference in the interaction energy when the kinase protein (1J1B) docked with geometrically optimized vinorelbine (-362.07 kcal mol^-1) (Fig 4.4a).
Fig 4.4a. Docking of optimised vinorelbine onto the crystal structure of kinase protein (PDB ID: 1J1B) with dock energy, -362.07 kcal mol\(^{-1}\)

However, the protein complexes with tamoxifen molecule shared a few residues (val\(^{267}\), lys\(^{271}\), asn\(^{564}\), gly\(^{565}\), ser\(^{566}\)) that were interacting with the refined vinorelbine structure (Fig 4.4b). This result was not obtained with the unrefined vinorelbine structure.
Fig 4.4b. Active site residues of the crystal structure of kinase protein (PDB ID: 1J1B) interacting with optimized vinorelbine within a distance of 5Å. The images were generated using Deep View package.
Though geometrically optimized vinorelbine successfully docked onto the crystal structure of the estrogen receptor (3ERT), calculated interaction energy was considerably more (-270.50 kcal mol\(^{-1}\)) (Fig 4.5a) compared to the value of tamoxifen docked onto the same protein (-288.29 kcal mol\(^{-1}\)) (Fig 4.6a). An analysis of the residues of the protein interacting with these two ligands strongly suggests that they occupy two different binding sites (Fig 4.5b and 4.6b)
Fig 4.5b. Active site residues of the crystal structure of estrogen receptor protein (PDB ID: 3ERT) interacting with vinorelbine within a distance of 5Å
Fig 4.6a. Docking of tamoxifen onto the crystal structure of estrogen receptor protein (PDB ID: 3ERT), with dock energy, -288.29 kcal mol⁻¹
Fig 4.6b. Active site residues of the crystal structure of estrogen receptor protein (PDB ID: 3ERT) interacting with tamoxifen within a distance of 5Å

4.2 Plant Leaf Powder, Extraction and Purification

Three distinct spots were separated when the solvent extract of dried leaf powder from Catharanthus roseus was passed through TLC (Fig 4.7) and these compounds were subsequently purified by using the column chromatography.

4.3 Infrared Spectroscopy (IR)

The IR Spectra of compound B and C are provided as Appendix 2 and 3. In the compound isolated and purified from spot A of thin layer chromatography, 3 carboxylic ester (-COO) and one free hydroxyl (-OH) functional group were attached. Owing to the presence of keto group (C=O) in the ester the absorption was recorded at 1,722 cm\(^{-1}\). This absorption confirms the presence of carboxylic ester group in compound. It was also, noticed that a broad absorption at
~3,300 cm\(^{-1}\) resulted because of hydroxyl group (Fig 4.8). All these results strongly suggest that the purified compound could be vinorelbine.

Fig 4.7. Separation of alkaloids from the solvent extract of dried leaf powder from *Catharanthus roseus* using thin layer chromatography (A, B and C to be identified)

4.4 NMR Spectroscopy

From the three unidentified compounds resulted from the TLC, upon subjecting to IR spectroscopy, broad absorption of compound A was similar to vinorelbine. Hence, this compound was further subjected \(^1\)H NMR analysis, for confirmation of vinorelbine properties. In the \(^1\)H NMR analyses, 8 scans (Appendix 4) were acquired with a 2-s relaxation delay using 128K data points. Chemical shifts (d) expressed in ppm and coupling constants (J) in Hz \(^1\)H NMR (Fig 4.9) were as follows;

10.48(s,1H), 7.69(d,1H, j=7.6Hz), 7.42(d,1H,j=12.4), 7.14-7.08(m,2H), 6.38(s,1H), 6.19(s,1H), 5.779(m,2H), 5.22(d,j=10.4)5.08(s,1H) 4.09-3.09(m,1H), 3.88(s,3H), 3.67(s,3H), 3.66(s,3H), 3.56(m,2H), 3.21(m,2H), 3.24(m,1H), 3.15(m,1H), 2.86(m,1H), 2.86(m,1H), 2.66(s,1H)2.59(m,1H), 2.33-2.31(bm,1H), 2.04-2.06(m,3H), 1.95(s,3H), 1.68(bm,1H), 1.59(bm,1H), 1.48(m,1H), 1.31(m,1H), 1.03(S,3H), 0.56(s,3H). It was observed that 3,370 cm\(^{-1}\)(OH &NH stretching), 2,950 cm\(^{-1}\)(C-H stretching), 1,728 cm\(^{-1}\)(C=O stretching) and 1,226 cm\(^{-1}\)(C-O stretching), The spectral data were consistent with reference NMR spectra of vinorelbine.
$3,370 \text{ cm}^{-1}$(OH & NH stretching), $2,950 \text{ cm}^{-1}$(C-H stretching), $1,728 \text{ cm}^{-1}$(C=O stretching) and $1,226 \text{ cm}^{-1}$(C-O stretching)

Fig 4.8. IR Spectra of TLC purified compound of *Catharanthus roseus* (spot A, unidentified methanol extract)
Fig 4.9. $^{1}{^1}H$NMR Spectra of TLC purified compound of *Catharanthus roseus* (spot A)
Fig 4.10a. Chemical structure drawn from $^1$H NMR Spectra of TLC purified compound of *Catharanthus roseus* (spot A)

Note: Vinorelbine original Molecule, Pubchem CID-60779, with Molecular Formula $C_{53}H_{66}N_4O_{20}$, have Molecular Weight of 1079.119 g/mol

Fig 4.10b. Chemical structure of vinorelbine
Using $^1$H NMR data structure was drawn for TLC purified compound from *Catharanthus* extract and same was compared with original vinorelbine. It was observed that drawn structure was comparable with original structure of vinorelbine (Fig 4.10a and Fig 4.10b).

### 4.5 Animal Experiments

The vinorelbine obtained from TLC purified compound of *Catharanthus roseus* was taken for *in vivo* studies.

![Fig 4.11. Tumor growth of vehicle control and vinorelbine (compound purified from *Catharanthus roseus* leaf), treated nude mice bearing MDAMB 231 xenograft, at end of treatment](image)
Table 4.2a. Individual Body Weight of Animals During the Treatment Period in Vehicle Control Group

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Body weights of MDAMB231 Mice (g) on Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>23.4</td>
</tr>
<tr>
<td>2</td>
<td>23.5</td>
</tr>
<tr>
<td>3</td>
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<td>±sd</td>
<td>0.16</td>
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<tr>
<td>SEM</td>
<td>0.06</td>
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<tr>
<td>Mean Body weight change</td>
<td>0</td>
</tr>
<tr>
<td>SEM</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Table 4.2b. Individual Body Weight of Animals During the Treatment Period in Vinorelbine (Compound Purified From *Catharanthus roseus* Leaf Treated @30 mg/mg, i.p.; Once Weekly for 4 Weeks) Treated Group

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Body weights of MDAMB231 Mice (g) on Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>22.6</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>7</td>
<td>22.5</td>
</tr>
<tr>
<td>8</td>
<td>23.4</td>
</tr>
</tbody>
</table>

Mean: 23.04 ± 0.40

Sem: 0.14

Mean Body weight change: 0 ± 0.28

SEM: 0.00
It was observed that the growth of the tumor reduced in the vinorelbine treated animals against increased growth in the control animals against their respective controls (Fig 4.11). At the start of the treatment, the body weight of the mice in control group ranged between 23.1 and 23.6 g, with the mean body weight being 23.41 g (Table 4.2a).

Similarly, at the start of treatment, the body weight of the mice in the vinorelbine treated group ranged between 22.5 and 23.6 g, with mean body weight being 23.04 g (Table 4.2b). While, the body weight in all control group mice, increased constantly (with mean body weight on day 30 of dosing being 25.21 g), the body weight of all vinorelbine treated animals reduced (with mean body weight on day 30 of dosing being 21.9 g) (Table 4.2a, Table 4.2b and Fig 4.12).

Fig 4.12. Mean body weight of nude mice bearing MDA MB 231 xenograft, treated with vehicle control and vinorelbine (compound purified from Catharanthus roseus leaf) treated nude mice, during the treatment
This accounted for an overall, 5 % reduction in mean body weight of vinorelbine treated animals against their body weight on day 1 and an 8 % increase in the control group at the end of the treatment (Fig 4.13).

**Fig 4.13.** Per cent body weight change of nude mice bearing MDA MB 231 xenograft treated with vehicle control and vinorelbine (compound purified from *Catharanthus roseus* leaf treated) nude mice, during treatment

Tumor volume of the mice in the control group, at the start of the treatment was within 120.18 and 169.21 mm², with the mean tumor volume being 148.46 mm² (Table 4.3a). The corresponding, tumor volume of the group of mice treated with vinorelbine was amid 114.37 and 167.53 mm², with a mean tumor volume of 148.65 mm² (Table 4.3b).
<table>
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<th>9</th>
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<td>38.57</td>
<td>45.06</td>
<td>42.59</td>
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</table>
Table 4.3b. Individual Tumor Volume of Animals During the Treatment Period in Vinorelbine (Compound Purified from *Catharanthus roseus* Leaf Treated- @ 30 mg/kg, i.p.; once weekly for 4 weeks) Treated Group

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Tumor Volume in MDAMB231 Mice (mm$^3$) on Day</th>
</tr>
</thead>
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</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>136.31</td>
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<td>3</td>
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<tr>
<td>4</td>
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<tr>
<td>SEM</td>
<td>5.27</td>
</tr>
</tbody>
</table>

*** p<0.001 when vinorelbine group was compared with vehicle group
The enlarged tumor in the control group mice was evident through the regular rise in tumor volume which attained 1718.17 mm² at the end of treatment. Conversely, in the vinorelbine treated group, tumor volume of each animal decreased, with the average tumor volume on day 30 being 374.39 mm² (Table 4.3a, 4.3b and Fig 4.14).

![Tumor growth kinetics of MDAMB231 xenograft bearing mice](image)

**Fig 4.14.** Tumor growth kinetics of nude mice bearing MDAMB 231 xenograft treated with vehicle control and vinorelbine (compound purified from *Catharanthus roseus* leaf treated), during treatment

The data when subjected to Students-t test, suggests a significant reduction in tumor volume from the 18th day onwards in the group treated with vinorelbine, in relation to the control group (Table 4.3b). The average volume of the tumor as observed was compared between the control group and the group treated with vinorelbine during the 30 day treatment period. Additionally this is supported by the
fact that the extent of tumor growth inhibition reached a maximum of 80 % in nude mice treated with vinorelbine at the end of the treatment (Fig 4.15).

Fig 4.15. Tumor growth inhibition in nude mice bearing MDAMB 231 xenograft treated with vehicle control and vinorelbine (compound purified from Catharanthus roseus leaf treated), during treatment

4.6 Comparison of estimated cost of cultivation and extraction of vinorelbine from Catharanthus roseus with synthetic vinorelbine

Based on the data presented in Table 4.4, it is evident that the cost of producing vinorelbine in its native state from Catharanthus roseus is much lesser (Rs 600.00 per mg) compared to vinorelbine produced through a synthetic route (Rs 1200.00 per mg).
Table 4.4 Comparison of Estimated Cost of Cultivation and Extraction of Vinorelbine from *Catharanthus roseus* with Synthetic Vinorelbine

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<tr>
<th>PARTICULARS</th>
<th>COST (INR)</th>
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<tbody>
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<td>Cost of cultivation <em>Catharanthus roseus</em> per hectare</td>
<td></td>
</tr>
<tr>
<td>Labor- sowing to plant dry product (all production expenses)</td>
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</tr>
<tr>
<td>Fertilizers/ organic matter</td>
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</tr>
<tr>
<td>Chemicals/organic</td>
<td>3,500.00</td>
</tr>
<tr>
<td>Stakes and twines</td>
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</tr>
<tr>
<td>Other inputs</td>
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</tr>
<tr>
<td>Miscellaneous</td>
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</tr>
<tr>
<td><strong>Total cost of cultivation</strong>*</td>
<td><strong>1,54,500.00</strong></td>
</tr>
<tr>
<td>Extraction of vinorelbine</td>
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</tr>
<tr>
<td>Methanol for extraction -125 L</td>
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</tr>
<tr>
<td>Acetic acid – 2 L</td>
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<tr>
<td>Sodium hydroxide -1 kg</td>
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<tr>
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<tr>
<td>Lab charges</td>
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<tr>
<td><strong>Total extraction charges</strong></td>
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</tr>
<tr>
<td><strong>Total expenditure to produce 500 g of vinorelbine</strong> @ (A) + (B)</td>
<td><strong>3,15,550.00</strong></td>
</tr>
</tbody>
</table>

Based on this the cost of production of commercial vinorelbine through plant extract (approximately) 600.00

Cost of synthetic vinorelbine through synthetic mean (approximately) 1,200.00

*: cost of cultivation as provided by University of Agricultural sciences, Bengaluru
@: expected vinorelbine yield per kg of dried leaf is, 200 mg
4.8 DISCUSSION

Natural compounds isolated from medicinal herbs and dietary plants play an important role in cancer prevention and treatment. Their function in cancer management is noteworthy as substantial anticancer agents used in the clinic. They are either natural or products derived from natural sources such as plants, animals and microorganisms. The conventional use of plant based medicine has been a major source of discovery of novel anti-cancer agents. These natural compounds used in cancer treatment or management include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and so on. Natural products extracted from different plant parts are useful herbal compounds. In silico studies are useful in screening the effect of ligands against different diseases. Docking the ligands helps to know their affinity towards diseases. Further, a ligand is docked to the protein of interest which helps in calculating the total energies depending on the shape of the molecule as well as electrostatics.

In the present study among the various herbal ligands that were screened for anti-tumor properties, exclusively against breast cancer, vinorelbine, present in *Catharanthus roseus*, got docked onto the crystal structure of kinase protein (1J1B) with the lowest calculated interaction energy. Since the vinorelbine molecule was present in its unrefined form, it was further refined using a geometrical optimization technique as implemented in the GAUSSIAN software package. There was no substantial difference in the calculated interaction energy between the unrefined and refined vinorelbine structure when docked onto the kinase protein; however, the protein complexed with the tamoxifen molecule shared a few residues that were interacting with the refined vinorelbine structure. This was not seen in the unrefined one. In a study conducted by Suvannang et al. (2011), aromatase inhibitors were geometrically optimized using the appropriate model chemistry implemented in the GAUSSIAN software, before attempting molecular docking. This explains the fact that docking program needs to have the ligands with the right molecular mechanical parameters and atom types or the results may not be accurate enough. Vinca plants are known to exhibit potential anti-tumor properties (Gordan and David 2005 & Joyce Nirmala et al. 2011) have reported that the vinorelbine extracted from *Vinca rosea* is active against the breast cancer protein, transferase.
Though the refined vinorelbine structure got successfully docked onto the crystal structure of estrogen receptor, the tamoxifen drug molecule showed more affinity towards the protein. However, the dock output shows that these two ligands occupy two different binding sites. The structural analogues of Adenosine Triphosphate (ATP), Adenosine Diphosphate (ADP) and phosphoaminophosphonic acid-adenylate ester (AMP-PNP), complexes with protein, 1J1B (Aoki et al. 2004). Active site residues of this ligand are almost the same as that of Tamoxifen when docked on the crystal structure of 1J1B protein. Since vinorelbine has docked onto a different binding site, different binding modes of vinorelbine and tamoxifen docking onto the kinase were noticed. Therefore, vinorelbine being a herbal based molecule, can serve as a better alternative to synthetic drugs, docetaxel or doxorubicin, which show side effects in patients. Hematological adverse effects of docetaxel are neutropenia, anaemia, febrile neutropenia and thrombocytopenia (Taxotere Docetaxel concentrate for infusion, 2016). As per the National Cancer Institute (NIH), the use of a commonly used breast cancer drug, pertuzumab, results in heart problems, diarrhea, nausea, vomiting, tiredness and so on (http://www.rxlist.com/perjeta-side-effects-drug-center.htm, 2016). Similarly, adriamycin (doxorubicin) another breast cancer drug confirmed to illustrate side effects such as alopecia, cardiotoxicity and so on (Jönsson et al. 1991). Further, Paganini-Hill and Clark (2000) have reported that tamoxifen-treated breast cancer patients illustrated condensed cognition, which is believed as a major side effect.

Leaves from *Catharanthus roseus* were subjected to an extraction procedure, using methanol as a solvent. The mixture was shaken and extracted using the Soxhlet extractor at 70-80 °C. The dried extract was placed in acidified distilled water, mixed and heated, leaving behind a rich alkaloid residue. A warm non-polar organic solvent, hexane, was added to this and maintained with constant stirring to form a thick emulsion. The solvent finally evaporated to yield alkaloids free from base. The alkaloid was isolated and purified using TLC. This sample was identified using IR and NMR techniques.

Leaves from *Catharanthus roseus* were subjected to an extraction procedure, using methanol as a solvent. Use of organic solvent for extraction of different alkaloids from plant material is reported as a universal practice. A range of solvents
were used to obtain several extracts from *Acorus calamus*. These extracts were reported to consist of a property to restrain diverse symptoms such as epantiepileptic (Jatinder et al. 2012), anti-inflammation (Lad et al. 2010) and anti-diarrheal potential (Shoba and Thomas 2001). In another experiment, vinblastine in its sulfate form and vinca alkaloid as dimethyl sulfonate salt, were isolated using acetonitrile as a solvent (Olaf van Tellingen et al. 1993). Three different alkaloids were effectively separated from the leaves of the *Catharanthus roseus* using TLC and these were subsequently purified with the help of column chromatography. Separation of compounds from plant extracts through TLC and column chromatography is a broadly used procedure. For instance, Abeysinghe et al (2011) used TLC and column chromatography methods for extracting secondary metabolites from an *Avicennia marina* plant to be tested for an antibacterial compound. In the present study, among various compounds that were extracted, the existence of vinorelbine was substantiated by performing IR and $^1$H-NMR spectral analyses. Hazra et al (2007) used similar techniques for identifying two antibacterial compounds from the seeds of *Mimusops elengi*.

Among the 3 TLC purified compounds obtained from the extracts of *Catharanthus roseus*, one of the compounds revealed the presence of C=O group and broad absorption when subjected to IR spectroscopic analysis. These structural results are comparable to the vinorelbine structure, suspecting the compound may be vinorelbine. Ashok Kumar and Ramaswamy (2014) used FT-IR spectroscopy method to analyse leaf extracts from petroleum ether, chloroform, ethyl acetate and methanol of leaves from medicinal plants.

Further, $^1$H-NMR analyses confirmed that the spectral data of the TLC purified compound from the *Catharanthus* extract was consistent with reference to the NMR spectra of vinorelbine. The successful identification of therapeutically essential bioactive chemical compounds from *Zehneria scabra* by Anand et al. (2011) through $^1$H-NMR analyses demonstrates the significance of NMR in plant extract identification. Veena Sharma and Pracheta Janmeda (2014) have applied extraction, purification and identification procedures for identifying flavonoids from the leaves of *Euphorbia neriifolia*. The study involved application of the soxhlet apparatus for extracting leaf extract, followed by TLC and HPLC to purify the compound. The
purified compound was confirmed as a flavonoid based on IR, $^{1}$H-NMR and MS analysis.

*In vivo* studies conclusively prove that, the animals xenografted with breast cancer cell lines and treated with vinorelbine purified from a plant extract, started healing after 18 days of treatment. The average tumor volume as well as the weight of the vinorelbine treated animals reduced persistently. Elisha Solowey et al. 2014 examined the effects of three different whole plant extracts on human tumor cells. A plant extract from *Urtica membranacea* showed strong anticancer capabilities since it inhibited actual tumor progression in a breast adenocarcinoma mouse model. Therapeutic properties of vinca alkaloids in subsidizing leukemia’s, lymphomas, advanced testicular cancer, breast cancer and lung cancer were experimentally confirmed in studies carried out by Joyce Nirmala et al. (2011). Similarly, in another review it was revealed that within a number of plant-derived compounds, vinca plants are known to exhibit potential anti-tumor properties, which have been an important source of many clinically useful, anti-cancer agents (Gordan and David 2005).

In addition to low toxicity of the herbal medicines, Kaefer and Milner (2011) have also reported that various bio-activities of phenolic compounds are responsible for their chemo-preventive properties such as anti-oxidant, anti-carcinogenic, or anti-mutagenic and anti-inflammatory effects. Gordan and David (2000), in their review exposed that, nearly 60 % of the drugs now in clinical trials for the multiplicity of cancers are either natural products or their derivates.

The projected cost of producing vinorelbine in its native state from *Catharanthus roseus* is nearly half of producing the same compound using a synthetic route. This suggests that producing vinorelbine in its native form directly from the *Catharanthus* extract is more economical. The low cost coupled with the anticipation of a fewer side effects makes vinorelbine extracted from *Catharanthus roseus* an ideal compound for replacing the existing breast cancer drug “doxorubicin”. Other advantages of using plant based extracts for treating cancer and other related diseases include:
• The possibility of increasing the area of cultivation of the proposed plant, *Catharanthus roseus*.

• Developing new cultivars and hybrids to increase the yield of vinorelbine in *Catharanthus roseus*.

• Creating better employment opportunities for the rural sector and the betterment of farming communities.

DaSilva et al. (2002) have proved that the synthetic medicines are extremely expensive and out of reach from widest range of world’s poor population. In contrast, the herbal medicines extracted or collected from nature directly are very cheap or available at no cost.

### 4.8 CONCLUSION

Herbal extracts are considered as one of the cheap sources of medicine for treating an innumerable number of diseases. A few of the herbal extracts are active against the dreaded disease of cancer, particularly against breast cancer. In this context, vinorelbine extracted from *Catharanthus roseus* may be a good alternate for the presently marketed drugs “docetaxel or doxorubicin” for the breast cancer. The vinorelbine ligand, upon geometrical optimization, had the lowest calculated interaction energy which also shared the binding site occupied by tamoxifen, another marketed drug for breast cancer. This indicates that the optimization of the vinorelbine crystal structure is helpful in improving the binding site of the ligand. Optimized vinorelbine can be used as an alternate to existing breast cancer drugs. Vinorelbine can be extracted from the widely and easily grown plant *Catharanthus roseus* using methanol as an extractant. The *in vitro* and *in vivo* studies have shown that this vinorelbine is effective in controlling breast cancer and successfully controlled xenografted breast cancer cells.

The vinorelbine extractant was dosed to treat breast cancer xenografted mice through an intra-peritoneal route, once weekly for four weeks. The outcome from these results infer that vinorelbine extracted from the dried leaf of *Catharanthus roseus* may effectively control the breast cancer. The synthetic drug presently used to supervise breast cancer, doxorubicin, has been confirmed to comprise of several
adverse effects. In contrast, vinorelbine extracted in its native form directly from the plant source is not only cost effective but is also likely to have fewer side effects. This distinctiveness, along with its confirmed breast cancer plummeting property in xenografted mice models certainly specifies extracted vinorelbine as a suitable alternate for existing marketed breast cancer drugs.

4.9 FUTURE WORK

- The present study was performed in a very small scale. The process can be optimised to increase the extract production on a large scale.

- The optimised process can be very useful for better productivity and a product of good quality.

- The produced Vinorelbine may be used for controlling the breast cancer.
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