A STUDY ON THE EFFECT OF HOMEOPATHIC MEDICINES ON THE INHIBITION OF TUMOUR PROGRESSION EMPHASIZING BREAST CANCER METASTASIS IN EXPERIMENTAL ANIMALS

Thesis submitted to

THE UNIVERSITY OF CALICUT
KERALA, INDIA

for the fulfilment of the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY
(FACULTY OF SCIENCE)

By

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CERTIFICATE

This is to certify that the thesis entitled **"A STUDY ON THE EFFECT OF HOMEOPATHIC MEDICINES ON THE INHIBITION OF TUMOUR PROGRESSION EMPHASIZING BREAST CANCER METASTASIS IN EXPERIMENTAL ANIMALS"** is a bona fide record of research work carried out by **Ms. REMYA V.**, under my guidance and supervision at Amala Cancer Research Centre, Thrissur, Kerala, India, and no part thereof has been presented for the award of any other degree, diploma or other similar titles of any University or Institute. The thesis has been checked for plagiarism and the similarity indices are within the allowed limits as recommended by the University of Calicut.

Place: Thrissur
Date: 10-05-2017

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DECLARATION

I hereby declare that the thesis entitled **"A STUDY ON THE EFFECT OF HOMEOPATHIC MEDICINES ON THE INHIBITION OF TUMOUR PROGRESSION EMPHASIZING BREAST CANCER METASTASIS IN EXPERIMENTAL ANIMALS"** is based on the original research work carried out by me at Amala Cancer Research Centre, Thrissur, Kerala, India, under the guidance and supervision of **DR. Girija Kuttan, Ph.D.**, Professor, Dept. of Immunology, Amala Cancer Research Centre, and no part thereof has been presented for the award of any other degree, diploma or other similar titles of any University of Institute.

Place: Thrissur
Date: 10.05.2017

Remya V.

ACKNOWLEDGEMENT

From the first and foremost word, I express my sincere and whole-hearted gratitude to my supervising guide, **DR. Girija Kuttan, Ph.D.**, with all my respect and obligation. Her expertise in the research field, timely suggestions and support were immensely helpful to handle the hurdles all along the way of past five years of my career till the submission of this thesis. Moreover, beyond the role of a research guide, she always used to deliver sensible advice, moral and emotional support during every tough situation throughout my life. I am really grateful to my dear supervisor to offer such a wonderful makeup, which will surely be an everlasting memory in my life.

I am deeply thankful to **DR. Ramadasan Kuttan, Ph.D.**, Research Director, Amala Cancer Research Centre, whose well-timed involvement and support is unforgettable at any point of my research career, without which it could not be possible for me to complete my Ph.D. studies at this time. He owns such a gentle, approachable and intellectual personality who always offer open helping hands for any student who approaches him.

I would like to acknowledge the respected academic faculties, **DR. Jose Padikkala, Ph. D.**, **DR. K.K. Janardhanan, Ph. D., FRCS**, **DR. T. D. Babu, Ph. D.**, **DR. Achuthan C. Raghavamenon, Ph. D.** and **DR. Jose Chungath** for their unconditional support with needful suggestions and criticisms during my period of research.

I wish to express my sincere thanks to **Padmabhooshan Rev .Fr. Gabriel CMI**, Founder Director, **Rev. Fr. Walter Thelappilly CMI**, Former Director, **Rev. Fr. Francis Kurisserry**, Managing Director, **Rev. Fr. Julius Arakkal**, Joint Director, Amala Institute of Medical Sciences, all academic and non-academic staffs for their kind co-operation and support to carry out my research at Amala Cancer Research Centre.

I would like to acknowledge, **University Grants Commission, Govt. of India**, for the fellowship grant provided during my Ph.D. studies.

It is my immense pleasure to acknowledge my dear lab mates, Mrs. Gilcy George K., Mrs. Vishnu priya Murali and Mrs. Sowmya T.M. for all the great time we had together. Moreover, I sincerely remember their mutual support, friendly criticisms and co-operation which were very helpful to manage the academic matters and to maintain a pleasant and comfortable atmosphere in the lab. I would also like to thank

the technical staffs in our lab, Mr., Arunachalam, Mr. Vivek, Mrs. Deepa Kewin, Mrs. Nirmala Sankar and Mrs. Santha for their kind attention and concerns for helping our lab research.

It would be very formal if I acknowledge my good friend circle at Amala. But as part of the formalities, I heartily thank Ms. Veena Ravindran, Ms. Tinchu Thankachen, Mr. Liju V.B., Mrs. Chubicka Thomas, Mrs. Salini Sasidharan, Mr. Arunaksharan N., Ms. Soorya Narayanan, Mrs. Sumitha Thushar and everyone who was there with me for any single occasion, for accepting me as one of you and for filling my life much enjoyable and energetic with many memorable moments and events at Amala.

I wish to acknowledge all other colleagues of Amala Cancer Research Centre, DR. Pratheesh Kumar P., DR. Hamsa T.P., DR. Siveen K.S., DR. Sindhu E.R., DR. Firdous A.P., DR. Jeena K., Mrs. Binitha P.P., Mrs. Lincy Lawrence, Mrs. Seema Menon, Mr. Vipin Sivaram, Mrs. Indu M.S., Mrs. Divya Menon K., Mrs. Meera R. Nair, Mrs. Jeksy Jos, Mrs. Smitha K.R., Mr. Shaji E.M., Mr. Ravikumar and Mrs. Greeshma for their good wish, support and kind approach towards me.

I am sincerely thankful to the non-academic staffs of Amala Cancer Research Centre, Mrs. Hemalatha, Mrs. Preetha, Ms. Thankammani and Mrs. Sumathy for all the help provided by them.

Most importantly, I wish to remember my most beloved friends, Ms. Arya Chandran L., Mrs. Reshma Sujith, Mr. Nikhil Anto P., Dr. Sruthy Anto P., Dr. Aravind S., DR. Dileep K.V., Mr. Yathi Premchand, Mrs. Revu S., DR. Indu S., Ms. Shameem A. and DR. Sreeja S. Thank you all for being with me during every moment of my difficulties and happiness. It is a real fact that without you people I could not be able to successfully complete this effort.

I would like to affectionately convey my heart felt gratitude to DR. Ruby John Anto and DR. Anto P.L. for all their support, care and loving concerns, even though it is definitely beyond words could say.

I am deeply committed to my parents, sister and brother in-law for their loving concerns, support and criticisms during the period of my research career. Especially, special thanks to my dear Achan for your daily prayers, well wish and passionate support for my higher studies. Your life really inspired me a lot.

Last but not least, I am sincerely obliged to the God Almighty for treating me with a mishmash of immense blessings, lots of happiness, confusing obstacles, worries and tears, helping people, wonderful friends and so on. It is because of all your blessings that I could do something for which I wish to achieve.

Remya V.

Dedicated to my dear Achan…

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Introduction

Introduction

The term cancer became as common as anything in our day to day life, simply because of the increased rate of cancer related diagnosed and deceased people around us. Scientifically, cancer refers not only to a single symptomatic disease but should be defined as the cumulative result of a group of correlated diseases that can affect any part of the body whose genetic causes and clinical effects are much diverse at the individual level. The recent global cancer statistical estimates published by the International Association of Research on Cancer (IARC) in the GLOBOCAN report 2012 states that there are 14.1 million newly diagnosed cases, 8.2 million cancer related deaths and there will be an expected rise of 70% new cases by the next two decades. Out of this, more than 60% of total new cases and 70% of deaths account for Asia, Africa, Central and South America (GLOBOCAN 2012).

To the present knowledge, the carcinogens which are physical (Ultra-Violet rays, ionising radiation etc.), chemical (tobacco smoke, asbestos, aflatoxin, arsenic etc.) or biological agents (infections by HPV, HBV etc., bacteria and parasites) to which the humans exposed accidentally or habitually, along with the hereditary triggering factors, have different mechanisms to initiate carcinogenesis, inducing dissimilar combination of genetic changes. Apart from accidental or involuntary exposure to carcinogens, there are risk factors that can be purposefully avoided which will definitely helpful to manage cancer incidence. Those factors such as alcohol abuse, tobacco smoking, obesity, lack of physical activity, unhealthy diet, HPV and HBV infections, air pollution, indoor smoke from fuels etc., when make avoidable at the individual or social level, will help to prevent about 30% of cancers. Out of these, tobacco remains as the single key risk factor that accounts for 20% of global cancer deaths and 70% of global lung cancer deaths (GLOBOCAN 2012).

However, in spite of the varying mechanisms of different carcinogens to start the multistage process of carcinogenesis that includes initiation and promotion, its ultimate result is the deregulated and excessive proliferation of cells starting from a pre-malignant lesion and result in malignant tumours that can display

immune surveillance and escape mechanisms to well establish in their microenvironment. This is then followed by the invasion-metastatic cascade, where various factors associated with the primary tumour microenvironment promotes the invasive phenotype of the tumour cells resulting in their ability to dislodge from the primary tumour, get intravasated to the circulation overcoming the shearing forces during their traverse, extravasated at favourable distant microenvironments and localization at their homing sites to from secondary metastases. Moreover, angiogenesis is also a complementary key phenomenon, where the solid tumour utilises body's own mechanism for their existence and development, as the solid tumour gets enlarged. The proliferating tumour cells secrete angiogenic factors that stimulate surrounding blood vessels to get directed and sprout neovessels towards tumour sites. Metastasis and angiogenesis are the major hurdles against cancer treatment strategies as they are the key promoters of systemic spread of cancers and responsible for 90% of cancer related deaths worldwide.

Hence, the status quo can be called as just manageable with conventional and developing modern therapies of cancer, even though they do not precisely promise a complete cure and definitely contribute unavoidable side effects. The recent cancer treatment strategies include a combination of surgery, chemotherapy, radiotherapy, adjuvant therapy or hormone therapy depending on the age and health status of the patient, position, stage, grade and receptor status of tumour etc. Surgery is implemented as a safe management that surgically removes the tumour tissue along with some peripheral normal tissue which will prevent the further dissemination of tumour cells from the primary tumour. Radiotherapy, using gamma rays, X-rays or other sources to kill the cells by the generation of free radicals, is meant for localised solid tumours prescribed before or after surgery or chemotherapy depending upon the decided treatment modality. Chemotherapy involves the injection of chemotherapeutic drugs regionally or systemically that prevents the survival of rapidly proliferating cells by different signalling mechanisms. The combination of chemotherapy drugs depends on the type of cancer, its surface markers, patient's health status etc. Immunotherapy is yet another strategy that uses antibodies against particular

molecules associated with cancer cells that may be given alone or along with chemotherapy. Hormone therapy is specifically used for cancers of hormone dependant tissues such as breast and prostate that uses hormone inhibitors to prevent hormone dependant proliferation of cancer cells. Besides these, several targeted combination therapies, stem cell transplant therapies etc. are under advanced clinical trials, and are reported to become very promising in near future.

Apart from these modern therapies of cancer, complementary and alternative medicines such as Ayurveda, Homeopathy, Chinese medicine, Unani, Acupuncture etc. are also being more pronounced nowadays, as research evidences are suggesting the therapeutic interventions of several natural products, polyherbal combinations and traditional medicines. Even if they may not serve as stand-alone efficient therapies to combat cancer, several of them were proved to be potent enough to prevent cancer, inhibit cancer cell proliferation without side effects, and sometimes could also cure the side effects such as immune suppression or genotoxicity caused by conventional therapies. Hence, the utilisation of the unique capability of these natural products and alternative medicines can be explored along with conventional therapies to develop an innovative integrative platform to defend against the traumatic disease, cancer.

Since the present study deals with homeopathic medicines, it is significant to discuss more about the Homeopathic System of Medicine. Homeopathy is a holistic discipline of alternative medicine, rooted long back in 18^{th} century, which conventionally comprises a vast array of mother tinctures and their ultra-diluted potentized forms as remedies for various types of transient and chronic ailments. Hence, this system of medicine also suggests remedies for malignant conditions in combinations, in a precise view of individualised medicines. The classical homeopathy relies on the principle of ‘like cures like’, proposed by the German physician Samuel Hahnemann, which denotes that those medicines that can impose a set of symptoms in healthy individuals can be used to cure similar symptoms in ill individuals. Moreover, potentization is regarded as the vital factor that determines the efficacy of a medicine which involves ultra-dilutions

of the starting material along with vigorous shaking in each step. Furthermore, it proposes a curing mechanism which is implemented through the body's own defence arm, which is the immune system. But, scientific evidences are not much accountable to support the classical basis, mechanism, application and popularity of this ultra-dilution therapy.

The last two decades are renowned for some outstanding experimental and clinical evidences that support homeopathic treatment of various diseases including cancer, even though there are opposing isolated reports which are controversial and declaring that the scientific evidences are just overstating the placebo effect. In this scenario, it is an urgent need to clinically evaluate and scientifically validate the efficacy of these medicines with a clear line of distinction between yes or no possibilities, without compromising the basic principles of homeopathy. Even though there are methodological constraints while dealing with pre-clinical experiments, a good number of scientific publications do proclaim the capability of homeopathic remedies to delay or inhibit the progression of cancer in several *in vitro* and *in vivo* experimental models. A number of results which should be mentioned include the effect of *Sabal serrulata* to reduce prostate cancer (Mac Laughlin et al., 2006), *Chelidonium* to inhibit chemically induced hepatocarcinoma (Biswas and Khuda-Bukhsh, 2002), *Ruta* 200C and *Phosphorous* 1M to inhibit chemically induced hepatocarcinoma and sarcoma respectively (Kumar et al., 2007), preliminary results that *Thuja occidentalis* inhibit metastasis (Sunila et al., 2006), *Ruta graveolens* to inhibit glioblastoma in animal models and the potential of drugs such as *Carcinosin*, *Phytolaca*, *Conium* and *Thuja* to exert cytotoxic effects in breast cancer cell lines (Frenkel, 2010). Moreover, the efficiency of potentized form of *Hydrastis candensis*, *Lycopodium clavatum*, *Carcinosinum* etc. to inhibit cancer development was also studied (Sunila et al., 2007). Some others such as *Calcarea carbonica*, *Sulfur*, *Lachesis*, *Kali carbonicum*, *Cobaltum* 30C, *Causticum* 30C, *Traumeel S*, *Belladonna* 7C were reported to reduce the side effects of conventional therapies in cancer survivors. *Ruta* 6C and *Calcarea phosphorica* caused the regression of intracranial tumours in patients without any additional conventional therapy (Oberbaum et

al., 2001; Jacobs et al., 2005; Balzarini et al., 2000). However, solid clinical evidences which point out the specific effects of homeopathy against cancer have not evidenced till date. But the available evidences from multiple research disciplines suggest two significant opinions that the remedies are safe without undesirable side effects and also they are having important roles at least to improve the health status of the patients.

In the light of these background evidences, we have investigated the possible potential of some selected homeopathic drugs to interfere with the key stages of cancer progression, such as metastatic episode, angiogenesis and the defence status of immune system, emphasizing breast cancer metastasis in experimental animals. The drugs selected for the study were potentized forms of *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum* at their 1M, 200C and 30C potency. As mentioned earlier, the selected drugs were being prescribed by homeopathic practitioners in combination remedies for cancer and also shown to have significant activity against different types of cancers in experimental and clinical stages.

Chapter 1 - Review of Literature

1.1. Epidemiology and Etiology of Cancer

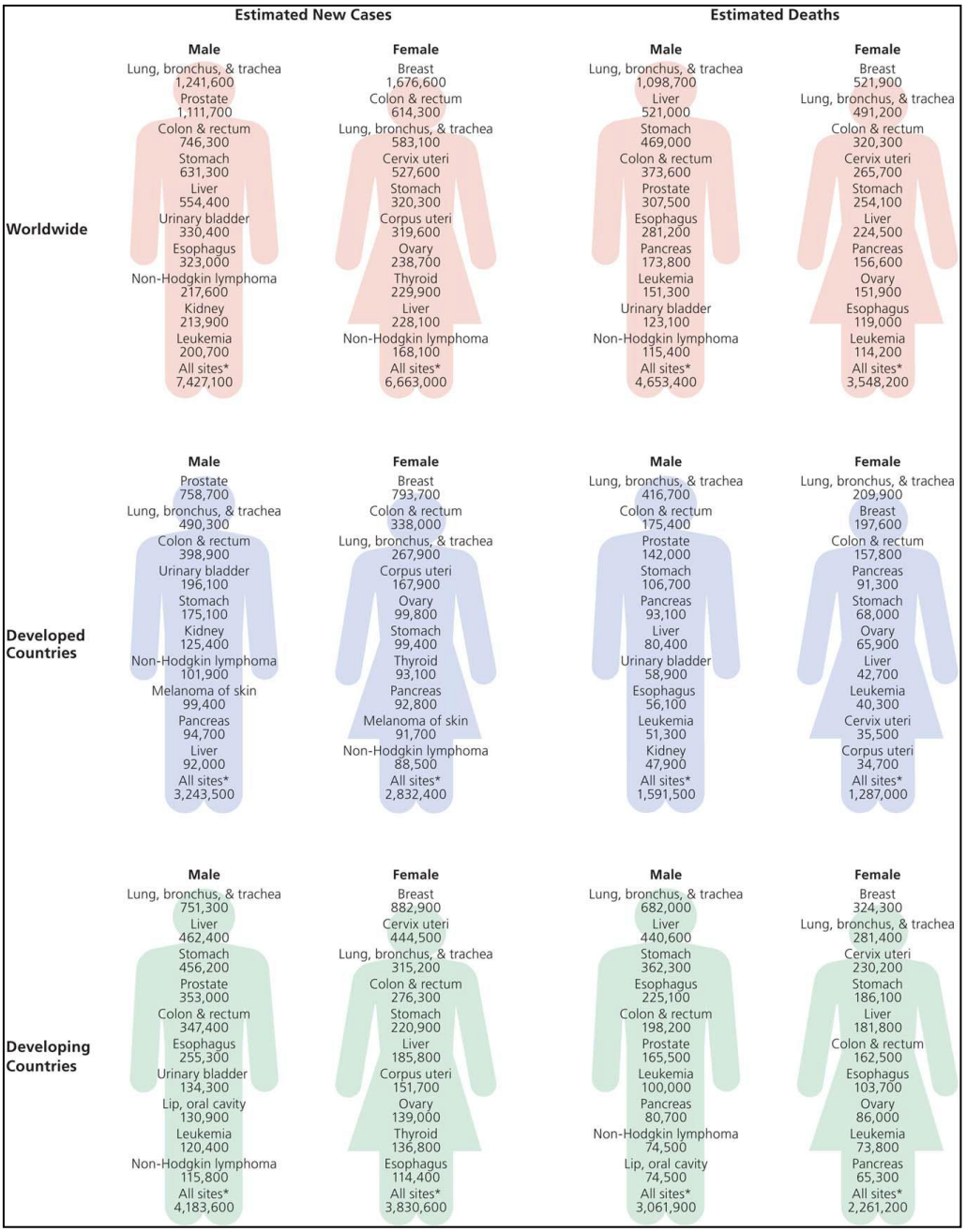
1.1.1. Cancer –The global scenario

The World Health Organisation (WHO) through its special cancer agency called IARC, which primarily aims to promote international collaboration in cancer research, provides the statistical estimates of global cancer burden with the assistance to cancer registries world-wide. The reports were periodically included in key publications such as Cancer Incidence in Five Continent series and the database, GLOBOCAN. GLOBOCAN provides incidence, mortality and prevalence of major types of cancer in about 184 countries worldwide. According to GLOBOCAN 2012, cancer is one of the leading causes of morbidity and mortality world-wide, which accounts for 14.1 million new cases and 8.2 million deaths in 2012 (Fig. 1). Lung cancer and breast cancer are the frequently diagnosed and also the leading cause of death in men and women respectively in global and in less developed countries. Other cancers which are frequently diagnosed globally include that of liver, stomach and colorectum among males and stomach, cervix, uteri and colorectum in females. But in more developed countries, those frequently diagnosed ones also include bladder cancer in males and uterine cancer in females. Meanwhile, prostate cancer is the most frequently diagnosed cancer in males and lung cancer remains the leading cause of deaths in females here. The burden of cancer continue to shift to the less developed countries with an incidence rate of 57% while the mortality rate of 65% of global cancers. Also, combined incidence rates of all cancers are twice as higher in more developed countries but the mortality rates were only 8-15% than less developed countries. This difference may be due to the late detection, unavailability of proper treatment, and increasing prevalence of risk factors in less developed regions (Torre et al., 2015).

1.1.2. Causative agents of cancer

Cancer is defined as a group of related diseases which is caused by the combined causative effects of exogenous and endogenous factors. Exogenous factors include physical, chemical or biological agents which can cause cancer

Figure 1.1. Estimated number of new cases and deaths by sex and level of economic development, 2012



(*excluding melanomas). Source: GLOBOCAN 2012

or increase the incidence of cancer are called as carcinogens. Endogenous factors include the genetic, hereditary or age-related causes that can lead to carcinogenesis. In addition these, there are several risk factors which can contribute to cancer, such as dietary habits, alcohol abuse etc. and it has been reported by WHO that about 30% of all cancers can be preventable by strictly controlling these risk factors (Pitot and Dragan, 1991; IARC 2012).

1.1.2.1. Exogenous Factors

1.1.2.1.1. Physical carcinogens

The physical carcinogens include ionising (X-rays, gamma rays radioactive sources such as radon etc.) and non-ionising radiation (Ultraviolet (UV) rays) which can affect any part of the body and the carcinogenic effects are induced by direct or indirect action as particles or photons. They can interact with any biological molecule creating ionisation or excitation of orbital electrons and cause the formation of high energy free radicals which can interact with biomolecules of the cell subsequently creating multiple cellular damages. Among them, most harmful ones include that on the existing DNA molecules resulting in damage of nucleotide bases, single strand and double strand breaks (SSBs and DSBs) in DNA which may lead to chromosomal abnormalities and genetic mutations. Most DSBs are likely to undergo illegitimate recombination and joining processes of repair, which are error-prone and hence multiple DSBs contribute to vast and stable chromosomal aberrations, reciprocal translocations and serious mutagenic lesions. Radiation can often induce point mutations, deletions and chromosome rearrangements which can cause loss of heterozygosity (LOH) of genes, which is an important cause of inactivation of tumour suppressor genes such as rather than activation of proto-oncogenes (Little, 2000).

Experiments in a good number of *in vitro* systems evidenced the alterations in members of *RAS* family, *c-MYC, MDM2, p53* etc., subsequent deregulations in cell cycle checkpoints, suggesting that multiple pathways are involved in radiation induced genetic instability which is transmissible for a number of generations of the cell lineage which is required to initiate the carcinogenic

process (Little, 2000, Ward, 1995, Li et al., 1992). Other than sunlight, radiation associated with occupational hazards such as from mines, nuclear plant, medical diagnosis and therapies may increase the risk of lung cancer, multiple myeloma, brain tumours, skin cancers, thyroid cancer etc. and recurrence of cancer in survivors according to recent literature (Cardis et al., 2007; Muirhead et al., 2009; Meulepas et al., 2016; Kim et al., 2016; Teng et al., 2016).

1.1.2.1.2. Chemical carcinogens

The chemical carcinogens include various natural and synthetic compounds which may be natural sources, pesticides, chemicals used in industries, waste products and effluents of various manufacturers etc. and can be grouped into different types as shown in table 1. Besides, IARC has reassessed the chemical and occupational circumstances of a group of chemicals enlisted as 'Group I carcinogens' in order to identify the additional tumour sites and mechanism of carcinogenesis.

The grouped list includes aromatic amines, PAH a related exposures, other chemicals and other complex exposures. Four aromatic amines and two others related to auramine and magenta production industries are evidenced to causes urinary bladder cancer. PAHs from exposure to chimney sweeping, coal gasification, coal-tar distillation, coke production, aluminium industry were reported to cause skin and lung cancer. Exposure to other chemicals such as aflatoxins and vinyl chloride can cause hepatocellular carcinoma; sulfur mustard can cause lung cancer whereas formaldehyde can cause nasopharyngeal cancer and leukaemia, all of them having strongly evidenced to cause genotoxicity. Other complex exposures include iron and steel industry which causes lung cancer, paints cause cancers of lung, urinary bladder and pleural mesothelioma; rubber manufacture industry causes leukaemia, lymphoma, cancers of urinary bladder, lung and stomach etc. (Bann et al., 2009).

1.1.2.1.3. Biological carcinogens

Biological carcinogens include infectious agents such as bacteria, fungi, virus or some parasites, whose chronic infections may lead to of carcinogenesis.

Table 1.1. Chemical Carcinogens

Group	Compound	Mechanism of action	Affected organ/ Cancer type
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene, Polychlorinated biphenyls (Luch, 2005)	Form adducts with purine bases of DNA, mainly resulting on transversions	Skin, Lungs, Stomach Liver, Skin
Aromatic amines/amides	2-Acetylaminofluorene 4-Aminobiphenyl 2-Naphthylamine (Luch, 2005)	Genotoxic compounds, increase the rate of cell duplication	Liver, Bladder Bladder Bladder
Aminoazo dyes	o-Aminoazotoluene N,N-dimethyl-4-aminoazobenzene (Golka et al. 2004)	Forms adducts with DNA and with hemoglobin	Liver, lungs, bladder Lungs, Liver
N-nitroso compounds	N-itrosodimethylamine (Hasegawa et al. 1998)	Form adducts at N- and O-atoms of DNA bases	Liver, lungs, kidneys
Carbamates	N-methylcarbamate esters (Wang et al. 1998)	Chromosome aberration, gene mutation, cell transformation	Experimental results showed liver, kidneys and tests degeneration
Halogenated Compounds	Trichloroethylene (Lock et al. 2007)	Somatic mutations, modification of cell cycle pathways	Experimental results showed kidney, liver and lung cancer
Natural Carcinogens	Aflatoxin B1 (Wild et al. 1986) Asbestos (Luch 2005)	Form adducts with guanine, react with RNA and proteins	Liver Lungs
Metals	Arsenic (Shi et al. 2004) Cadmium (Hartwig et al. 2002) Nickel (Costa et al. 2003)	Oxidative stress Inhibit DNA repair pathways and nucleotide-excision repair Histone acetylation And DNA hypermethylation	Skin, lungs, liver Lungs, prostate, kidneys Lungs, nasal cavity
Anticancer Drugs	Alkylating agents (Luch 2005)	Interstrand and/or intrastrand cross-links	Leukaemia

(Source: Oliveira et al., 2007)

A large number of scientific studies have proved that the pathogenesis caused by different bacterial strains is strongly correlated to carcinogenesis. The best example is the ulcer formation and chronic gastritis caused by *Helicobacter pylori* which can increase the risk of gastric cancer, mucosa associated lymphoid tissue (MALT) lymphoma and other inflammatory cancers (Crowe et al., 2005). Other species include *Salmonella typhi* and gall bladder cancer, *Streptococcus bovis* and colon cancer, *Chlamydia pneumoniae* and lung cancer etc. Bacterial agents follow different mechanisms such as chronic infections, immune evasion and immune suppression to induce carcinogenesis. Studies proved that these bacterial agents or their toxins can alter the cell cycle regulatory pathways or can promote other pathways that can lead to the proliferation, survival, invasion and even metastasis of transformed cells (Mager, 2006).

Different viruses that cause cancer include Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Papilloma Virus (HPV), Epstein-Barr virus (EBV), Human herpes virus 8 (HHV8), Human thymus-derived-cell leukaemia/lymphoma virus-1 (HTLV-1), Human Immunodeficiency Virus (HIV) etc. Chronic infection with HBV is a major risk factor for hepatocellular carcinoma, where the integration of viral genome results in insertional mutagenesis which results in deletions, translocations, transversions or duplications in the host genome. The genomic instability results in chronic inflammation that leads to liver cirrhosis and finally leads to hepatocellular carcinoma. HCV doesnot integrate into the genome, but induce the carcinogenic effects through chronic inflammation and stimulation of humoral and cell mediated immune responses. The direct cellular effects include interaction with proto oncogenes, transformation of *ras* gene, inhibition of apoptosis and inhibition of Tumour Necrosis Factor-α (TNF- α) signalling pathways. EBV is an established carcinogen of non-Hodgkin's lymphoma, where the viral infection results in the interaction of viral proteins with signalling molecules within the cell, interact with *Rb* gene and inhibit *p53* mediated apoptosis. HPV infection is sexually transmitted and the infection is followed by genome integration to the host cells which then interact with cell signalling pathways by inhibiting *p53* mediated apoptosis, disruption of mitotic check points and

accumulating genetic mutations. They are more significant to cause cancers of cervix and others such as of vulva, anus, penis, head and neck etc. HHV8 viral infection is associated with Kaposi's sarcoma, non-Hodgkin's lymphoma of B cells, primary effusion lymphoma and Castleman's disease, where the infection results in inhibition of *Rb* and *bcl-2* mediated apoptosis, stimulation of cytokines and angiogenic factors etc. which promotes carcinogenesis. HTLV-1 infections are transmitted through breast milk, sexual contact or blood transfusions and the infection process include interaction of vial transcription factors may promote the genes for cell proliferation and inhibit cell-cycle inhibitors (Kuper et al., 2000).

Apart from bacterial and viral carcinogens, certain parasites such as blood flukes and liver flukes, when initiate chronic infection in the host, are capable of increased production of NOS and RNOS and also can promote the release of endogenous carcinogens. The blood fluke *Schistosoma hematobium* is a definite cause of bladder cancer and the liver fluke *Opisthorchis viverrini* cause an increased risk of cholangiocarcinoma (Kuper et al., 2000).

The role of fungi in the etiology of cancer is also much pronounced in recent years as the studies on carcinogenic mycotoxins has been very much discussed this time through various literature. They may induce carcinogenesis either by altering the host antibody responses, preventing the host defences or producing carcinogenic mycotoxins. Recent studies have shown that *Candida albicans* and other yeasts are capable of predominantly causing oral cancers. Certain species such as *Aspergillus*, *Fusarium* etc. may produce metabolites such as aflatoxins, fumonisines, ochratoxins, zeraleone and are linked with cancers of liver, oesophagus, prostate etc. Certain other human pathogenic such as *Candida parapsilosis*, *Trichophyton*, *Epidermophyton* and *Scopulariopsis* are known to produce carcinogens. *Alternaria alternate* can produce alternariol and alternariol monomethyl ether is associated with high risk of oesophageal cancer. Similarly, *Aspergillus versicolor* was reported to cause gastric cancer and this is correlated by the production of N-nitroso compounds and mycotoxins produced by the fungus. Aflatoxins, cyclosporine A, fungal metabolites such as oxalic acid, calcium oxalate, were also verified to be linked with cancer (Wainwright, 2002).

1.1.2.1.4. Habitual and life style factors

Tobacco use: According to WHO reports, tobacco use accounts for the major cause of about 20% of all cancers and the cause of 70% of lung cancer deaths world-wide. It gradually kills 50% of its users that accounts for the death of about six million people per year world-wide, out of which about five million are direct smokers while the remaining are victims of second-hand exposure. This abuse is strongly related to the stimulating and addictive property of nicotine, which can be released by the use of any form of tobacco products. Cigarettes or their alternative forms are the major used forms of tobacco which contains about 60 known carcinogens in the main stream smoke and the same in the side-stream smoke too. Even though lung cancer is considered strongly associated with tobacco use, it can cause all histological types of cancers such as squamous cell carcinoma, small cell carcinoma, adenocarcinoma (including bronchiolar–alveolar carcinoma) and large cell carcinoma, cancers of bladder, uterus, renal pelvis, oral cavity, liver, larynx, stomach, oesophagus, cervix etc (IARC, 2004). The carcinogens include strong ones such as such as polycyclic aromatic hydrocarbons (PAH), N-nitrosamines and aromatic amines and weak ones such as acetaldehyde and isoprene. PAH are usually locally acting carcinogens and they are strong carcinogens such as Benzo[a]pyrene (BaP) (Dipple et al., 1984). N-Nitrosamines are a large group of systemic carcinogens including N-nitrosamines 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN), which are found only in tobacco (Hecht and Hoffmann, 1988). Combustion products of aromatic amines include bladder carcinogens such as carcinogens 2-naphthylamine and 4-aminobiphenyl (Luch, 2005). Up on metabolic activation in the physiological system, the tobacco-specific NNK forms DNA adducts, increase the formation of hydroxyl radicals and other free radicals, which further damage the DNA forming single strand breaks, increase oxidative stress, lipid peroxidation and form protein adducts. Moreover, NNK can mutate Kras and p53, can modulate various signal transduction pathways modulated through PI3K-Akt, Erks, IGF-IR which can mediate cell proliferation, survival and metastatic growth of tumours (Yalcin and de la Monte, 2016).

Alcoholism: Chronic consumption of alcohol is associated with risk of cancers of oral cavity, larynx, pharynx, oesophagus, liver, pancreas, colorectum and breast. Alcohol can also act as a co-carcinogen along with HBV and HCV infections, oestrogen dependant breast cancers as well as tobacco induced carcinogenesis. Studies on the mechanism of alcohol-induced carcinogenesis suggest that alcohol is not a carcinogen, but a co-carcinogen whose metabolic products such as acetaldehyde and free radicals may interact with DNA and proteins; interfere with folate metabolism and DNA repair. Moreover, induction of cytochrome P4502E1, alterations in cell cycle, and alterations in NF-κB proinflammatory pathways by alcohol metabolites which are prominent leads of carcinogenesis (Anand et al., 2008; Boffetta and Hashibe, 2006).

Obesity: The American Cancer Society reports that obesity is linked with 14% of cancers in males and 20% in females, including cancers of breast, endometrium, colon, kidney, oesophagus, prostate, pancreas, gall bladder and liver. Increased modernization, adopting western diet and sedentary life styles are majorly contributing to increased prevalence of obese people in developing countries. Reports from various studies signify that insulin, adiposity, neurochemicals, hormones, insulin resistance, inflammation etc are main intermediates of obesity and carcinogenesis through multitargeting signalling pathways such as IGF/insulin Akt pathway, leptin/JAK/STAT pathway, hyperglycemia or inflammation linked NF-κB pathway (Anand et al., 2008).

Physical inactivity: Increased risk of cancers of breast, colon, prostate, pancreas and melanoma has been found associated with physical inactivity among men and women. In women, breast cancer risks are contributed by increased estradiol levels, lower concentration of hormone binding globulin, larger fat masses and higher levels of insulin in serum. On the other hand, incidence risk of colon cancer is associated with increased insulin levels, altered prostaglandin levels, immunosuppression and altered bile acid metabolism. Similarly, higher testosterone level and IGF-1 in the blood and lack of immunity due to physical inactivity accounts for prostate cancer in men. Besides these, another study indicated that sedentary men and women have about 56% and

72% of higher incidence of melanoma respectively than those exercising 5-7 days/week (Anand et al., 2008).

Diet: The extent to which diet influences cancer depends up on the type of cancers, but most of cancers are prone to occur by modified and unhealthy dietary habits, which has been seen very prominent in various countries or regions. Food carcinogens include macro components which act directly and micro components which act directly such as genotoxic agents (Sugimura, 2002; Anand et al., 2008). The daily food that we consume contains significant types of carcinogens which will be food additives or toxic compounds generated by cooking methods, such as in the form of nitrosamines, nitrates, pesticides, dioxins etc. For, instance, the consumption of red meat such as beef has been extensively reported to produce carcinogenic substances upon cooking and is known to linked with cancers of oesophagus, stomach, colorectum, prostate, breast, pancreas and oral cavity. The aromatic hydrocarbons and heterocyclic amines (HCAs) produced during cooking of meat, the pyrolysates and amino acids produced by charcoal or smoke processing of meat are strong carcinogens (Bingham et al., 2002; Chao et al., 2005; Hogg, 2007; Rodriguez et al., 2006; Garcia-Closas et al., 2007; Tappel, 2007; O'Hanlon, 2006; Toporcov et al., 2004). PhIP (2-amino—methyl-6-phenyl imidazo [4,5-b] pyridine) is reported to be the most abundant carcinogen by mass of cooked beef and accounts for about 20% of mutagenicity (Lauber and Gooderham, 2007). Moreover, food with high calorie content such as fat-rich food has a positive correlation with cancer incidence while healthy food rich with vegetables, fruits and antioxidants has negative correlation and preventive action on cancer. Long term exposure to nitrile preservatives of food products, bishenol content in plastic food containers which will be contaminated with the food are associated with different types of cancers including cancers of breast and prostate (Sasaki et al., 2002, Durando et al., 2007, Ho et al., 2006). Moreover, arsenic content in food increase the risk of bladder, kidney, liver and lung cancers (Szymanska-Chabowska et al., 2002). Other components such as saturated fatty acids, trans fatty acids, refined sugars and flour are also associated with different types of cancers, several of them are reported to activate inflammatory pathways. Also, recent case-control and

cohort studies suggest that higher salt and salted food intake is associated with increased incidence of gastric cancer and has a synergistic effect on *H.pylori* mediated inflammation and malignancy (Tsugane, 2005).

1.1.2.2. Endogenous Factors

1.1.2.2.1. Genetic factors

It has been scientifically evidenced that heredity and other genetic factors are playing key roles in the incidence of certain types of cancers such as colorectal, prostate, breast ovarian cancer etc. The familial incidence of these cancers is noticeably prevalent nowadays and scientific studies are progressing to explain the influences at the molecular level (Bale and Li, 1997). The genetic susceptibility of breast cancer is an autosomal dominant penetrant mutation of genes such as *BRCA1* and *BRCA2* which may be deletions or insertions probable to take place anywhere within the gene. Besides, familial inheritance of mutations in *p53*, *PTEN* etc. were also associated with genetic syndromes, Li-Fraumeni and Cowden's respectively, which are linked to high risk of cancer. The risk of susceptibility of breast cancer to a woman is two or more times greater if she has a first degree relative who developed the disease before 50 years of age, and the risk is even more higher if the relative developed the disease at her younger age. Moreover, family history of a particular type of cancer is also linked to risks of other cancers of colon, ovary, prostate etc. which can follow similar mutation patterns (McPherson et al., 2000). Colon cancer is also associated with inherited syndromes (2-5%) such as Lynch syndrome, familial adenomatous polyposis, MUTYH-associated polyposis, certain hamartomatous polyposis conditions and one-third of colon cancer exhibit increased familial risks, with a number of less penetrant but more frequent susceptibility genes have been identified (Jasperson et al., 2010). It has also been evidenced that genetic factors are significantly contributing to the occurrence of lung cancer. The risks are higher in individuals whose close relatives were diagnosed by the disease at their young age and in those with multiple affected family members, along with the triggering co-factors of smoking and other environmental exposures (Matakidou et al., 2005). There are

also reports that signify the elevated hereditary risk factors associated with pancreatic and prostate cancer incidence and mortality (Klein et al., 2000, Bratt, 2002). However, even though genetic factors can be a stand-alone reason for carcinogenesis, environmental and habitual risk factors significantly contribute complemented effects to promote the incidence of all these types of cancers (Lichtenstein et al., 2000).

1.1.2.2.2. Hormonal factors

Hormone related cancers of breast, endometrium, ovary, prostate, thyroid, osteosarcoma etc. has been vastly reviewed in literature which explains the hormone dependant cell proliferation and the randomly accumulating genetic errors occur during this excessive and deregulated proliferative cycle, contributing to one-third of all cancers. The resulting malignant phenotype depends up on the pattern of mutations and the various genes such as those in the endocrine pathway, tumour suppressor genes, oncogenes and DNA repair genes involved throughout these processes. *BRCA1* and *BRCA2* are the most studied tumour suppressor genes associated with the susceptibility to breast cancer, ovarian cancer and some other types of cancer (Miki et al., 1994; Wooster et al., 1995). Similarly, germ line mutations in *TP53* (Li, 1996) and overexpression of *HER2/neu* oncogene (Pietras and Pegram, 1999) are also studied to be linked to advanced breast cancer. Estrogen-induced breast cancer has been reported to be the result of molecular operations such as by the alkylation of cellular molecules, the generation of active radicals that can damage DNA together with the potential genotoxicity of estrogen and some of its metabolites (Nandi et al., 1995; Yager and Leihr, 1996). Estrogens usually increase the proliferations of cells of breast and endometrium, while progestogens increase the same in breast cells, but decrease proliferation in the endometrium. Hence addition of progestogens in estrogen therapy after menopause may decrease the risk of endometrial cancer, but increase the risk of breast cancer. Long term exposure to estrogen such as conditions of menarche at early age, late menopause, continuous estrogen therapy after menopause, sequential intake of oral contraceptives, obesity are significant factors that can contribute to increased risk of breast cancer or endometrial cancer (Henderson et

al., 1991; Harris et al., 1992; Colditz et al., 1992). On the other hand, pregnancy has a life time influence on breast cancer incidence, as the risk is comparatively high during the early period after pregnancy, due to hormonal stimulations, but later on due to the permanent differentiation of cells the risk will be getting lowered (Lambe et al., 1994; Rosner et al., 1994; Russo et al., 1993). Besides these, lactation also modestly reduces the risk of breast cancer (Newcomb et al., 1994). Apart from these, regular exercise, avoidance of alcohol consumption, first full term pregnancy at the young age, consumption of food substances such as soybeans, flax seed etc. that contain weakly estrogenic compounds are protective factors, that can be effectively considered to avoid the risk of hormone dependant cancers (Steinmetz and Potter, 1996; Henderson and Feigelson, 2000).

1.1.2.2.3. Chronic inflammation

It has been known for several years that inflammation has a close association with cancer. Even though inflammation is normally a part of defence response, chronic inflammation may lead to several chronic diseases such as cancer, cardiovascular diseases, diabetes etc. This is because most of the pro-inflammatory gene products, if maintain their persistent production along with the significant absence of anti-inflammatory factors, can modulate multiple signalling pathways of cell proliferation, invasion, metastasis, angiogenesis and apoptosis. These include the TNF- family members, interleukins, chemokines, MMPs, COX-2, LOX etc., through their regulation by transcription factors such as NF-κB, which were found to be associated with a wide variety of cancers (Aggarwal et al., 2006). Persistent infections may lead to chronic inflammation where the phagocytes and leukocytes may induce DNA damages to kill the cells by production of reactive oxygen and nitrogen species. These intermediates may react to form mutagenic agents such as peroxynitrite and thus persistent inflammation may lead to continuous interactions of these free radicals with the proliferating cells which may then lead to point mutations, deletions, and chromosomal rearrangements (Maeda and Akaike, 1998). *p53* mutations are most frequently associated with the inflammatory tumourigenesis similar to that in inflammatory bowel disease, rheumatoid arthritis etc (Yamanishi et al., 2002).

The strongest evidence came from the case of colon carcinogenesis where persons with inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are seen more prone to colon carcinogenesis. Similarly, Hepatitis C infection predisposes to liver carcinoma, schistosomiasis is associated to colon and bladder cancer and chronic *H.pylori* infection is strongly associated to stomach cancer (Ernst and Gold, 2000). After the transformation of cells, the inflammatory components of immune system with the help of regulatory molecules will cause the further progression of tumour, even though anti-tumour responses are happening on the other hand. For instance, tumour associated macrophages (TAMs) can kill neoplastic cells by the assistance of IL-2and IL-12, they produce potent angiogenic and lymphangiogenic factors including various cytokines and proteases which will promote tumour progression (Brigati et al., 2002; Tsung et al., 2002; Coussens and Werb, 2002).

1.1.2.2.4. Oxidative stress

Endogenous production of reactive intermediates of oxygen and nitrogen (ROI and RNI) is a normal physiological process associated mainly with mitochondria, cytochrome P450 and peroxisomes, which is essential for metabolism, cell cycle regulation and several signal transduction pathways (Nathan, 2003). The redox balance of useful and harmful effects of these intermediates inside the cell is balanced by endogenous antioxidant defence mechanisms which involve enzymes such as catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, proteins which can sequester transition metals, glutathione (GSH), cysteine, thioredoxin, vitamins etc (Valko et al., 2007). Thus oxidative stress is a result of incapability of endogenous systems to neutralise the reactive intermediates and free radicals (Sies et al., 1985; Sies, 1985). However, these free radicals are considered to the main link between inflammation and cancer, as they will be stimulated my various pro-inflammatory components and the resulting interactions with biomolecules leads to the accumulation of multiple mutations. The carcinogenic actions of ROI and RNI can be explained as direct or indirect. Direct action involves oxidation, halogenation, nitration etc. of DNA, proteins and lipids while indirect effects involve the interaction between these intermediates and the biomolecules to

form other compounds that can mediate various signalling events and carcinogenesis. However, the alterations include single- or double-stranded DNA breaks; modifications in the nucleotides or sugars; formation of DNA adducts and DNA protein crosslinks, which can result in various processes leading to carcinogenesis (Federico et al., 2007). Besides, the formation of several reactive peroxides, aldehydes, deactivation of various proteins involved in free radical scavenging, activations of cell survival pathways such as the NF-κB, AP-1, extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), and phosphoinositide 3-kinase/Akt8 virus oncogene cellular homolog (PI3K/Akt) pathways are also the major leads of oxidative stress-induced carcinogenesis (Reuter et al., 2010).

1.2. Stages of tumour progression

From the initial step of carcinogenesis till the advancement of tumour progression, there involves a series of steps which can be clearly defined by their molecular and clinical characteristics, even though they are not mutually exclusive.

1.2.1. Carcinogenesis

Carcinogenesis or the formation of cancer is the combinatorial effect of any among the vast array of extrinsic factors to which human are exposed and the intrinsic factors that modifies the normal physiological processes. It is evident from various cell culture and animal experiments that carcinogenesis is a multi-step process and the stages can be broadly divided as initiation, promotion and progression which are distinguished by the genetic and epigenetic events involved (Foulds 1954; Gutierrez and Salsamendi, 2001; Trosko 2001).

Initiation is characterised by irreversible genetic alterations in a susceptible normal cell to follow a malign evolution which make them prepare to take the first step to a neoplastic disease. This cell will be phenotypically similar to surrounding normal cells, and definitely not a cancer cell, which will undergo successive genotypic changes and mutations which are capable to induce

proliferation without differentiation (Trosko 2001; Trosko 2003). Although, there are efficient repair mechanisms within the cell, cells initiated to get proliferated will no longer wait for a repair and ensure the clonal expansion of these initiated cells with inhibition of apoptosis and differentiation (Shacter and Weitzman, 2002; Richardson et al. 1986; Frowein 2001; Trosko, 2001). Initiation is also considered as an additive process where the carcinogenic dose determines the incidence, multiplicity and latency of neoplastic manifestation. Also, not all the initiated cells may cause cancer, until the terminal differentiation genes were also get mutated. Furthermore, spontaneous initiations also exist which are caused by spontaneous mutations through depurination, deamination and errors in DNA replication process (Farber 1984; Trosko 2001; Pitot and Dragan; 1991).

Promotion stage involves the proliferation of initiated cells which also involves subsequent irreversible genotypic and phenotypic changes along with the fixing of mutations. Substances that can act as promoters need not to be potent carcinogens, rather they are enough capable to induce the proliferation of initiated cells, as demonstrated by various laboratory experiments (Mehta 1995; Gomes-Carneiro et al. 1997). They do not interact directly with the genetic material but alter the physiological processes, without being metabolically activated, by indirect oxidation and genetic changes (Butterworth et al. 1992; Weisburger 1998; Williams 2001; Gutierrez and Salsamendi, 2001). Their constant exposure and enough concentration in the target tissue is needed to effect stable promotion of initiated cells and hence the initial stage of promotion is a reversible process, in the sense, if the promoter is gradually withdrawn, there will be regression of the initiated cells, probably by apoptosis (Butterworth et al. 1992). Also, not all the cells exposed to promoters are induced to get proliferated. On the final stages of promotion, only those cells which are stimulated to proliferate without differentiation, which could resist apoptosis and could maintain the deregulated instability, are only capable to develop to a group of neoplastic cells, which can be called as pre-neoplastic lesions (Trosko, 2001).

Progression is the most extended and final stage of carcinogenesis where the pre-cancerous lesions or benign tumours that results on initiation and promotion will get transformed to malignant lesions characterised by genetic and epigenetic changes and independent of any stimulus of carcinogenesis (Klaunig et al. 2000, Williams 2001, Lutz 2000, Gutierrez and Salsamendi, 2001). Progression is characterised by irreversibility, genetic instability, rapid growth, invasion, metastasis and changes in the biochemical, metabolic and morphological features of cells. The acquisition of metastatic and angiogenic phenotypes are the most essential characteristics of cells of progressive stage of cancer whose inhibition will delay the neoplastic growth. All these changes are responsible for the clinical symptomatic manifestations of tumour progression which is mostly the diagnosable stage of cancer (Pitot and Dragan, 1991, Klaunig et al. 2000, Gutierrez and Salsamendi, 2001, Dixon and Kopras 2004).

1.2.2. Invasion and metastasis

The invasion–metastasis cascade which starts from the local invasion of primary tumour cells up to the establishment of distant metastases involves a series of complex succession of cell biological events, which are discussed below (Valastyan and Weinberg, 2011; Fidler, 2003) (Fig.1.2).

Local invasion: The tumour cells which acquired invasive phenotype in a primary tumour should first pierce the basement membrane to invade into the surrounding stroma. It is proposed that most carcinoma cells follow this process as cohesive multicellular units termed as collective invasion. Alternatively, individual tumour cells can undergo the protease-, stress-fiber-, and integrin-dependent mesenchymal invasion or the protease-, stress-fiber-, integrin-independent and Rho/ROCK-dependent amoeboid invasion depending up on the changing microenvironment, during which the cells may express varying molecular signatures (Friedl and Wolf, 2003; Wang et al., 2004). Apart from this, single cell invasive programs overcome the e-cadherin intercellular tight junctions by executing the epithelial-mesenchymal transition (EMT) which allow dissolution of adherens and tight junctions, followed by loss of cell polarity and transition of cells to mesenchymal phenotype resulting in increased

invasiveness (Thiery et al., 2009). These changes are facilitated by as set of transcription factors such as Slug, Snail, Twist, ZEB1 and ZEB2 which induce the expression of mesenchymal markers and suppress the ones which are associated with epithelial phenotype (Thiery et al., 2009).

Furthermore, the matrix metalloproteinases (MMPs), whose activity is tightly controlled in normal cells, were become activated by various factors secreted by tumour cells, resulting in lysis of the basement membrane and other ECM components and allow invasion of cancer cells to the stroma (Kessenbrock et al., 2010). The stroma is a site where tumour cells will encounter various types of cells including fibroblasts, macrophages, endothelial cells, adipocytes and other immue cells. This composition of stroma depends up on the aggressiveness of the tumour cells, the more aggressive tumour should confront a highly reactive stroma (Joyce and Pollard, 2009; Grivennikov et al., 2010). These stromal cells further enhance the tumour aggressiveness by mediated various signalling mechanisms (Gocheva et al., 2010) and this allow access to the systemic circulation and dissemination of tumour cells from the primary site (Valastyan and Weinberg, 2011).

Intravasation and Survival in Circulation: Intravasation refers to the entry of tumour cells into the blood or lymphatic circulation assisted by various molecular pathways mediated by growth factors such as TGF-β, EGF etc. that stimulate the trans-endothelial migration of cells (Gupta and Massague, 2006; Giampieri et al., 2009; Wyckoff et al., 2007). Moreover, the tumour associated microvasculature, resulted by VEGF induced neoangiogenesis, is leakier with very weak interactions between adjacent endothelial cells. This continuously reconfiguring microvasculature along with the absence of extensive pericyte coverage will definitely aids intravasation (Carmeliet and Jain, 2011; Valastyan and Weinberg, 2011).

The circulating tumour cells (CTCs) will undergo different kinds of stresses in the circulation before they can get arrested to the target site. As they are devoid of integrin-dependant adhesive mechanisms, which are essential for their survival, they will be challenged by anoikis alarms as long as they are retained in the circulation (Guo and Giancotti, 2004). But, it is the fact that most of the

CTCs will be trapped to capillary beds because of their large diameter than the capillary tubules and hence will be escaped before anoikis alarms get signalled. Apart from this, these CTCs will also undergo hemodynamic shear forces and predation by immune cells, especially the NK cells in circulation. This will be evaded by the tumour cells by forming a platelet coated emboli with effective diameter to escape from these hindrances until they get arrested at their favourable sites (Joyce and Pollard, 2009; Valastyan and Weinberg, 2011).

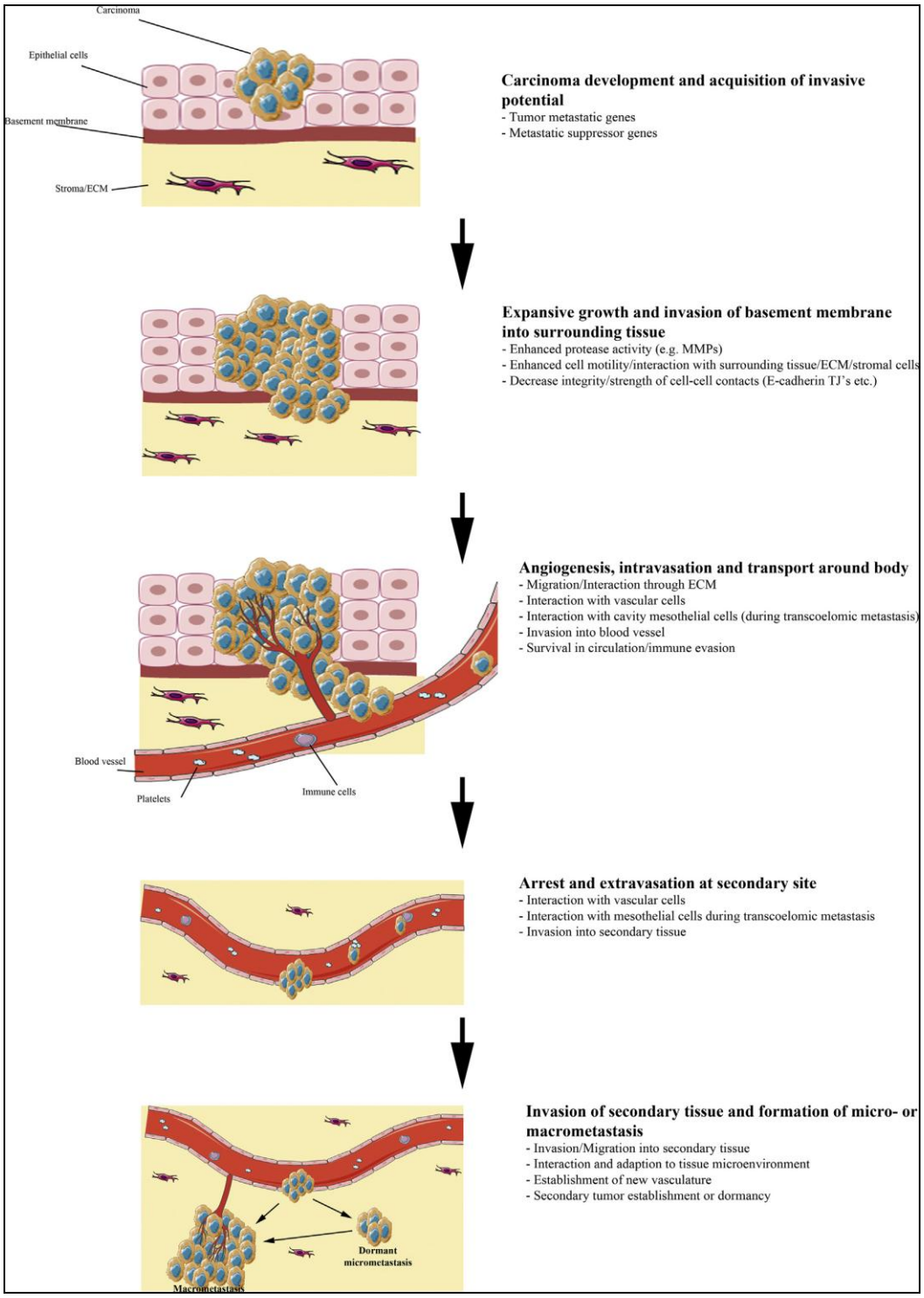
Arrest at distant site and Extravasation: The molecular basis of deposition of CTCs at specific sites is yet awaiting detailed study and the present literature proposes either of the two mechanisms to explain the specific organ targets for any metastasis, as follows. Firstly, the CTCs will be arrested at particular sites due to the anatomical lay out of the vasculature that directs from the primary tumour site so that those cells which will be settled at specific capillary beds and are further checked by the constricted luminals of the capillaries will obviously get extravasated to the adjacent tissues of these capillary beds (Gupta and Massague, 2006). Alternatively, the tumour cells can trigger expression of several adhesion molecules that can bind to organ specific vasculature and thus resulting in their arrest at these sites (Brown and Ruoslahti, 2004; Auguste et al., 2007).

The targeted arrest of primary tumour cells is followed by their extravasation from the endothelial walls towards the tissue parenchyma outside (Al-Mehdi et al., 2000). Intravasation is favoured by various factors secreted by tumour cells such as VEGF and other proteins sequestered around including MMPs, COX-2 etc. (Gupta et al., 2007; Padua et al., 2008). However, differing from intravasation, the capillaries and endothelial cells at distant sites are not unstable, but highly functional and will create physical barriers for the tumour cells to enter the nearby tissues. Hence, the characteristics of distant microenvironment strongly influence the extravasation of tumour cells and this is a determining factor of their secondary tumour sites (Nguyen et al., 2009; Valastyan and Weinberg, 2011).

1.2.3. Survival in foreign microenvironment and formation of micrometastases: Tumour cells at the secondary site should have to satisfy the demands of the foreign microenvironment where they encounter a different set of stromal cells, tissue architecture growth factors etc. Some have proposed that cancer cells will form a pre-metastatic niche to overcome this problem where the primary tumour cells produce systemic signals to their secondary sites and the successive molecular events create a hospitable environment to the tumour cells at their secondary sites even before the cells reach the secondary sites (Psaila and Lyden, 2009). Even though the model of pre-metastatic niche has been questioned, it is generally accepted that the tumour cells organize a wide range of mechanisms to adapt themselves to the new microenvironment to form micrometastases (Dawson et al., 2009; Valastyan and Weinberg, 2011).

1.2.4. Metastatic colonization: In fact, not all the disseminated cells can form metastases at secondary sites. These cells will suffer incompatibilities in the foreign micro environment resulting in a slow establishment or form micrometastases and retain a dormant period of several weeks to months and ultimately detectable metastases were only found in those sites where tumour cells are provided with favourable microenvironment for their survival and proliferation, which accounts for the tissue tropism of most metastases (Chambers et al., 2002; Fidler, 2003). This will be favoured by many proteins such as those transcription factors involved in the EMT pathways (Thiery et al., 2009). Moreover, out of the vast number of disseminated cells, the tumour initiating cells that can simultaneously overcome the incompatibilities and retaining high renewal capacity are only capable of completing the metastatic colonization and form macroscopic metastatic lesions, which represents the end point of invasion-metastasis cascade (Shackleton et al., 2009; Clevers, 2011; Valastyan and Weinberg, 2011).

Figure 1.2. Stages of invasion and metastasis



(Source: Jiang et al., 2015)

1.3. Angiogenesis

Angiogenesis, the process by which capillaries sprout from preexisting blood vessels, is tightly regulated by a large number of proangiogenic and antiangiogenic factors. Tumour cells have an absolute requirement for a persistent supply of new blood vessels to nourish their growth and to facilitate metastasis. Thus, tumour vascularization is a vital process for the progression of a neoplasm from a small localized tumour to an enlarging tumour with the ability to metastasize (Folkman, 1971; Liotta et al., 1974). The angiogenic cascade leading to tumour vascularization can be divided into two general phases, the prevascular phase (referred to as the "angiogenic switch") and the vascular phase (Pepper et al., 1995; Hanahan and Folkman, 1996). Once tumour cells undergo the transformation to an angiogenic phenotype, these malignant cells are capable of inducing phenotypic changes in endothelial cells as well as in other cell types (Norrby, 1997; Polverini, 1996). At that point, avascular tumours can acquire their own blood supply, which permits a rapid rate of growth. While tumours lacking adequate vasculature become necrotic (Brem et al., 1976) or apoptotic (Holmgren et al., 1995), tumours that have undergone neovascularization may not only enter a phase of rapid growth but may also have high metastatic potential (McMahon, 2000).

A large number of proangiogenic factors and their cognate receptors have been identified, including basic fibroblast growth factor (Rifkin and Moscatelli, 1989), platelet-derived growth factor (PDGF) (Nicosia et al., 1994), platelet-derived endothelial cell growth factor (Takahashi et al., 1996), fibroblast growth factor (Jouanneau et al., 1995), angiopoietin-1 (Suri et al., 1998), transforming growth factor beta-1 (TGF-β1) (Pepper et al., 1993), transforming growth factor alpha (TGF-α), and epidermal growth factor (EGF) (Gleave et al., 1993). Perhaps the best characterized of the proangiogenic factors is vascular endothelial growth factor (VEGF), which is relatively unique among growth factors in terms of its specificity for the vascular endothelium (Ferrara and Henzel, 1989; Leung t al., 1989; Ferrara and Alitalo, 1999).

The formation of new blood vessels is tightly regulated by specific growth factors that target receptor tyrosine kinases (RTKs). Vascular endothelial growth factor (VEGF) and the Flk-1/KDR RTK have been implicated as the key endothelial cell-specific factor signaling pathway required for pathological angiogenesis, including tumour neovascularization. Inhibition of the VEGF tyrosine kinase signaling pathway blocks new blood vessel formation in growing tumours, leading to stasis or regression of tumour growth. Advances in understanding the biology of angiogenesis have led to the development of several therapeutic modalities for the inhibition of the VEGF tyrosine kinase signaling pathway. A number of these modalities are under investigation in clinical studies to evaluate their potential to treat human cancers (McMahon, 2000).

Angiogenesis inhibitors have been developed to interrupt the angiogenic process at the growth factor, receptor tyrosine kinase and intracellular kinase levels. Other anti-angiogenic therapies alter the immune response and endogeneous angiogenesis inhibitor levels. Most anti-angiogenic therapies for malignant gliomas are in Phase I/II trials and only modest efficacies are reported for monotherapies. The greatest potential for angiogenesis inhibitors may lie in their ability to combine safely with chemotherapy and radiotherapy (Wong et al., 2009).

1.4. Cancer and the immune system

The immune system has the greatest potential for the specific destruction of tumours with no toxicity to normal tissue and for long-term memory that can prevent cancer recurrence. The last 30 years of immuno-oncology research have provided solid evidence that tumours are recognised by the immune system and their development can be stopped or controlled long term through a process known as immunosurveillance. Tumour specificity of the immune response resides in the recognition of tumour antigens. Viral proteins in tumours caused by viruses and mutated proteins from oncogenes or other genes, as well as non-mutated but abnormally expressed self-proteins found on all tumours, have been shown to be good antigens and good targets for immunosurveillance. In many

cancers, however, malignant progression is accompanied by profound immune suppression that interferes with an effective anti-tumour response and tumour elimination. Initially, most of the escape from immunosurveillance was ascribed to changes in the tumour cells themselves (loss of tumour antigens, loss of human leukocyte antigen molecules, loss of sensitivity to complement, or T cell or natural killer (NK) cell lysis), making them a poor target of an immune attack (Finn, 2012).

1.4.1. Immunosurveillence, immunoediting and escape of tumours

The immune system has three primary roles in the prevention of tumours. First, the immune system can protect the host from virus-induced tumours by eliminating or suppressing viral infections. Second, the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumourigenesis. Third, the immune system can specifically identify and eliminate tumour cells on the basis of their expression of tumour-specific antigens or molecules induced by cellular stress. The third process is referred to as tumour immune surveillance, whereby the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm. The idea that the immune system, which so effectively protects the host from microbial pathogens, might also recognize and destroy tumour cells was first discussed over a century ago and has recently been reviewed in detail (Dunn et al., 2002) (Swann and Smyth, 2007). The key cells of the immune system for tumour surveillance are T cells, which are part of the adaptive immune response. After recognition of an antigen on a tumour cell via the T cell receptor (TCR), activated CD8+ T cells can kill the tumour target cell and thus are called cytotoxic T cells (CTL). One subset of CD4+ T cells, T helper cell type 1 (Th1), provides “help” for the activation of CD8+ T cells. The other CD4+ subset, Th2 cells, stimulates a humoral immune response and suppresses the development of a Th1 response. CD4+ T cells can also display cytotoxic activity in some situations. CD8+ and CD4+ T cells recognize antigens presented as peptides by major histocompatibility complex (MHC) class I or class II molecules, respectively (Igney and Krammer, 2002).

T cells and NK cells use two major mechanisms to kill tumour cells: the death receptor pathway and the granule exocytosis pathway (Rouvier et al., 1993). In the death receptor pathway, the lymphocyte displays the death ligand CD95L on the cell surface, triggering apoptosis via the death receptor CD95 on the target cell (Walsh et al., 1994; Li et al., 1998). Moreover, for immune surveillance of tumours and metastases, NK cells also use the death ligand TRAIL [tumour necrosis factor (TNF)-related apoptosis-inducing ligand], which triggers apoptosis via the death receptors TRAIL-R1 or TRAIL-R2 (Smyth et al., 2001; Takeda et al., 2002; Takeda et al., 2001) (Igney and Krammer, 2002).

NK cell triggering is the result of a complex balance between inhibitory and activating signals and require not only a deficient MHC-I expression on target cells but also the expression of inducible ligands of activating NK cell receptors (Lanier, 2005). Consequently, these cells have the ability to recognize and destroy a wide range of abnormal cells (including tumour cells, virus-infected cells, cells bound by an antibody, allogeneic cells), as well as stressed cells, without damaging the healthy and normal "self " cells (Langers et al., 2012).

Most hematopoietic cells, except most T cell subsets, express Fcγ receptors (FcγRs) (Nimmerjahn and Ravetch, 2008). There are three types of FcγRs which recognize the Fc part of IgG antibody subclasses with different affinities. The activating FcγRI (CD64) binds to human IgG1 and IgG3 with high affinity, is expressed on macrophages and neutrophils, and mediates phagocytosis of target cells. The FcγRII (CD32) class comprises activating low affinity FcγRIIa (which binds human IgG1, IgG2, and IgG3) and inhibitory FcγRIIb (which recognizes human IgG1 and IgG3 with low affinity) and may attenuate signaling from activating receptors as FcγRI if engaged in phagocytes. FcγRIIIb is a protein expressed by neutrophils and may play a role in neutrophil activation. Activating low affinity FcγRIIIa (type III receptor for IgG; CD16) mediates antibody-dependent cellular cytotoxicity (ADCC) and is highly expressed on the cytotoxic CD56dim CD16+ NK cell subset as well as on other hematopoietic cells. Several mouse model studies postulated the impact of activating FcγRs on anti-tumour effects in antibody therapy indicating that ADCC has a substantial effect on tumour rejection (Seidel et al., 2015).

Despite tumour immune surveillance, tumours do develop in the presence of a functioning immune system, and therefore the updated concept of tumour immunoediting (Dunn et al., 2002) is a more complete explanation for the role of the immune system in tumour development. The tumour immunoediting concept is divided into 3 phases, designated elimination, equilibrium, and escape (Dunn et al., 2004). The elimination phase of cancer immunoediting is exactly the same process described in the initial theory of tumour immune surveillance, whereby the immune system detects and eliminates tumour cells that have developed as a result of failed intrinsic tumour suppressor mechanisms. The elimination phase can be complete, when all tumour cells are cleared, or incomplete, when only a portion of tumour cells are eliminated. In the case of partial tumour elimination, the theory of immunoediting is that a temporary state of equilibrium can then develop between the immune system and the developing tumour. During this period it is envisaged that tumour cells either remain dormant or continue to evolve, accumulating further changes (such as DNA mutations or changes in gene expression) that can modulate the tumour-specific antigens and stress-induced antigens that they express. As this process continues, the immune system exerts a selective pressure by eliminating susceptible tumour clones where possible. The pressure exerted by the immune system during this phase is sufficient to control tumour progression, but eventually, if the immune response still fails to completely eliminate the tumour, the process results in the selection of tumour cell variants that are able to resist, avoid, or suppress the anti-tumour immune response, leading to the escape phase. During the escape phase the immune system is no longer able to contain tumour growth, and a progressively growing tumour results (Swann and Smyth, 2007).

Owing to the abundant experimental and clinical evidence there is no longer any doubt for the existence of cancer immunoediting from immune surveillance to escape. Cancer cells are gradually able to gain several mechanisms of immune evasion during tumour progression, even though they are being pursued by the initial and continuing phases of immune surveillance. Rather, immunological sculpting contributes to immune selection pressure, which produces tumour cell variants that are resistant to immune effector cells because of their low

immunogenicity. In advanced cancers, the marked shifting to immunosuppressive conditions as the result of the constitution of the immunosuppressive network in tumours makes it more difficult to provoke an immune activation to eliminate cancer cells. Given that, adoptive immunotherapy using peptide vaccine and DC transfer is not sufficient to reduce tumour volume and their elimination by direct priming for T cells in such conditions, indirect cross-priming for T cells, which can be induced by massive cell death in combination with anticancer drugs, will be required. Indeed, not only modulation of anticancer drug-induced cell death, but also activation of antitumour immune responses by using molecular targeting drugs such as antibodies and small molecules may provide remarkable enhancement of chemotherapeutic effects in cancer therapy (Kim et al., 2007).

1.5. Homeopathy and Cancer- the scientific background

Homeopathy is a widely practiced system of holistic medicine rooted back in the 18th century, proposed by the German physician Samuel Hahnemann. It is one of the main complementary and alternative medicine (CAM) commonly followed in Europe, India, Israel and Latin America (Bellavite et al., 2005). Even though the use of homeopathic medicines was estimated to be increased in United States, only 1.8% of CAM users following the treatments, reflecting the low popularity of homeopathic practice in the US compared to Europe and Asia (Barnes et al., 2008). Despite these facts, homeopathic medicines in the US are regulated by FDA following the Homeopathic Pharmacopoeia (HPUS) of the United States established in 1897 and is still updating regularly. Accordingly, people can buy homeopathic medicines without a prescription for diseases which are self-limiting and are amenable to self-diagnosis if and only if these medicines contain no toxic substances (Pinco, 1996).

The classical homeopathy relies on the principle of similar or ‘like cures like’ which can be explained as highly diluted substances, which may be of plant, animal or mineral origin, that can cause disease symptoms in healthy volunteers, can be used to treat similar set of symptoms in ill individuals (Jonas et al., 2003; Hahnemann, 1982). But the same principle is at the centre of controversy, as the

scientific community has been questioning the clinical efficacy of these ultra-diluted remedies whose amount of substance may go beyond Avogadro number for any potency greater than 7C. Hence, the disputes consign that they donot have any more biological effects rather than the placebo effect (Swayne, 2000; Ernst, 2007).

Although the healing potential of homeopathic drugs is more or less widely accepted, the exact mechanism of action is still unclear. Hahnemann describes the mechanism of action through the 'primary action' of the medicine and the 'secondary and curative reaction' of the organism: 'Every agent that acts upon the vitality, every medicine, deranges more or less the vital force, and causes a certain alteration in the health of the individual for a longer or a shorter period. This is termed primary action. Although a product of the medicinal and vital powers conjointly, it is principally due to the former power. To its action our vital force endeavors to oppose its own energy. This resistant action is a property, is indeed an automatic action of our life-preserving power, which goes by the name of secondary action or counteraction' (Sunila et al., 2009). *In vitro* and *in vivo* analysis to elucidate the possible mechanism of action of the homeopathic drugs have been reported and a hypothetical conclusion that the drugs acts at molecular level through the activation/ inactivation of particular genes (Khuda- Bukhsh, 1997).

The recent years proclaim many scientific evidences from surveys, clinical and laboratory studies that support the curing efficiency of many homeopathic medicines for a wide variety of diseases such as infections, allergies, depression, and also life threatening diseases including cancer (Frenkel, 2002; Adler et al., 2011; Jacobs et al., 2005). Some hospital based surveys in France and Italy and some other European countries revealed the use of homeopathic medicines by a good percentage of patients out of the total that followed CAM therapies (Downer et al., 1994; Molassiotis et al, 2005; Johannessen et al., 2008). As part of the Best Case Series Program under NCI, a clinical report from India in 2008 proved the effectiveness of homeopathic remedies on cancer care, where 14 cancer patients were seen effectively treated with homeopathic medicines (Banerji et al., 2008). The best reports from India came with the NCI-BCS

program that evaluated the cancer treatment using Banerji protocols, developed by P. Banerji Homeopathic Research Foundation (PBHRF), Kolkata. These protocols are modifications of classical homeopathy and uses specific medicines for specific diseases instead of the concept of individualised medicines of classical homeopathy. While evaluating the patients having lung or oesophageal cancer, they did not receive any treatments such as surgery, chemotherapy or radiotherapy other than the medicines as per Banerji protocols. The rigorous evaluation of clinical results warranted the efficacy of these medicines and further recommended research based studies and the Office of complementary and Alternative medicine under NCI is currently collaborated with the All India Institute of Medical Sciences to obtain approval to monitor and evaluate the clinical cases of PBHRF clinic (Frenkel, 2010).

Clinical studies also contribute many supportive evidences for the use of homeopathic medicines for cancer and also against the adverse effects of cancer treatments even though none of them strictly point out their high potential or stand-alone effects against cancer. Medicines such as *Calcarea carbonica*, *Sulfur*, *Lachesis*, *Kali carbonicum*, *Cobaltum* 30C, *Causticum* 30C, *Traumeel S*, *Belladonna* 7C were reported to reduce the side effects of conventional therapies in cancer survivors. *Ruta* 6C and *Calcarea phosphorica* caused the regression of intracranial tumours in patients without any additional conventional therapy (Oberbaum et al., 2001; Jacobs et al., 2005; Balzarini et al., 2000). Even though there are methodological constraints while dealing with pre-clinical experiments, a good number of scientific publications do proclaim the capability of homeopathic remedies to delay or inhibit the progression of cancer in several *in vitro* and *in vivo* experimental models. Mac Laughlin and his group tried to prove the effect of *Sabal serrulata* to reduce prostate cancer and the results recommend further studies on this medication on prostate cancer pathology (Mac Laughlin et al., 2006). Another study revealed that *Chelidonium* could inhibit chemically induced hepatocarcinoma in mice (Biswas and Khuda-Bukhsh, 2002). Kumar et al proved the efficacy of *Ruta* 200C and *Phosphorous* 1M to inhibit chemically induced hepatocarcinoma and sarcoma respectively in mice models (Kumar et al., 2007). Preliminary results on the effect of *Thuja*

occidentalis to inhibit lung metastasis in mice model signify with a significant reduction in tumour nodules, other parameters of lung fibrosis and also an increased life span of metastasis induced animals (Sunila et al., 2006). Another study revealed the potential of drugs such as *Carcinosin*, *Phytolaca*, *Conium* and *Thuja* to exert cytotoxic effects in breast cancer cell lines. The results state that the drugs caused cell cycle arrest and activation of caspase mediated apoptosis in breast cancer cells (Frenkel, 2010). Moreover, the efficiency of potentized form of *Hydrastis candensis*, *Lycopodium clavatum*, *Carcinosinum* etc. to inhibit cancer development was also studied (Sunila et al., 2007). However, solid clinical evidences which point out the specific effects of homeopathy against cancer have not evidenced till date. But the available evidences from multiple research disciplines suggest two significant opinions that the remedies are safe without undesirable side effects and also they are having important roles at least to improve the health status of the patients (Frenkel, 2010).

1.6. Scope of the present study

The present study aims a scientific validation of the use of drugs used in classical homeopathy against malignant conditions. Based on the background literature, we have selected potentized forms of *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum* at their 1M, 200C and 30C potency for the study.Potentiation/succession/dynamization is the process in which a substance is diluted with alcohol or distilled water and then vigorously shaken at each step of dilution, which is believed by Hahnemann which stimulate the 'vital energy' of the basic material. Potency is the term used to denote the dilution scale of the homeopathic drug. Seveeral potency scales such as centesimal scale (C), decimal scale (X), quintamillesimal scale (LM) has been used in homeopathy. In the present study, centesimal scales are denoted for all the medicines used, where the basic material is diluted by a factor of 100 in each step of dilution. Thus 30C and 200 C end up with the original material diluted by a factor 100^{-30} and 100^{-200} respectively. 1M is equivalent to 1000C and hence it represents a form of original material diluted by a factor of 100^{-1000}. All the drugs selected for the present study are being used by homeopathic practitioners against benign

tumours, ulcer and some malignant conditions (Attarwala et al., 2006). Detailed studies were reported which could prove the immunopharmacological properties of *Thuja occidentalis*, whose aqueous-alcoholic extract or their fractions or extract containing herbal preparations, can induce antibody response, cytokine induction, T cell response and even toxicity against HIV virus in pre-clinical experimental conditions (Bodinet, 2002; Gohla et al., 1989; Gohla et al., 1992). Clinical data also showed the efficacy of the extract containing preparations against respiratory tract infections and common cold (Wustenberg et al., 1999; Kohler et al., 2002). Eventhough the fresh plant extract has intoxicating properties, the preparations that contain diluted amounts of the same was proved to be clinically safe. The toxic property has been explained owing to the presence of rich quantities of a monoterpene called thujone. But this compound was reported to be safe and non-toxic up to a daily dose of 75 mg in humans (Pinto-Scognamiglio, 1967). *Ruta graveolens*, is a traditionally used medicinal plant especially in alternative medicines. Its potentized preparation at 200c was reported to inhibit tumour development in N-nitrosodiethylamine (NDEA) induced hepatocarcinoma models in rats, DLA induced solid tumour model, EAC induced ascites tumour model and 3-methylcolanthrene induced sarcoma model in mice (Preethi et al., 2006; Sunila et al., 2007). There are other reports also which proved the clastogenic potential of *Ruta graveolens* and its homeopathic preparation on bone marrow cells in mice (Preethi et al., 2008). However a study on normal monkey kidney cell line, *in vitro*, showed that it is non-toxic to normal cells (Arora et al., 2014). *Carcinosinum*, the potentized preparations from carcinomas and has been used against malignant conditions, was also subjected to various scientific studies. It was reported that *Carcinosinum* 200c could induce apoptosis in DLA cells *in vitro*, mediated through the upregulation of p53 gene (Preethi et al., 2011). Another study checked the effect of intermittent use of *Carcinosinum* 200c along with *Natrum sulphuricum* 30c against azo dye induced hepatocarcinoma which resulted in synergistic therapeutic effects (Bhattacharjee et al., 2009). It has also been reported that potentized preparations of *Thuja occidentalis* and *Carcinosinum* could exert preferential cytotoxic effects against breast cancer cell lines by

altered expression of cell cycle regulatory proteins as well as induction of the apoptotic cascade.

These drugs were already been using alone or in combinations at their varying potencies for various malignancies and also their anti-tumour potential were already proved by different scientific studies as mentioned above. Hence, further studies should move on to investigate the capability of these drugs to interfere with any progressive stages of cancer, say the metastatic cascade , angiogenesis etc., since any therapeutic scope at these processes is having necessary significance in the current scenario of cancer. Moreover, homeopathic medicines are proposed to be act through the body's own defense system, it is an essential need to analyse the effect of these drug on the immune status during tumour progression. We have recently reported the immune stimulatory potential of these drugs in normal experimental animals and this may be one of the mechanism of action though which the pharmacological actions of the drugs being implemented in an organism. Hence, the present study investigated the possible potential of the selected homeopathic drugs; say *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum*, to interfere with the key stages of cancer progression, such as metastatic episode, angiogenesis and also their effect on the defence status of immune system during metastatic progression of the tumour with an emphasis to breast cancer metastasis in experimental animals.

Based on the background literature, we have focussed our studies on the following objectives in order to analyse the beneficial effects and the regulatory roles of the selected homeopathic remedies (1M, 200c and 30c potencies of Thuja, Ruta and Carcinosinum) against cancer progression and to further explain the underlying mechanism of therapeutic action.

1. Screening of the selected homeopathic remedies using *in vivo* tumour models.
2. Evaluation of the regulatory roles of drugs on the immune system.
3. Studying their effect on metastasis and angiogenesis using B16F10 melanoma model.

4. Studying their effect on breast cancer metastasis using 4T1 breast tumour model.

5. Assessment of the modulatory roles of drugs on the cell immune responses during tumour progression.

Chapter 2
Materials and Methods

2.1. MATERIALS

2.1.1. Homeopathic medicines and potentiated alcohol

Homeopathic preparations of 1M, 200c and 30c potencies of *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum* were purchased from Wilmer Schwabe, Germany. Potentiated ethanol at 30c potency was used as the vehicle control and was obtained from Simila Homeo Laboratories, Aluva, Kerala, India. As all the drugs have been potentized in ethanol, the vehicle control is set as the potentized form of ethanol.

2.1.2. Cell lines used in the study

Dalton's Lymphoma Ascites (DLA) cell line and Ehrlich ascites carcinoma (EAC) cell line were obtained from Adayar Cancer Institute, Chennai and maintained as ascites tumours in Swiss albino mice. B16F-10 metastatic mouse melanoma cell line, K-562 leukemic cell line and EL-4 thymoma cell line were obtained from National Centre for Cell Sciences, Pune, India. 4T1 mouse breast carcinoma cell line was a kind gift from DR. Preethi K.C., John Hopkins University, USA.

2.1.3. Animals used in the study

BALB/c mice and Swiss albino mice were obtained from small animal breeding section, Govt. Veterinary College, Thrissur. C57BL/6 mice were purchased from National institute of Nutrition, Hyderabad, India. The animals were maintained at $23^0C \pm 1^0C$ in controlled environment (12-h light/12-h dark), fed with normal mouse chow (Sai Durga Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were performed with prior permission from the Institutional Animal Ethical Committee (IAEC), and performed as per the rules and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

2.1.4. Chemicals

L-glutamine	: HiMedia Laboratories, Mumbai
Trypsin	: -do-
Fluid Thioglycollate Medium	: -do-
Rosewell Park Memorial Institute medium	

(RPMI-1640) : -do-

Hank's Balanced Salt Solution (HBSS) (1X) : -do-

Bovine Serum Albumin (BSA) : -do-

Cysteine : -do-

Carbazole : -do-

Foetal Bovine Serum (FBS) : Biological Industries, Israel

Dulbecco's Modified Eagles Medium

(DMEM) : Sigma Aldrich, USA

Collagenase Type IV

(from *Clostridium histolyticum*) : -do-

Collagenase Type I

(from *Clostridium histolyticum*) : -do-

Elastase (from porcine pancreas) : -do-

Hyaluronidase (from bovine testes) : -do-

Diethylpyrocarbonate (DEPC) : -do-

Dithiotheritrol (DTT) : -do-

Ethidium bromide : -do-

ECM-Gel

(from Engelbreth Holrn-Swarm

mouse sarcoma) : -do-

L-γ Glutamyl p-nitroanilide : -do-

Glycyl glycine : -do-

4, 6-Glucuronic acid lactone : -do-

Hydroxyproline : -do-

Chloramine T : -do-

Oligonucleotide primer sequences

(Tab 2.1) : -do-

Agarose : -do-

Phytohaemagglutinin A (PHA) : -do-

6-thioguanine : -do-

Acetyl acetone : Sisco Research Laboratories, India

1, 4 - Bis (phenyloxazol-2yl) Benzene

(POPOP)	: -do-
2, 5 - Diphenyl oxazole (PPO)	: -do-
Folin's Ciocalteau reagent	: -do-
Glucosamine hydrochloride	: -do-
Papain (Extracted from Papaya latex)	: -do-
Concanavalin-A (Con A)	: -do-
Thiobarbituric acid	: -do-
Tris-HCl	: -do-
Naphthalene (scintillation grade)	: -do-
Lipopolysaccharide (LPS)	: Difco Laboratories, USA
Pokeweed mitogen (PWM)	: -do-
Mouse monoclonal CD-31 antibody	: Abcam, Cambridge, UK
Crystal violet	: Romali, Mumbai, India
Trypan blue	: -do-
Sialic acid (n-acetyl neuraminic acid)	: -do-
DNase, RNase free water	: Genei, Bangalore
Trizol reagent	: -do-
AMV RT buffer	: -do-
AMV RT	: -do-
Oligo (dT)	: -do-
5X PCR buffer	: -do-
$MgCl_2$	: -do-
Gel loading dye	: -do-
Molecular weight marker	: -do-
dNTP mix	: -do-
Taq DNA polymerase	: -do-
Isopropanol	: Merck Specialities Pvt. Ltd., Mumbai
Formaldehyde	: -do-
Eosin	: -do-
Leishman's stain	: -do-
Pararosaniline	: -do-
Sodium citrate	: -do-

Sodium acetate	: -do-
Sodium bicarbonate	: -do-
EDTA	: -do-
Acetic acid	: -do-
Sodium nitrite	: -do-
Perchloric acid	: -do-
Sodium hydroxide	: -do-
Sodium-Potassium tartarate	: -do-
Copper sulphate	: -do-
Citric acid	: -do-
Sodium chloride	: -do-
Potassium hydroxide	: -do-
Sodium tetraborate	: -do-
Periodic acid	: -do-
Sodium arsenite	: -do-
Dimethyl sulfoxide (DMSO)	: -do-
Chloroform	: -do-
Dioxan	: -do-
Dextrose	: -do-
Disodium hydrogen phosphate	: -do-
Potassium dihydrogen phosphate	: -do-
Potassium chloride	: -do-
Potassium iodide	: -do-
Hematoxylin	: -do-
Glucose	: -do-
Conc. HCl	: -do-
Conc. H_2SO_4	: -do-
p-dimethyl amino benzaldehyde	: Qualigens Fine Chemicals, Mumbai
Alpha-naphthyl acetate	: Loba Chemie, Mumbai, India
Ferric chloride	: -do-
Cedar wood oil	: Nice Chemicals, India
Potassium alum	: -do-

2.1.5. Diagnostic Reagents and Kits

Drabkin's reagent	: Haemochek, India
Mouse ELISA Kits of IL-1β, IL-6, TNF-α, IL-2, IFN-γ, GM-CSF	: Pierce Biotechnology, USA
Mouse ELISA kits of VEGF	: R&D Systems, USA
DAB system kit	: Genei, Bangalore

2.1.6. Radioactive Materials

^{3}H-Thymidine	: BARC, Mumbai, India
Sodium Chromate ($Na_2Cr^{51}O_4$)	: -do-

2.1.7. Tissue Culture Wares

Medium filtering assembly	: Millipore, USA
Cellulose syringe filtering apparatus	: Sartorius, Germany
Tissue culture flask (25 cm^2)	: Tarson, India
Tissue culture petri dish (10 cm and 6 cm)	: -do-
96-well flat bottom culture plate	: -do-

2.1.8. Instruments

Automatic Gamma Counter	: PerkinElmer, USA
Rack Beta Liquid Scintillation Counter	: Wallac, Finland
CO_2 Incubator	: Napco, Canada
Deep Freezer (-80ºC)	: Remi, Chennai, India
Deep Freezer (-20ºC)	: -do-
Vortex mixer	: -do-
High speed cooling centrifuge	: -do-
Incubator	: Beston Industries, India
Electronic Balance	: Schimadzu, Japan
ELISA-Reader	: Awareness Technology, Gujarat
Gel Documentation system	: Vilber Lourmat, France
Bench-top centrifuge	: Rotek Instruments, India
Distillation Unit	: -do-
Inverted Microscope	: Leica, Germany

Lyophilizer	: Labconco Inc, USA
Minicycler–Thermo cycler	: MJ Research, USA
Spectrophotometer	: Elico, India
Haemocytometer	: Rohem Instruments Pvt. Ltd, India
Hot air oven	: Amur Instrumentation, India
Autoclave	: Kemi, India
Laminar Air Flow chamber	: -do-
Water bath	: -do-
Colony counter	: Medica Instruments, Mumbai
Spinwin Microcentrifuge	: Tarson, India
Submerged electrophoresis unit	: Bangalore, Genei, India
Tissue homogenizer	: Yorco Scientific Industries, Delhi
Transilluminator	: Vilber Lourmat, France

2.1.9. Reagents

2.1.9.1. Phosphate Buffered Saline (PBS)

NaCl	-	8.00 g
KCl	-	0.20 g
Na_2HPO_4 $2H_2O$	-	1.44 g
KH_2PO_4	-	0.20 g
Distilled Water	-	1000 mL

pH was adjusted to 7.2 with I N HCl or NaOH

2.1.9.2. PBS-EDTA Solution

EDTA	-	20 mg
PBS	-	100 mL

Sterilized by autoclave

2.1.9.3. Trypsin Solution

Trypsin	-	200 mg
Glucose	-	20 mg
PBS-EDTA	-	100 mL

Sterilized by filtration

2.1.9.4. Alsevier's Solution

Dextrose	-	2.05 g
Sodium citrate	-	0.80 g
NaCl	-	0.42 g
Distilled water	-	100 mL

pH adjusted to 6.1 with 10% citric acid

2.1.9.5. Scintillation Fluid

PPO	-	2.5 g
POPOP	-	0.25 g
Naphthalene	-	100 g
Dioxan	-	1000 mL

2.1.9.6. Ehrlich's reagent

A: p-dimethylaminobenzaldehyde	-	1 g
Conc. HCl	-	50 mL
Absolute ethanol	-	50 mL
B: $FeCl_3$. 6 H_2O	-	2.03 g
Water	-	500 mL
Conc. H_2SO_4	-	300 mL

Mix **A** and **B** (1:3)

2.1.10. Stains

2.1.10.1. Trypan Blue

Trypan blue	-	100 mg
Saline (0.9%)	-	100 mL

Trypan blue was dissolved in saline by overnight stirring. Any suspended particles were removed by filtration

2.1.10.2. Eosin

Eosin	-	500 mg
Ethanol	-	100 mL (Final volume)

2.1.10.3. Harris Haematoxylin

Haematoxylin	-	5g
Ethyl alcohol	-	50 mL
Potassium alum	-	50 mg

Potassium iodide	-	50 mg
Distilled water	-	950 mL

Haematoxylin was dissolved in alcohol using gentle heat. The alum was dissolved in distilled water by heating with frequent stirring and keep overnight at 4^0C. Alcoholic haematoxylin was added to the alum solution. The mixture was cooled and potassium was added and filtered.

2.1.10.4. Crystal Violet

Crystal violet	-	50 mg
Methanol	-	20 mL
Distilled water	-	80 mL

2.2. METHODS

2.2.1. Tissue Culture

2.2.1.1. Sterilization of tissue culture wares

All the glass wares and filtration assembly were washed using 1% extran and thoroughly rinsed with distilled water followed by overnight drying in a hot air oven at 40^0C. Autoclaving of these culture wares was done at 15 pounds/square inch pressure for 15 min, followed by drying in a hot air oven to make them ready to use for experiments.

2.2.1.2. Preparation of tissue culture media

DMEM (9.98 g/L) and RPMI (10.3 g/L) were prepared in autoclaved double distilled water and supplemented with L-glutamine (2 mM). The pH of the media solution was adjusted to 7.2 using sodium bicarbonate followed by membrane filtration using 0.22 μm cellulose filter applying negative pressure in the filtration apparatus. Fluid thioglycollate medium (29.96 g/L) was used to check the sterility of the medium, where 1 mL of the filtered medium was suspended in 10 mL of sterile thioglycollate medium and incubated at 37^0C for 7 days to observe any visible contamination. The medium was again supplemented with 10% foetal bovine serum, and broad spectrum antibiotics such as penicillin (100 units /mL) and streptomycin (100 μg /mL) prior to use.

2.2.1.3. Maintenance of cell lines *in vitro*

Adherent cell lines such as B16F-10 mouse melanoma cell line and 4T1 mouse breast carcinoma cell line were maintained as monolayer in $25cm^2$ tissue culture flasks using DMEM containing 10% FBS and antibiotics, provided with the removal of spent medium every third day. As and when the cell density reaches about 80% confluency, the medium was removed and the cell layer was washed with 2 mL of PBS solution and then with PBS-EDTA solution. This was followed by the addition 100 μL of trypsin solution (0.2%) and incubated for 2-3 minutes at 37^0C. Then, 5 mL of 10% complete DMEM was added, cell suspension was dispersed by repeated pipetting without frothing, and an aliquot of the single-cell suspension was added to fresh tissue culture flasks containing

5mL of complete DMEM. The flasks were incubated at 37^0C in CO_2 incubator provided with 5% CO_2.

Suspension cultures of K-562 and EL-4 cell lines were maintained in RPMI-1640 supplemented with 10% FBS and antibiotics. The cell suspension was dispersed by repeated pipetting without frothing, cell density was estimated and $1x10^6$ cells were seeded into fresh culture flasks containing 10 mL of RPMI-1640. The flasks were then kept at 37^0C in shaker incubator provided with 5% CO_2. The confluent flasks were sub-cultured every third day.

2.2.1.4. Preparation of tumour cell suspension from *in vitro* cultures

In the case of adherent cultures, monolayers with 70-80% confluency were used. After 2-3 steps of washing with PBS, cells were harvested using cell scraper and suspended in PBS. Meanwhile, suspension cultures were centrifuged at 3000 rpm for ten minutes, cell pellet was washed with PBS and finally the cell pellet was suspended in PBS. The suspension was dispersed without frothing and the required cell density was adjusted. The viability of the cell suspension was determined by trypan blue exclusion method (Kuttan et al., 1985) and the suspension is ready to seed for experiments if it satisfies the viability greater than 95%.

2.2.1.5. Determination of cell viability by trypan blue exclusion method (Kuttan et al., 1985)

The cell suspension (100 µL) was mixed with 100 µL of 1% trypan blue solution and incubated for 2-3 minutes. The cells were loaded on to the haemocytometer and the viable and dead cells were counted under the 10X objective of a light microscope. Dead cells are permeable to trypan blue while viable cells do not take up the dye. Hence the number of blue-coloured dead cells and the unstained viable cells can be counted and the percentage viability was determined using the formula,

$$\% \text{ of viable cells} = \frac{\text{Number of viable cells}}{\text{Number of viable cells + Number of dead cells}} \times 100$$

2.2.2. Determination of solid tumour development in experimental animals (Atia and Weiss, 1966)

Animals were induced with solid tumour on the right hind limb by subcutaneously injecting a viable cell suspension of the required number of tumour cells in PBS. Diameter of the developing tumour was measured using a vernier calipers and the tumour volume was calculated using the formula,

Tumour volume (cc) = $4/3\pi r_1^2 r_2$, where r_1 and r_2 are the radii of the tumour along the vertical and horizontal planes respectively.

2.2.3. Determination of survival rate of ascites tumour bearing experimental animals

Animals were induced with ascites tumour intraperitoneally by injecting a viable cell suspension of the required number of tumour cells in PBS. The death pattern of the animals was observed and the survival rate was calculated which was expressed in terms of mean survival days. The percentage increase in life span (%ILS) was calculated using the formula,

$$\% \text{ ILS} = \frac{T - C}{C} \times 100$$

Where, 'T' is the number of days survived by the animals treated with the test material and and 'C' is the number of days survived by the control animals.

2.2.4. Determination of haematological parameters in experimental animals

2.2.4.1. Determination of Haemoglobin (Drabkin and Austin, 1932)

Principle

Ferricyanide in the Drabkin's solution reacts with haemoglobin to form met-haemoglobin which then reacts with cyanide and get converted to cyanmethaemoglobin, which is having an absorption maximum at 540 nm.

Procedure

Blood sample (20 µL) was added to 5 mL of Drabkin's reagent taken in a test tube, mixed well and allowed to stand for 5 min at room temperature. The

optical density (OD) of the solution was measured at 540 nm in a spectrophotometer against reagent blank. The haemoglobin content was calculated using the formula,

$$\text{Haemoglobin (g/dL)} = \frac{\text{OD of the test sample}}{\text{OD of the standard solution}} \times 60 \times 0.251$$

2.2.4.2. Determination of total White Blood Cell count (Cheesbrough and McArthur, 1976)

Principle

Turks fluid contains acetic acid, which lyses the RBCs and crystal violet, which stains the WBCs. The sample, when mixed with Turks fluid and loaded on to a haemocytometer, the number of stained cells can be counted from the four corner squares.

Procedure

Blood sample (20 µL) was mixed with 380 µL of Turk's fluid, mixed well and allowed to stand at room temperature for 2-3 min. The cell suspension was then mixed gently and loaded on to the haemocytometer and counted under a light microscope using 10X objective. The total WBC count was determined using the formula,

$$\text{Total leukocytes/mm}^3 = \frac{\text{No. of cells (N)} \times \text{dilution factor} \times \text{depth factor}}{\text{Area counted}}$$

where, Dilution factor = 20 µL; Depth factor = 10 mm; Area counted = 4 sq.mm

Therefore, Total leukocyte counts/mm^3 = (N × 20 × 10)/4

Total leukocyte counts/mm^3 = N × 50

2.2.4.3. Differential Count of Leucocytes (Cheesbrough, 1976)

Procedure

A drop of blood sample was placed on one end of a clean glass slide and an even smear was made using a glass spreader and air dried. A few drops of Leishman's stain was evenly dropped over the smear and kept for 3 min.

Distilled water was then slowly added on to the stain in order to dilute it, followed by a rinse under tap water and air dried. Various leucocytes were differentially counted, out of a total of 100 cells, based on the morphology of their nucleus under 100X objective using immersion oil.

2.2.5. Determination of immunological parameters in experimental animals

2.2.5.1. Preparation of SRBC suspension (Mehra and Yaidya, 1993)

Sheep blood was collected in Alsever's solution (1:1) from the sheep house of Government Veterinary College, Thrissur, Kerala. This can be stored at 4^0C for not more than one week. Blood was washed thrice with equal volume of PBS (pH 7.2) and the cell pellet was suspended in Hanks balanced salt solution (HBSS). 10 parts of 4% SRBC and one part of 1% trypsin solution (0.2% in PBS-EDTA) were mixed and incubated at 37^0C for 30 min. Afterwards the cells were washed twice with PBS to remove the trypsin content and resuspended in PBS to a concentration of 2%.

2.2.5.2. Preparation of bone marrow cell suspension

The animals were sacrificed by cervical dislocation and fixed on a wax board. The skin and flesh overlying the femur bone were surgically removed and the femur bone was separated from the shaft. The bone marrow was flushed out into a test tube by carefully passing a jet of RPMI-1640 medium containing 2% FBS using a 26G needle and syringe. The cell suspension was gently dispersed by repeated pipetting without frothing, centrifuged and suspended at required cell concentrations in RPMI-1640 medium containing 10% FBS. All these procedures should be done in sterile conditions.

2.2.5.3. Preparation of spleen cell suspension

The animals were sacrificed and spleen was collected aseptically without any adherent tissue. Spleen was minced into pieces in PBS and processed into cell suspension using a stainless steel mesh. The whole suspension was transferred to a centrifuge tube and allowed to settle in an ice bath for 2 min. The supernatant was then centrifuged at 3000 rpm for 10 min. The cell pellet was

washed thrice with the PBS and resuspended in RPMI-1640 medium containing 10% FBS at required concentrations.

2.2.5.4. Preparation of thymus cell suspension

The animals were sacrificed and bilobed thymus was collected aseptically without any adherent tissue. It was processed to single cell suspension as the same way as that of the spleen cells and finally resuspended at required concentrations in RPMI-1640 medium containing 10% FBS.

2.2.5.5. Determination of bone marrow cellularity (Sredni et al., 1992)

Bone marrow cells were collected from both femurs of the animals as described above. The cells were dispersed well and resuspended in known volume of RPMI-1640 medium containing 10% FBS. The cell density was determined by serial dilution of this cell suspension and counting of the cells from the diluted suspension using a haemocytometer. Cell density was expressed in terms of total number of cells/femur.

2.2.5.6. Determination of α- naphthyl esterase activity (Bancroft, 1984)

Principle

The esterase enzyme present in the cytoplasm of differentiating monocytes will dissociate the α-naphthyl acetate present in the reaction mixture releasing α-naphthol. It will readily combine with the coupling agent para-rosaniline to produce an insoluble azodye at the site of enzyme activity. Haematoxylin will stain the nucleus blue against the yellowish-brown coloured cytoplasm.

Procedure

Bone marrow cells were collected from both femurs of the animals as described above. The cells were dispersed well and resuspended in minimum known volume of RPMI-1640 medium containing 10% FBS. The cell suspension was spread into a smear on a clean glass slide and air dried. The slide was then fixed using fixative solution containing 37% formaldehyde and air dried overnight. The slide was then immersed in a reaction solution containing α-naphthyl acetate, sodium nitrite and para-rosaniline in a coupling jar for 45 min. This was followed by thorough rinse with distilled water and staining with haematoxylin for 1-2 min. After rinsing again with distilled water, the slide was air dried and observed under a 100X objective of a light microscope using immersion oil.

2.2.5.7. Determination of circulating antibody titre (Singh et al., 1984)

Principle

SRBC will be agglutinated in presence of anti-SRBC antibody to from visible and diffused 'mat'. If the test sample is devoid of any anti-SRBC antibody, the non-agglutinated SRBC will settle as a clear 'button'. The maximum dilution of anti-sera sample at which we can observe for a clear agglutination gives the titre of the antibody.

Procedure

Anti-sera samples (100 µL) were serially diluted in round bottom 96-well tissue culture plates containing 100 µL of PBS/well (pH 7.2). 100 µL of trypsinized SRBC was then added to each well, mixed gently and incubated at room temperature for 3 hr. The maximum dilution of the sample at which clearly visible agglutination of SRBC observed was noted.

2.2.5.8. Jerne's plaque assay (Jerne and Nordin, 1963)

Principle

SRBCs dispersed in the vicinity of anti-SRBC antibody producing cells (contained in the sample) in a semi-solid matrix, will be lysed to form clear zones (plaques) in the presence of a complement source. The number of plaques correlates to the number of antibody producing cells.

Procedure

A mixture of 500 µL of agarose (0.5%), 50 µL of SRBC (7%) and 50 µL of spleen cell sample suspension (8×10^6 cells/mL) was distributed into tubes kept at 45^0C. The mixture was poured over a clean glass slide and allowed to solidify. The slides were incubated with fresh rabbit serum (1:10 diluted with PBS, pH 7.2) for 1 hr at 37^0C. The rabbit serum acts as a source for complement and hence the slides were placed in such a manner that the solidified gel remains in contact with the complement source. After incubation, the number of plaques were counted using a colony counter and represented as number of plaque forming cells/10^6 spleen cells.

2.2.5.9. Proliferation assay for splenocytes, thymocytes and bone marrow cells (^{3}H Thymidine incorporation assay) (Justo et al., 2003)

Principle

Specific mitogens can stimulate the proliferation of lymphoid cells. This rate of proliferation can be correlated to the rate of incorporation of ^{3}H Thymidine (added to the medium) to the DNA of the cells, which was estimated by measuring the radioactivity of the precipitated DNA.

Procedure

Sterile conditions should be maintained throughout the procedure. The cell suspension of spleen, thymus or bone marrow containing 5×10^4 cells (prepared as described in section) were seeded in to 96-well round bottom titre plates in RPMI-1640 medium supplemented with 10% FCS and antibiotics. The cells were incubated with or without mitogens (PHA-2.5 μg/mL; Con-A-10 μg/mL; PWM-10 μg/mL and LPS-10 μg/mL) in a final volume of 200 μL in a humidified atmosphere containing 5% CO_2 at 37^0C for 48 hrs. After incubation, 1 μCi of ^{3}H thymidine was added to each well and incubated for further 18 hr, provided the same conditions. Afterwards, DNA of the cell suspensions were precipitated using 10% perchloric acid and the pellets were dissolved in 0.5 mL of 0.5 N NaOH. The contents of each well was then transferred to 5 mL scintillation fluid and allowed to stand in dark, overnight. The radioactivity of the solution was measured using a liquid scintillation counter and expressed in units of counts per minute (CPM).

2.2.6. Determination of cell mediated immune responses in experimental animals

2.2.6.1. Determination of NK cell activity and ADCC by ^{51}Cr-release Assay (Kim et al., 2000)

Principle

When the cells were incubated with ^{51}Cr, it diffuses through the cell membrane and binds to the cytoplasmic proteins, which will then only be released when the cell membrane is ruptured.

2.2.6.1.1. Determination of NK cell - mediated cytotoxicity (Tsavaris et al., 2002)

Labelling of target cell

K-562 was used as the target cell. The cells ($1x10^6$) were washed in RPMI-1640 and were resuspended in a few drops of FBS. $Na_2{}^{51}CrO_4$ (100 µCi) was added and incubated in a shaker incubator at 37^0C for 1 hr. After incubation, cells were washed twice, resuspended in 5 mL of in RPMI medium and incubated for 1 hr at 4^0C. The cell suspension is then washed and resuspended in complete medium at a concentration of 1×10^5 cells/mL.

4hr ^{51}Cr release assay

The labelled cells (100 µL) were mixed with an equal volume of various dilutions of splenocytes to yield an effector: target ratio of 100:1 in 96-well round bottom titre plates in a final volume of 200 µL with RPMI-1640 and incubated at 37^0C for 4 hr. After incubation, the titre plates were centrifuged at 5000 rpm for 15 min and the supernatant was collected. Radioactivity of the supernatant was measured in a gamma ray spectrometer. In order to calculate the percentage of lysis caused by NK cell activity, the values of the following control tubes were monitored along with each experiment. Spontaneous release (SR) - wells contained only target cells and medium. Total release (TR) - wells contained target cells, medium and 0.1 mL of 1N HCl. Radioactivity of the samples were termed as experimental release.

$$\% \text{ Lysis} = \frac{\text{Experimental release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

2.2.6.1.2. Determination of antibody-dependent cell-mediated cytotoxicity (ADCC) (Kim et al., 2000)

SRBC was used as the target cell. The cells ($1x10^7$) were labelled as described above and 0.1 mL of the suspension is mixed with 100 µL of spleen cells (effector cells) to get an effector-target ratio of 100:1. This is followed by the addition of 50 µL of anti-sera against SRBC, raised in rabbits, and incubated for 4hrs at 37^0C. The final volume was made up to 200 µL with complete medium and the ^{51}Cr release assay was performed as explained above.

2.2.6.2. Determination of antibody-dependent complement-mediated cytotoxicity (ACC) (Singh et al., 1984)

Principle

When tumour cells were incubated with specific antibodies along with a complement source, the classical complement pathway will get activated resulting in tumour cell lysis. The percentage lysis of cells was estimated by trypan blue exclusion method.

Procedure

Antiserum was serially diluted in RPMI-1640 to get 1:1, 1:2 and 1:4 dilutions. An aliquot of 100 µL from these serum dilutions was mixed with $1x10^6$ 4T1 cells. 50 µL of the complement solution (1:10 diluted fresh rabbit serum) was added to make up the final to 2 mL and incubated at 37^0C for 3 hr. After incubation, the cells were centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the cell pellet was suspended in 100 µL of medium and cytotoxicity was assessed by Trypan blue exclusion method. Samples with cells alone, cells treated with complement alone, and cells treated with antibody alone were kept as controls (Kuttan et al., 1985).

2.2.7. Anti-metastatic studies on B16F10 melanoma-induced metastasis in experimental animals

2.2.7.1. Determination of B16F10 melanoma-induced pulmonary metastasis in experimental animals (Liotta, 1986)

C57BL/6 mice were used for the study. B16F-10 cells ($1x10^6$ cells/100 µL PBS) were injected through the lateral tail vein of mice. Animals were sacrificed on the 21^{st} day of tumour induction and the lung tissue was observed for the presence of metastatic tumour nodules. The lung tissue was then used for the estimation of various biochemical parameters such as lung collagen hydroxyproline, lung uronic acid, lung hexosamine. Blood was collected and the separated serum was used for the estimation of sialic acid and γ-glutamyl transpeptidase. Moreover, the lung tissue was also subjected to

histopathological analysis. A fixed number of metastasis-induced animals were maintained to observe their death pattern in order to determine the survival rate.

2.2.7.1.1. Estimation of biochemical parameters

2.2.7.1.1.1. Estimation of Hydroxyproline (Bergman and Loxley, 1970)

Principle

Hydroxyproline present in samples were oxidized to pyrrole derivatives which then react with p-DAB in acidic medium to produce a coloured product. The product is more stable in the presence of high concentrations of isopropanol and gives absorbance at 560 nm.

Reagents

1. Oxidant solution buffer

Sodium acetate	-	5.7 g
Trisodium citrate	-	3.75 g
Citric acid	-	0.55 g
Isopropanol	-	38.5 mL
Distilled water	-	61.5 mL

2. Chloramine T oxidant solution

Chloramine T - 1.75 g/25 mL distilled water

3. Ehrlich's reagent

Mix chloramine T solution and oxidant solution buffer in the ratio 1:4 on the day of use.

Procedure

Lung tissue (1 g) was homogenized using 4 mL isotonic saline and hydrolyzed using 6 N HCl. The tubes were sealed and incubated at 110^0C for 24 hr. The hydrolysate (1 mL) was neutralized with KOH and made up to 5 mL with distilled water. The neutralized sample (0.5 mL) was then mixed with 2.5 mL of isopropanol and 1 mL of oxidant solution with mixing and incubated at room temperature for 4 min. Ehrlich's reagent (2 mL) was added to the tubes and incubated at 60^0C in a water bath for 21 min. The tubes were then allowed to cool at room temperature for 1 hr. The absorbance of the solution was read at

560 nm. The concentration of hydroxyproline in the sample was determined with respect to a reference graph of hydroxyproline standard.

2.2.7.1.1.2. Estimation of Uronic acid (Bitter and Muir, 1962)

Reagents

Sulphuric acid reagent - 0.952 g sodium tetraborate in 100 mL of conc. H_2SO_4 (0.025 M) Carbazole reagent - 0.125 g carbazole in 100 mL absolute alcohol

Acetate Buffer (0.1M) - Solution A (0.2 M solution of acetic acid) + Solution B (0.2 M solution of sodium acetate)

Procedure

The lung tissue was digested using crude papain (10 mg /g dry weight of tissue) in 5 mL of 0.5 M acetate buffer (pH 5.5) containing 0.005 M cysteine and 0.005 M disodium salt of EDTA at 65^0C for 24 hr. Sulphuric acid reagent was prepared and 5 mL of the reagent was taken in pre-cooled tubes. The sample (1 mL) or standard glucuronolactone solution was layered on to the acid solution. The tubes were then closed with ground glass stoppers and the rack was shaken gently and then vigorously. The rack containing the tubes was then kept in boiling water bath shaker for 10 min and cooled at room temperature. Carbazole reagent (0.2 mL) was added to the tubes and incubated in a boiling water bath for 15 min with shaking followed by cooling. The pink colour thus developed was read at 530 nm. Uronic acid content of the tissues were estimated against the standard graph of glucuronolactone and expressed in terms of µg /100 mg wet weight.

2.2.7.1.1.3. Estimation of Hexosamine (Elson and Morgan, 1933)

Reagents

Preparation of 2% acetyl acetone in 0.5 M Na_2CO_3: 2 mL of acetyl acetone in 100 mL of 0.5 M Na_2CO_3

Preparation of Ehrlich's reagent Dissolve 1.33 g of p-dimethyl amino benzaldehyde (PDAB) in 100 mL of 1:1 ethanol: conc. HCl.

Principle

Hexosamine present in the samples react with acetyl acetone at 100^0C. The resulting product will again react with Ehrlich's reagent to form a coloured product which can be read at 530 nm.

Procedure

Lyophilized tissue samples (5 mg) were hydrolyzed with 2N HCl (5 mL) at 100^0C for 6 hr. HCl was then removed by evaporation, the residue was dissolved in water and made up to a known volume. Sample aliquots were added to 1 mL of freshly prepared 2% acetyl acetone in capped tubes and kept in boiling water bath for 15 min. After cooling in tap water, 5 mL of 95% ethanol and 1 mL of Ehrlich's reagent were added and mixed thoroughly. The purple red colour developed was read after 30 min at 530 nm. Water blank and standard glucosamine solutions of various concentrations were also treated similarly to get a standard curve. Hexosamine contents of tissues were expressed as μg /100 mg dry weight.

2.2.7.1.1.4. Estimation of protein bound serum sialic acid (Skoza and Mohos, 1976)

Principle

Acid hydrolysis of sample causes the liberation of sialic acid, which then forms a coloured product with thiobarbituric acid.

Reagents

H_2SO_4	-	0.2 N
Periodic acid	-	25 μM in 62.5 mM H_2SO_4
Sodium arsenite	-	0.2% in 0.5 M HCl
Thiobarbituric acid	-	6% (pH 9.0)

Procedure

Sample (200 μL) was mixed with equal volume of 0.2N H_2SO_4 and hydrolyzed for 1 hr at 80^0C. To this hydrolysate, 50 μL of periodic acid (25 μM) was added and incubated for 30 min at 37^0C. This is followed by the addition of 50 μL of sodium arsenite and then 100 μL of thiobarbituric acid. The solution is heated in a boiling water bath for 7.5 min and 400 μL of DMSO was added to intensify

the colour and read at 549 nm and 532 nm. Sialic acid standard solutions were used to plot the standard graph.

2.2.7.1.1.5. Estimation of γ-glutamyl transferase (Szasz, 1976)

Principle

γ-glutamyl transferase catalyses the transfer of γ-glutamyl moiety of a γ-glutamyl donor to the acceptor.

Gamma-glutamyl-p-nitroanilide+acceptor ⟶ gammaglutamyl-acceptor + p- nitroaniline

The release of p-nitroaniline was determined from the increase in absorbance at 410 nm.

Reagents

L- γ-glutamyl-p-nitroanilide - 2.5 mM
Glycyl glycine - 20 mM
Tris-HCl (pH 8.0) - 0.05 M
NaCl - 75 mM

Procedure

The standard assay mixture comprises all the regents as described above in a final volume of 1 mL, to which 25 µL of the sample was added. The rate of release of p-nitroaniline was measured at an optical density of 410 nm using a spectrophotometer.

2.2.7.1.2. Histopathological Analysis (Culling et al., 1976)

The tissue was fixed in 10% neutral formalin for at least 4 hr. The tissues were then dehydrated by passing through alcohol series, cleaned in xylene and embedded in paraffin. The paraffin embedded tissue was then sectioned to obtain sections of about 5-6 µm thickness which were then mounted on a clean glass slide. The sections are then stained with haematoxylin and eosin and visualized under the microscope to observe any histological changes.

2.2.7.1.3. Determination of the rate of survival of experimental animals

The animals were injected with B16F-10 melanoma cells ($1x10^6$ cells /100 µL) intravenously through the lateral tail vein (Liotta, 1986). The number of survival days of each animal was noted. The percentage increase in life span (% ILS), caused by treatment with any test material was calculated using the formula,

$$\% \text{ ILS} = \frac{T - C}{C} \times 100$$

Where, 'T' is the number of days survived by the animals treated with the test material and and 'C' is the number of days survived by the control animals.

2.2.8. Anti-angiogenic studies in experimental animals

2.2.8.1. Capillary counting assay (Kishi et al., 2000)

C57BL/6 mice were used for the study. The animals were shaven at the ventral skin and were intradermally inoculated with B16F10 melanoma cells ($1X10^6$ cells /animal) to develop solid tumours. On the 11^{th} day of tumour induction, animals were again shaven at their ventral skin and sacrificed. The skin around the developed tumour was dissected out and the number of tumour directed capillaries per cm^2 was counted using a dissection microscope.

2.2.8.2. Determination of VEGF and GMCSF levels

Blood was collected from the caudal vein of animals at specific time points; serum was separated and used for the estimation of VEGF and GMCSF levels by specific quantitative ELISA kits.

2.2.9. Studies on the expression of marker genes

2.2.9.1. RNA isolation from tissue (Chomczynski and Mackey, 1995)

All the glass wares and plastic wares used for RNA isolation should be rinsed with DEPC treated water, autoclaved twice and dried in hot air oven at 40^0C. Lung tissues (100 mg) were collected from the animals; washed with PBS and

minced well and disperse the cells in cold conditions. Trizol reagent (1 mL) was added, dispersed well by pipetting and incubated at room temperature for 5 min. The solution was aspirated out by pipetting and transferred to a centrifuge tube placed on ice bath. The same step was repeated with 250 µL of trizol reagent and the cell suspension was collected in the same tube. The suspension was centrifuged at 10,000 rpm for 10 min at 4^0C. The supernatant was collected in another tube and kept at room temperature. Chloroform (300 µL) was added to the supernatant and mixed well for 3 min at room temperature. The resulting milky pink solution is then mixed by inverting the tube and it was centrifuged at 10,000 rpm for 10 min at 4^0C. The upper aqueous layer was carefully collected to an appendorf tube and 600 µL of ice cold isopropanol was added to it. The solution was mixed well and allowed to stand at room temperature for 10 min. After incubation, the tubes were centrifuged at 12,000 rpm for 10 min at 4^0C. The supernatant was discarded; the pellet was dissolved in 70% ethanol (prepared in DEPC-treated water) and kept at room temperature for 10 min. It was then centrifuged at 13,000 rpm for 10 min at 4^0C. The supernatant was discarded; the pellet was dried and dissolved in 50µL of DEPC-treated water and stored at -20^0C. The qualitative checking of RNA samples were done by agarose gel electrophoresis. RNA was quantified by measuring the absorbance ratios of 260/280 and 260/230.

2.2.9.2. cDNA synthesis from RNA (Sambrook and Russel, 2001)

An aliquot of RNA sample containing 4 µg of RNA was used for cDNA synthesis.

The reaction mixture consists of the following components.

RNase free water	–	4.4 µL
AMV RT buffer	–	1 µL
Oligo (dT)	–	0.6 µL
dNTPs	–	1 µL
RNA sample	–	1 µL (vary depending up on the concentration of the RNA sample)
Total	=	8 µL

The reaction mixture was incubated at 65^0C for 5 min. This was followed by cooling at room temperature for 5 min. DTT (100 mM, 1 µL) was added, followed by addition of 1 µL of AMV RT. The tubes were kept at 42^0C for 30 min. The prepared cDNA was stored at -20^0C.

2.2.9.3. Amplification of genes using specific primers

Amplification of marker genes were done using specific primers, whose sequences are given below (Tab.2.1).

The master mix was prepared as described below.

Components	**Quantity/sample**
5X PCR Buffer	– 5 µL
Molecular grade water	- 13 µL
dNTPs	– 1 µL
25mM Mg Cl_2	– 2.5 µL
Forward Primer	– 1 µL
Reverse Primer	– 1 µL
cDNA sample	– 1 µL
Taq Polymerase	– 0.5 µL
Total	= 25 µL

The master mix was then subjected to amplification in the minicycler with the following reaction profile.

Steps	**Temperature**	**Time**
Step 1	94^0C	4 min
Step 2	94^0C	1 min
Step 3	58.5^0C	30 sec (for GAPDH)
Step 4	72^0C	1 min
Go to step 2 and repeat 40 cycles		
Step 5	72^0C	7 min

The temperature and time duration in Step 3 will vary depending up on the primers used, which is noted as follows.

Gene	Temperature	Time	No: of cycles
MMP-2	57.5^0C	30 sec	40
MMP-9	65.0^0C	30 sec	40
ERK-1	63.5^0C	30 sec	40
ERK-2	62.5^0C	30 sec	40
VEGF	61.5^0C	30 sec	40
TIMP-1	58.0^0C	30 sec	40
TIMP-2	60.5^0C	30 sec	40

2.2.9.4. Detection of PCR products

The PCR product (10 µL) was resolved in 1.5 % agarose gel by electrophoresis.

Reagents

a) 5X TAE buffer

   Tris-base – 24.2g

   Acetic acid – 5.71 mL

   0.5 M EDTA – 10 mL

Add 800 mL of double distilled water, adjust the pH to 8.3 and made up to 1L

b) 10X gel loading dye

c) DNA molecular weight marker

Procedure

The electrophoresis apparatus was cleaned well and the edges of the gel tray were sealed. The gel comb was placed appropriately. 1.5% agarose gel was prepared in 1X TAE buffer and 0.5µg/mL ethidium bromide (1 µL) was added to it. The gel was mixed well without air bubbles; carefully poured into the gel tray and allowed to solidify. Then the comb was carefully removed and the electrophoresis tank was filled with 1X TAE buffer until the gel is fully immersed in the buffer. The molecular weight marker as well as the amplified DNA samples (10 µL) were separately mixed with the gel loading dye (2 µL) and loaded into individual wells of the gel. Electrophoresis was carried out at 70V until the dye front reaches $3/4^{th}$ portion of the gel. Afterwards, the gel was photographed under UV illuminator chamber using the gel documentation system.

2.2.10. Studies on 4T1 mammary carcinoma-induced spontaneous metastasis in experimental animals (Pulaski and Ostrand-Rosenberg, 2001)

2.2.10.1. Solutions and Reagents

1. 1X Hank's balanced salt solution (HBSS)
2. Deionized distilled water for making reagent solutions
3. Collagenase type I cocktail

   Collagenase type I (50 mg) was dissolved in 25 ml of 1X HBSS. The solution is filter- sterilized and stored as aliquots containing 2.5 ml per tube at -20^{0}C. The aliquots were thawed in a 37^{0}C water bath, when needed.
4. Collagenase type IV/elastase cocktail

   Collagenase type IV (50 mg) was dissolved in 25 ml of 1X HBSS. The solution is filter- sterilized and stored as aliquots containing 2.5 ml per tube at -20^{0}C. The aliquots were thawed in a 37^{0}C water bath, when needed, and 30 units elastase was added per aliquot.
5. Hyaluronidase cocktail

   Hyaluronidase (50 mg) and BSA (0.025g, preservative for storage) was dissolved in 25 ml 1X HBSS; filter sterilized and stored as 2.5 mL aliquots at -20^{0}C. The aliquots were thawed in 37^{0}C water bath, when needed.
6. 6-Thioguanine, 60 mM and 60 µM

   6-thioguanine (0.1 g) was dissolved in 10 ml of 0.01 M NaOH to make the 60 mM stock solution and stored at 4^{0}C. The solution was thawed in 37^{0}C water bath, when needed, and diluted to 60 µM in culture media.

2.2.10.2. Determination of 4T1 induced mammary tumour development and metastasis in BALB/c mice

Female BALB/c mice (8 weeks old) were used for the study. The animals were injected with 7x10^4 4T1 mammary carcinoma cells subcutaneously into the fourth mammary gland by gently piercing the skin using a 27G needle with the bevel of the needle facing up and injecting the required volume. The tumour diameters were measured using vernier calipers, every week starting from the 7th

day up to the 42nd day of tumour induction. The measurement was done in such a way that one should measure the length wise and width wise diameter of the tumour which are perpendicular to each other. The tumour volume was calculated using the parabolic equation,

Tumour volume (cc) = $(LW^2)/2$, where L is the length and W is the width, where L>W.

The metastasis of these tumour cells to their secondary sites was studied as follows.

2.2.10.3. Determination of 4T1 mammary carcinoma-induced spontaneous metastasis to multiple organs (lymph nodes, lungs and liver) in BALB/c mice

Female BALB/c mice (8 weeks old) were induced with 4T1 mammary tumour as described above. The metastatic organs such as draining lymph nodes, lungs and liver were separately collected by sacrificing the animals at different time points. The organs were processed to cell suspensions and the metastatic cells were screened using 6-thoguanine. All these procedures should maintain sterile conditions.

2.2.10.3.1. Preparation of lymph node samples

The tumour-induced animals were sacrificed on the 18th day of tumour induction and the draining lymph nodes were aseptically collected and washed in a petri dish containing 1X HBSS. The lymph node was then transferred to a 10 cm tissue culture dish containing 10 mL of culture media supplemented with 60 µM 6-thioguanine. The lymph node was teased well and the cells were dispersed into the medium using a cell strainer. The suspension was then incubated for 48 hr at 37^0C provided with 5% CO_2 atmosphere.

2.2.10.3.2. Preparation of lung samples

The lung tissue was aseptically collected on the 25th day of tumour induction and transferred to a 6 cm culture dish containing 5 mL of 1X HBSS. The tissue was washed properly to remove any remaining blood and transferred it to

another culture dish. Then, the tissue was minced well and transferred to a centrifuge tube containing 2.5 mL collagenase type IV/elastase cocktail. The culture dish was again rinsed with 2.5 mL of HBSS which was also transferred to the above tube. The samples were incubated at 4^0C for 75 min with mixing at intervals. After incubation, the samples tubes were diluted to 10 mL using 1X HBSS and filter the cell suspension through a nylon strainer to avoid chunks of the tissue. The resulting sample solution was centrifuged at 1500 rpm for 5 min at room temperature. The pellet was washed with 1X HBSS for two-three times and finally it was suspended in 10 mL culture medium containing 60 µM, 6-thioguanine. The suspension was then incubated for 48 hr at 37^0C provided with 5% CO_2 atmosphere.

2.2.10.3.3. Preparation liver samples

The liver was aseptically collected on the 32^{nd} day of tumour induction and transferred to a 6 cm culture dish containing 5 mL of 1X HBSS. The tissue was washed properly to remove any remaining blood and transferred it to another culture dish. Then, the tissue was minced well and transferred to a centrifuge tube containing 2.5 mL collagenase type I cocktail. The culture dish was again rinsed with 2.5 mL of hyaluronidase cocktail which was also transferred to the above tube. The samples were incubated at 37^0C for 25 min with mixing at intervals. After incubation, the enzyme-digested samples were diluted to 10 mL using 1X HBSS and filtered through a nylon strainer to avoid chunks of the tissue. The resulting sample solution was centrifuged at 1500 rpm for 5 min at room temperature. The pellet was washed with 1X HBSS for two-three times and finally it was suspended in 10 mL culture medium containing 60 µM, 6-thioguanine. The suspension was then incubated for 48 hr at 37^0C provided with 5% CO_2 atmosphere.

2.2.10.3.4. Screening of metastatic cells using 6-thioguanine

4T1 carcinoma cells are resistant to 6-thioguanine. Hence the cells can be detected easily from the organ tissues where the 4T1 tumours establish their metastases. When the processed tissue samples were incubated with 6-thioguanine, only the metastatic 4T1 cells will survive and grow attached to the

culture dish. After incubation of the sample suspensions in 6-thioguanine containing medium, the supernatant was poured out and the inner surface of the culture dishes was washed with PBS. The cells were then stained using 1% crystal violet solution and viewed under the light microscope. Besides this, duplicate samples of the same organ were incubated with 6-thioguanine for 48 hr. The cells attached on the culture dish were then trypsinized and counted on to a haemocytometer in order to estimate the cell density.

2.2.10.3.5. Determination of biochemical parameters of lung tissue and serum

The lung biochemical parameters such as hydroxyproline, hexosamine and uronic acid and serum parameters such as sialic acid and GGT content were estimated as per the methods described in section 2.2.8.1.

2.2.10.3.6. Determination of expression of marker genes of primary tumour

The RNA of the tumour tissue was isolated, cDNA was prepared and the amplification of genes such as MMP-2, MMP-9, TIMP-1, TIMP-2, VEGF and GAPDH were performed using specific primers. The amplified products were then resolved in 1.5% agarose gels and viewed under UV using the gel documentation system. The procedure was explained in detail in section 2.2.10.

2.2.10.3.7. Determination of survival rate of animals

The animals were induced metastasis using 4T1 cells as described in section and the survival rate of animals were determined as described in section 2.2.8.1.8.

2.2.11. Statistical analysis of data

All data were expressed as mean ± S.D. The statistical analysis of the data of independent experiments were done by one way ANOVA using Graphpad InStat version 3.00 for Windows 95, GraphPad Software, California, USA.

Table 2.1 Oligonucleotide primer sequences used to study expression patterns of the genes

Gene	Primer sequence	base pairs
MMP-2	Forward 5′-GAGTTGGCAGTGCAATACCT-3′ Reverse 5′-GCCGTCCTTCTCAAAGTTGT-3′	354
MMP-9	Forward 5′-AGTTTGGTGTCGCGGAGCAC-3′ Reverse 5′-TACATGAGCGCTTCCGGCAC-3′	327
ERK-1	Forward 5′-GCTCGACCACACTGGCTTTC-3′ Reverse 3′-GATCAACTCCTTCAGCCGCTC-3′	512
ERK-2	Forward 5′-ACAGGACCTCATGGAGACGG-3′ Reverse 5′-GATCTGCAACACGGGCAAGG-3′	216
VEGF	Forward 5′-TGCTCACTTCCAGAAACACG-3′ Reverse 5′-GGAAGGGTAAGCCACTCACA-3′	453
TIMP-1	Forward 5′- CTGGCATCCTCTTGTTGCTA-3′ Reverse 5′- AGGGATCTCCAGGTGCACAA-3′	414
TIMP-2	Forward 5′-AGACGTAGTGATCAGGGCCA-3′ Reverse 5′-GTACCACGCGCAAGAACCAT-3′	525
GAPDH	Forward 5′-CGTCCCGTAGACAAAATGGT-3′ Reverse 5′-CCTTCCACAATGCCAAAGTT-3′	527

Chapter 3
Screening of potentiated homeopathic medicines for their anti-tumour activity using in vivo tumour models

3.1. Introduction

Use of complementary and alternative therapies is widespread among cancer patients. Throughout the world cancer patients try many questionable or unproven treatment methods. The reasons for adopting these therapies are complex and are related to the social and cultural contexts of their geographical locations (Shukla and Pal, 2004). Homeopathy is a holistic system of alternative medicine in which the medicines are proposed based on individualisation rather than the concept of specific remedy for any diseases. As described by Hahnemann, "Every powerful medicinal substance produces in the human body a kind of peculiar disease, the more powerful the medicine, the more particularly marked and violent the disease. We should imitate nature, which sometimes cures a chronic disease, by superadding another, and employ in the disease (especially chronic) we wish to cure, that medicine which is able to produce another very similar artificial disease, and the former will be cured similia similibus." This principle is likely to be explained as every medicinal substance that can produce a set of disease symptoms in the healthy body can be used to cure those particular symptoms exhibited by a diseased individual. He published his findings in 1810, as the book, The Organon of the Rational Art of Healing, wherein he described the methods and principles of a system of medical treatment to which he had given the name of "Homeopathy" (Hahnemann, 1982).

Even though homeopathy prescribes many drugs for malignant conditions, it is yet unproven scientifically to accept their efficacy and reliability. Nowadays, it is mainly used in supportive cancer care and hence suggestions to use them in integrative therapy are documented based on scientific studies (Kassab et al., 2009). Several studies supported that CAM could improve the ill effects of the patients and can partly or wholly substituted conventional medicines (Ernst, 2005; Frenkel, 2010).

Our present study aims to screen the selected homeopathic medicines, say *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum* for their activity to inhibit or delay the tumour development in *in vivo* transplanted tumour models, which will be subsequently used for further studies.

3.2. Materials and Methods

3.2.1. Homeopathic drugs, dosage and administration

1M, 200c and 30c potencies of the homeopathic medicines of *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum* (succused in 90% ethanol) were orally administered at a dose of 10 µl per dose for ten consecutive days. In order to optimise the ethanol concentration, 10 µl of each drug was diluted to 100 µl using sterile water and administered to each animal. Potentiated ethanol was used as the vehicle control.

3.2.2. Cell lines

DLA and B16F10 cells were used to induce solid tumours while EAC and EL-4 cell lines were used to induce ascites tumours in mice models.

3.2.3. Animals

Inbred male Swiss albino mice (6-8 weeks old, 25-30 g body weight) were used as model for DLA-induced solid tumour, EAC and EL-4-induced ascites tumours. C57BL/6 mice were used as models for B16F10 melanoma induced solid tumours.

3.2.4. Effect of potentiated medicines on DLA-induced solid tumour

Eleven groups (12 animals /group) of Swiss albino mice were used for the experiment. All the animals were induced solid tumour by injecting Dalton's lymphoma ascites tumour cells ($1x10^6$ cells/animal), subcutaneously on the right hind limb of mice. Drug treatment was started on the 7^{th} day after tumour induction. Group I was kept as untreated tumour control. Group II was treated with potentiated ethanol. Groups III, IV and V were treated with 10 doses *Thuja* 1M, *Thuja* 200c and *Thuja* 30c respectively. Groups VI, VII and VIII were treated with *Ruta* 1M, 200c and 30c respectively. Groups IX, X and XI were treated with *Carcinosinum* 1M, 200c and 30c respectively. The radii of developing tumours were measured every third day starting from the seventh day of tumour inoculation using Vernier calipers for 30 days and tumour volume

was calculated using the formula $V = 4/3\ r_1^2\ r_2$, where r_1 and r_2 are the radii of the tumour along two directions.

3.2.5. Effect of potentiated medicines on EAC-induced ascites tumour

Swiss albino mice were grouped as above and were induced ascites tumour by injecting EAC cells ($1x10^6$ cells/animal), intraperitoneally. Drug treatment was started on the 7^{th} day after tumour induction. Group I was kept as untreated tumour control. Group II was treated with potentiated ethanol. Groups III, IV and V were treated with 10 doses *Thuja* 1M, *Thuja* 200c and *Thuja* 30c respectively. Groups VI, VII and VIII were treated with *Ruta* 1M, 200c and 30c respectively. Groups IX, X and XI were treated with *Carcinosinum* 1M, 200c and 30c respectively. The death pattern of all the animals was noted and the mean survival days were calculated.

3.2.6. Effect of potentiated medicines on EL-4 induced ascites tumour

Swiss albino mice were grouped as above and were induced ascites tumour by injecting EL-4 cells ($1x10^6$ cells/animal), intraperitoneally. Drug treatment was started on the 7^{th} day after tumour induction. Group I was kept as untreated tumour control. Group II was treated with potentiated ethanol. Groups III, IV and V were treated with 10 doses *Thuja* 1M, *Thuja* 200c and *Thuja* 30c respectively. Groups VI, VII and VIII were treated with *Ruta* 1M, 200c and 30c respectively. Groups IX, X and XI were treated with *Carcinosinum* 1M, 200c and 30c respectively. The death pattern of all the animals was noted and the mean survival days were calculated.

3.2.7. Effect of potentiated medicines on B16F10-induced solid tumour

Animals were grouped as described above and were induced solid tumour by injecting B16F10 melanoma tumour cells ($1x10^6$ cells/animal), subcutaneously on the right hind limb of mice. Drug treatment was started on the 7^{th} day after tumour induction. Group I was kept as untreated tumour control. Group II was treated with potentiated ethanol. Groups III, IV and V were treated with 10 doses *Thuja* 1M, *Thuja* 200c and *Thuja* 30c respectively. Groups VI, VII and VIII were treated with *Ruta* 1M, 200c and 30c respectively. Groups IX, X and

XI were treated with *Carcinosinum* 1M, 200c and 30c respectively. The radii of developing tumours were measured every third day starting from the seventh day of tumour inoculation using Vernier calipers for 30 days and tumour volume was calculated using the formula $V = 4/3\ r_1^2\ r_2$, where r_1 and r_2 are the radii of the tumour along two directions.

3.3. Results

3.3.1. Effect of potentiated medicines on DLA-induced solid tumour

Figure 3.1a shows the effect of different potencies of *Thuja occidentalis* on the development of DLA-induced solid tumour in Swiss albino mice. It was found that the 1M potency of *Thuja* could significantly inhibit the tumour development when compared to the 200c and 30c potencies. The tumour volume on the last day of the study was 1.04 ± 0.05 cc ($P<0.001$), 2.77 ± 0.25 cc ($P<0.001$) and 3.57 ± 0.12 cc for *Thuja* 1M, 200c and 30c potencies respectively, which should be compared to the tumour control group which showed a tumour volume of 3.89 ± 0.16 cc on the same day.

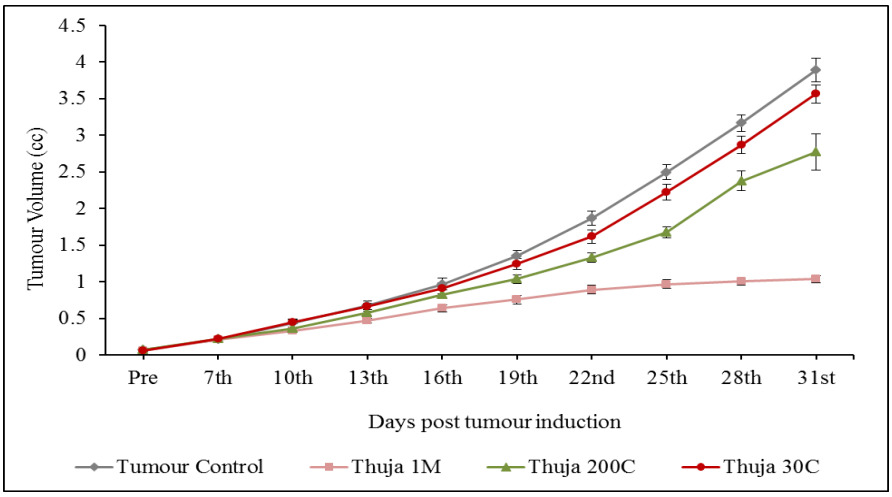
Figure 3.1b depicts the effect of different potencies of *Ruta graveolens* on the development of DLA-induced solid tumour. Out of the three potencies of *Ruta*, the 1M potency did not showed any anti-tumour effects when compared to the control. Meanwhile, the 200c potency as well as the 30c potency showed significant inhibition, both at the same level when compared to the tumour control group. The tumour volume of the control group was 3.89 ± 0.16 cc on the 31st day, whereas the volume was 1.69 ± 0.11 cc and 1.89 ± 0.21 cc for *Ruta* 200c and 30c respectively ($P<0.001$), which represents a significant effect. It is important to note that the groups treated with the 200c and 30c potencies did not showed any significant difference in tumour volume when compared to each other.

Figure 3.1c represents the effect of 1M, 200c and 30c potencies of *Carcinosinum* against DLA-solid tumour in mice. It was found that *Carcinosinum* 1M only showed significant effect against the tumour development when compared to the control ($P<0.001$). The 200c and 30c

potencies showed a relatively reduced tumour volume on the final day, but it is less statistically significant, when compared to the control. The tumour volume for the 1M treated group was 1.77 ± 0.20 cc on the final day whereas the control group showed a tumour volume of 3.89 ± 0.16 cc on the same day. As shown in Figure 3.1d, potentiated ethanol, which was used as the vehicle control did not showed any effects when compared to the tumour control.

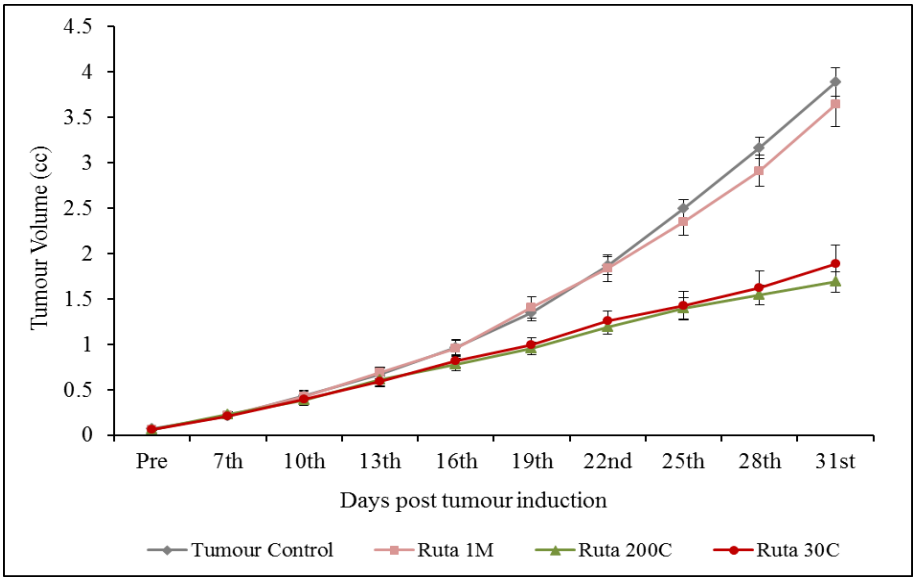
Figure 3.1

a. Effect of potentiated *Thuja* on DLA-induced solid tumour bearing mice



Values are mean ± standard deviation.

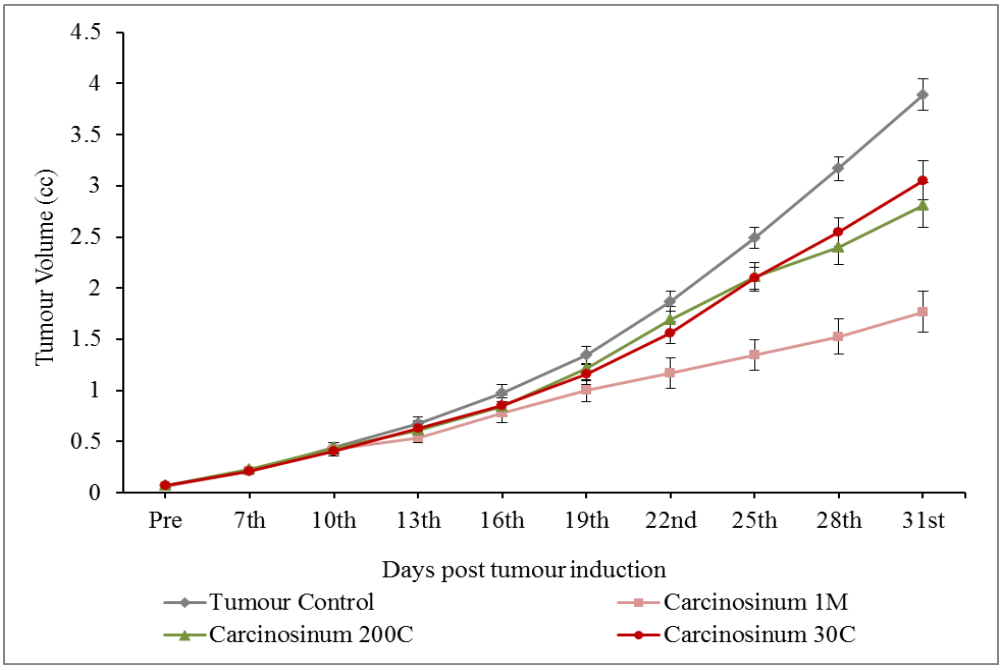
b. Effect of potentiated *Ruta* on DLA-induced solid tumour bearing mice



Values are mean ± standard deviation.

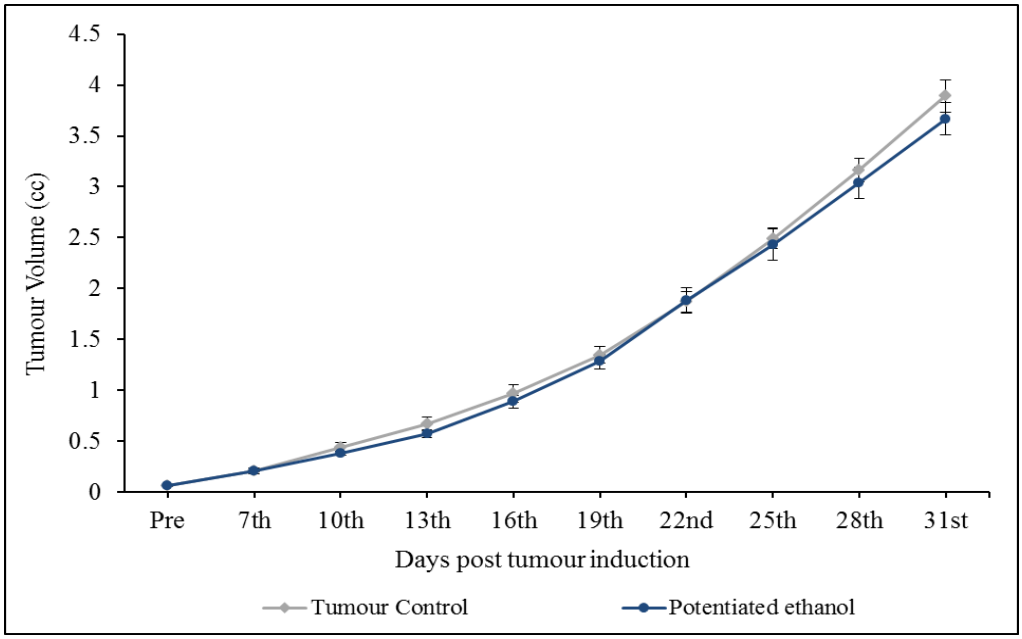
Figure 3.1 continued…

c. Effect of potentiated *Carcinosinum* on DLA-induced solid tumour bearing mice



Values are mean ± standard deviation.

d. Effect of potentiated ethanol on DLA-induced solid tumour bearing mice



Values are mean ± standard deviation.

3.3.2. Effect of potentiated medicines on EAC-induced ascites tumour

As shown in Table 3.1, *Thuja* 1M, *Ruta* 200c and *Ruta* 30c only showed statistically significant increase in the life span of EAC-induced ascites tumour bearing animals. The remaining potencies of the three drugs did not affect the ascites development and the life expectancy of the tumour bearing animals. It should be stated that treatment with *Thuja* 1M, *Ruta* 200c and *Ruta* 30c increased the life span of ascites bearing animals to 62.05 %, 47.14 % and 29.35 % respectively, when compared to the tumour control where the mean survival days was only 17.33±1.67 days. It should be noted that the anti-tumour effects of *Ruta* 200c and *Ruta* 30c did not vary significantly when compared to each other. Figure 3.2 depicts the death pattern of all animals in the groups treated with *Thuja* 1M and *Ruta* 200c compared to the tumour control group.

Table 3.1

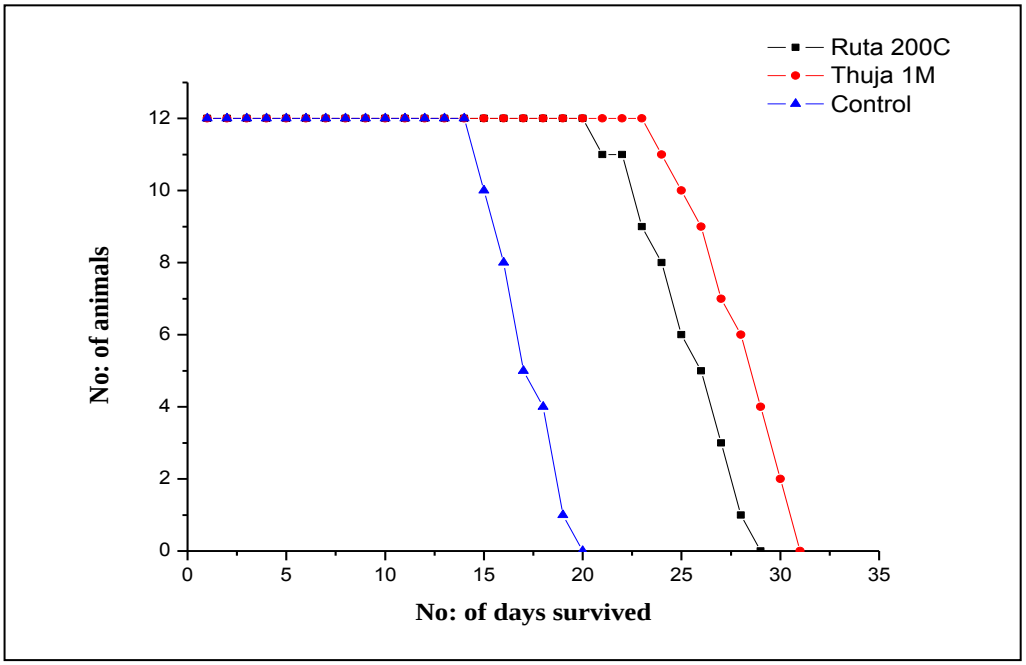
Effect of potentiated homeopathic medicines on EAC-induced ascites tumour bearing mice

Group	Mean Survival Days	% Increase in Life Span
Tumour Control	17.33 ± 1.67	ns
Potentiated ethanol	18 ± 1.41	ns
Thuja 1M	28.08 ± 2.31[a]	62.05
Thuja 200c	19.33 ± 1.83	ns
Thuja 30c	18.08 ± 2.11	ns
Ruta 1M	18.92 ± 1.38	ns
Ruta 200c	25.5 ± 2.43[a]	47.14
Ruta 30c	22.42 ± 2.39[a]	29.35
Carcinosinum 1M	17.58 ± 2.15	ns
Carcinosinum 200c	19 ± 1.81	ns
Carcinosinum 30c	18.42 ± 1.73	ns

Values are mean ± standard deviation. [a]$P < 0.001$, ns - not significant with respect to the tumour control.

Figure 3.2

Effect of *Thuja* 1M and *Ruta* 200c on EAC-induced ascites tumour bearing mice



Grouped animals were induced with ascites tumour and treated with or without the drug. Death pattern of the animals were noted and expressed graphically. Values are individual observations of the death pattern of animals.

3.3.3. Effect of potentiated medicines on EL-4 induced ascites tumour

As shown in Table 3.2, out of the three potencies of all the selected drugs, only 1M potency of *Thuja* could increase the life span of EL-4 induced ascites tumour bearing animals when compared to the tumour control group. *Ruta* and *Carcinosinum*, as well as the vehicle control, did not showed any increase in the survival of tumour bearing animals. Figure 3.3 depicts the death pattern of all animals in the *Thuja* 1M treated group compared to the tumour control group.

Table 3.2

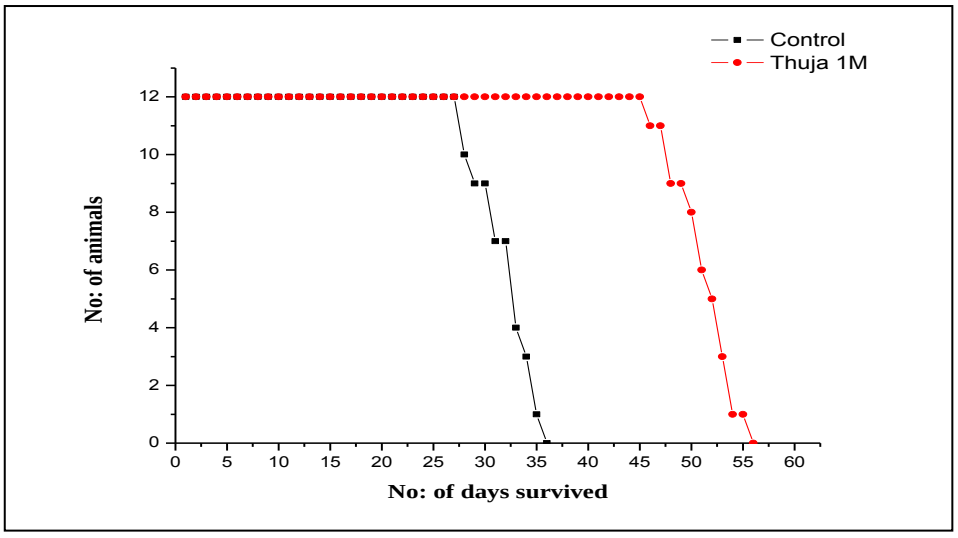
Effect of potentiated homeopathic medicines on EL-4-induced ascites tumour bearing mice

Group	Mean Survival Days	% Increase in Life Span
Tumour control	32.16 ± 2.76	ns
Potentiated ethanol	33.08 ± 2.15	ns
Thuja 1M	51.33 ± 2.93[a]	59.57
Thuja 200c	37.5 ± 2.15	ns
Thuja 30c	30.17 ± 2.12	ns
Ruta 1M	33 ± 1.95	ns
Ruta 200c	32.58 ± 2.11	ns
Ruta 30c	33.08 ± 2.35	ns
Carcinosinum 1M	34.17 ± 2.52	ns
Carcinosinum 200c	33.25 ± 2.53	ns
Carcinosinum 30c	35.75 ± 2.49	ns

Values are mean ± standard deviation. [a]$P < 0.001$, ns - not significant, with respect to the tumour control.

Figure 3.3

Effect of *Thuja* 1M on EL-4-induced ascites tumour bearing mice



Grouped animals were induced with ascites tumour and treated with or without the drug. Death pattern of the animals were noted and expressed graphically. Values are individual observations of the death pattern of animals.

3.3.4. Effect of potentiated medicines on B16F10-induced solid tumour

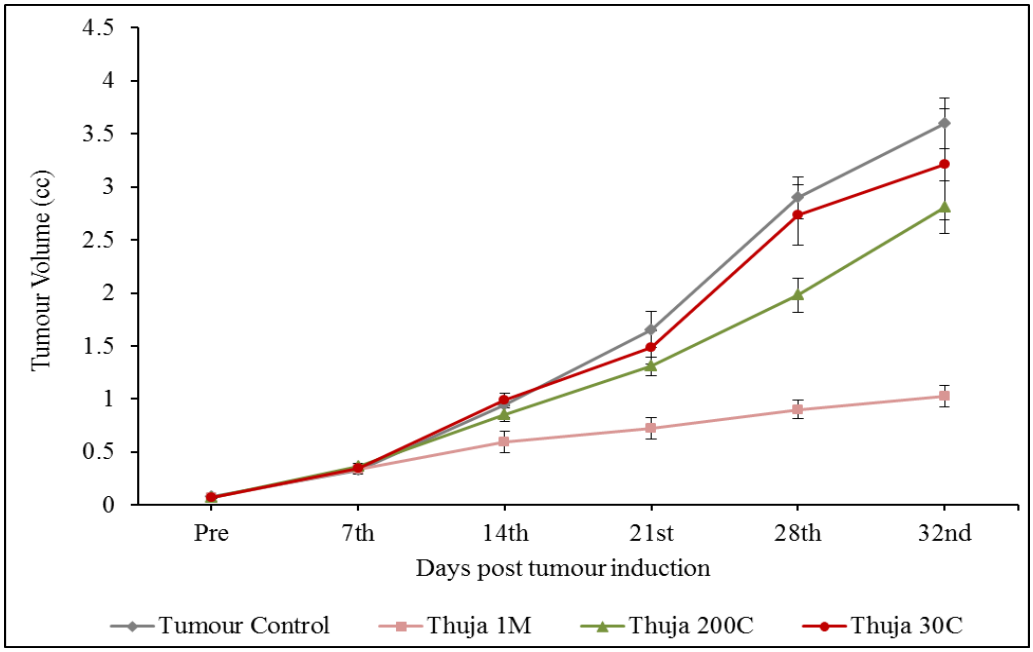
The effect of 1M, 200c and 30c potencies of *Thuja* on the development of B16F10-induced solid tumour in C57BL/6 mice is shown in Figure 3.4a. It was evident from the figure that the drug showed a significant effect against the solid tumour development not in a concentration dependant manner, but in a potentiation dependant manner. 1M potency of *Thuja* was showing the maximum activity with a tumour volume of 1.03 ± 0.10 cc on the 32nd day of tumour induction which was found very much reduced when compared to the tumour control group showing a tumour volume of 3.60 ± 0.24 cc. The 200c potency treated group showed a tumour volume of 2.81 ± 025 cc and the 30c potency group did not showed a significant effect when compared to the tumour control group.

Out of the three potencies of *Ruta*, the 200c and 30c potencies showed a significant reduction in the tumour volume compared to the tumour control group. The tumour volume for *Ruta* 200c, *Ruta* 30c and the tumour control were 1.97 ± 0.28 cc, 2.08 ± 0.18 cc and 3.60 ± 0.24 cc respectively. The 1M potency did not showed any significant difference in tumour volume (3.69 ± 0.35 cc) compared to the control (Fig 3.4b). Also, the 200c and 30c potencies showed similar effects and their values did not show any significant statistical difference with respect to each other.

Figure 3.4c showed that any of the potencies of *Carcinosinum* could not delay or inhibit the development of B16F10-induced solid tumour in mice. The values of tumour volume lies very close to that of the tumour control during all the time points of the study. As shown in Figure 3.4d, the vehicle control did not show any anti-tumour effects on B16F10-induced solid tumour in mice.

Figure 3.4

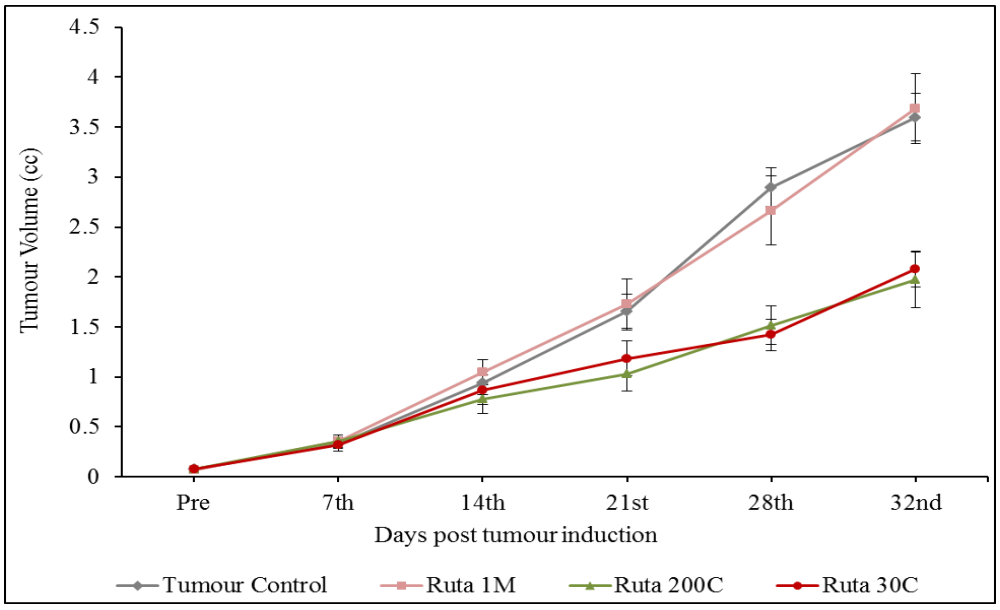
a. Effect of potentiated *Thuja* on B16F10-induced solid tumour bearing mice



Values are mean ± standard deviation.

Figure 3.4 continued…

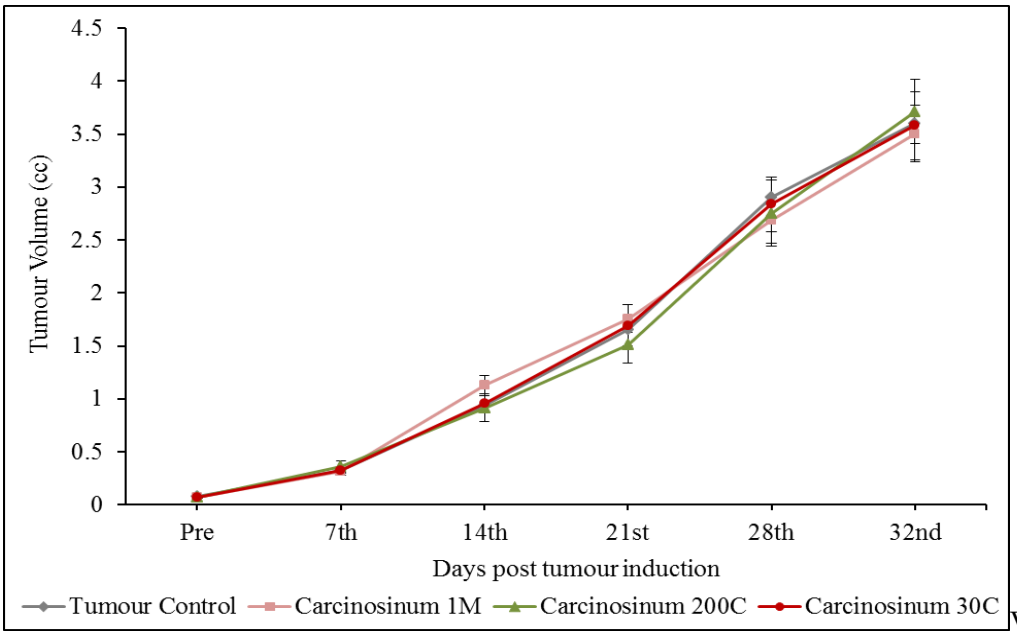
b. Effect of potentiated *Ruta* on B16F10-induced solid tumour bearing mice



Values are mean ± standard deviation.

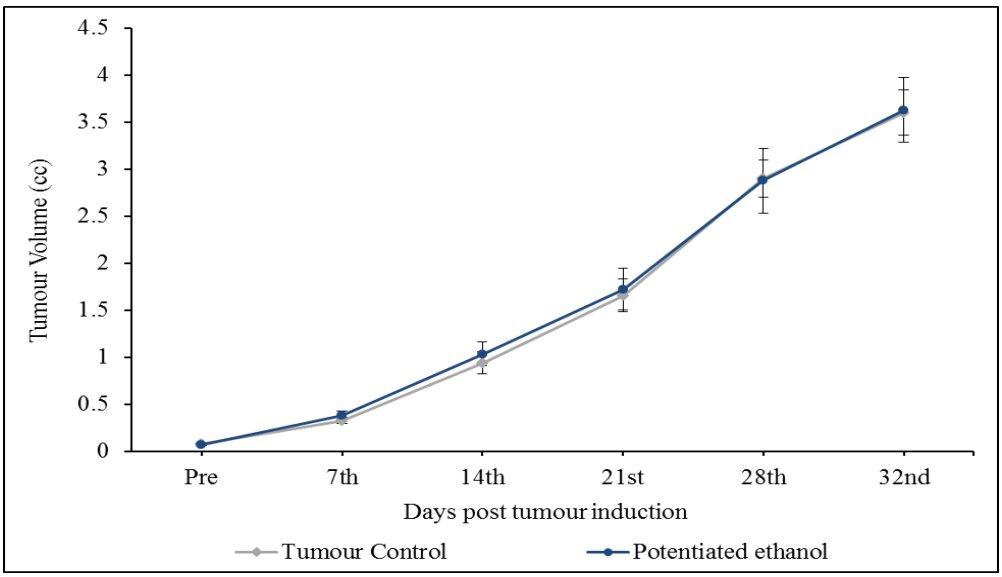
Figure 3.4 continued…

c. Effect of potentiated *Carcinosinum* on B16F10-induced solid tumour bearing mice



Values are mean ± standard deviation.

d. Effect of potentiated ethanol on B16F10-induced solid tumour bearing mice



Values are mean ± standard deviation.

3.4. Discussion

Various homeopathic drugs have undergone clinical trials in human patients and are reported to be effective against various diseases (Rastogi et al., 1993; Banerji et al., 2012; Pathak et al., 2003; Rajendran, 2004; Sunila et al., 2007). All the drugs selected for the present study are being used by homeopathic practitioners against cancer. We found that the potentiated preparations of *Thuja*, *Ruta* and *Carcinosinum* exhibit varying effects on tumour development in different tumour models.

Out of the three potencies of *Thuja*, the 1M potency was found to be highly active against solid tumours induced by DLA and B16F10 as well as ascites tumours induced by EAC and EL-4. The 200c potency of *Thuja* showed a little reduction in the tumour volume of solid tumours whereas they did not affect the development of ascites tumours or the survival of ascites tumour bearing animals. Significant reduction in the tumour volume was observed from the very next week of the commencement of drug administration and further reduction relative to the tumour control was observed thereafter. Previous reports regarding the anti-tumour potential of *Thuja* in animal models also evidenced the efficacy of higher potencies to inhibit chemically induced carcinogenesis, the capability to induce up-regulation and down-regulation of different set of genes in tumour cell lines and some preliminary report regarding the anti-metastatic effects (Kumar et al., 2007; Preethi et al., 2011; Sunila et al., 2007). However, within the limits of the present study, we have selected the 1M potency of *Thuja* for further studies using the above mentioned transplanted tumour models.

Differing from *Thuja*, the three potencies of *Ruta* showed a different pattern in their anti-tumour effects. *Ruta graveolens* extract as well as the dynamized preparations was already reported to possess anti-tumour activity in DLA models, chemically-induced sarcoma and hepatocarcinoma models and also could induce apoptosis mediated through the intrinsic pathway (Preethi et al.,2006; Preethi et al., 2011; Kumar et al., 2007). The present results showed that among the tumour models used, *Ruta* 200c and *Ruta* 30c exhibited higher inhibition of tumour development in solid tumour models and EAC induced

ascites tumour models while the 1M potency did not show any significant effect in any of them. Moreover, both the 200c and 30c potency was observed to inhibit the tumour development at the same level with significant reduction observed just after nine days of drug administration. Since the 200c potency is more diluted, it was selected for further studies rather than the 30c potency.

Potentiated preparation of *Carcinosinum* was also reported to have cytotoxic effects on breast cancer cell lines mediated through cell cycle arrest and induction of apoptosis (Frenkel et al., 2010). Out of the four tumour models studied, we have found that, only the 1M potency of *Carcinosinum* could inhibit DLA-induced solid tumour whereas all the other tumours remain unaffected by all the potencies. This indicates that *Carcinosinum* is more selective in its anti-tumour potential when compared to *Thuja* and *Ruta*.

However, the conclusive results from all the tumour models indicates that *Thuja* and *Ruta* showed less specific and relatively higher potential to inhibit the development of all the tumours (except EL-4 in the case of *Ruta*) at their higher potencies, which is in concordance with the previous reports. Even though the results of the present study signify the higher effect of *Thuja* 1M potency, *Ruta* 200c/30c potency and *Carcinosinum* 1M potency, it is evident from the results that the comparative activities of different potencies may not be the same in every case of malignancy.

Hence, in clinical cases also, the efficacy of different potencies may vary depending up on the type of cancer, stage of tumour and the health status of the patient. Hence it cannot be stated that the pharmacological activity of these drugs are either concentration dependant or dilution dependant.

Insufficient knowledge regarding the mechanism of action of these homeopathic preparations limits much more explanations regarding the higher activity of certain potencies compared to the other potencies. However, the present study which was aimed to screen the potentiated homeopathic medicines is concluded as follows. *Thuja* 1M, *Ruta* 200c and *Carcinosinum* 1M exhibited relatively higher anti-tumour potentials among the *in vivo* tumour models studied and hence these potencies will be used for further studies in these tumour models.

Chapter 4
Evaluation of the regulatory roles of homeopathic drugs on the immune system

4.1 Introduction

Homeopathic remedies are known to be the cure for symptoms of a disease and not for a particular disease. Mostly these ultra-diluted remedies may strengthen the physiological system of the organism to effectively fight against the pathological condition, thus might be significantly targeting the immune system for their action. But scientific studies are not well validated to explain their efficacy, reproducibility and mechanism. Very few experimental and clinical level studies were reported which can be considered as promising cases. For instance, controlled clinical trials proved the immunomodulatory activity of *Echinacea* containing homeopathic preparations, which when used alone or in combination with other homeopathic dilutions, could reduce the side effects of antineoplastic therapies along with the enhancement of various immune parameters (Melchart et al., 1994). Another report regarding the complex homeopathic medication named Canova, revealed that *in vitro* treatment on bone marrow cells could stimulate the differentiation of mono nuclear cells and activation of progenitor cells and stromal cells (Cesar et al., 2008). Preclinical animal model studies on ultra-dilutions of thymic hormones and cytokines proved their immunostimulatory roles under immunosuppressed condition (Bastide et al., 1987). A different study reported the specific modulation of IgG and IgM mediated antibody responses in experimental animals by a homeopathic preparation of keyhole limpet hemocyanin (KLH) antigen (Bentwich et al., 1993). Similarly various animal experimental studies regarding the capability of many homeopathic remedies to modulate the immune effectors were also documented (Conforti et al., 1997; Macedo et al., 2004; Lussignoli et al., 1999; Davenas et al., 1987). However, drugs possessing immunomodulatory effects will be demanded during every pathological condition including malignancy and various immunosuppressed conditions like in the course of chemotherapy or radiotherapy. Recently, a few immune therapies are being widely used but they are also capable to create anaphylactic reactions, adverse and even fatal side effects (Pearson et al., 1995).

Here comes the importance of natural and non- toxic immunomodulators which can boost up the immune system thereby rendering a powerful defense mechanism to the body. Several plant products were reported to act as immunomodulators by regulating the expression of various cytokines required for differentiation, maturation and activation of various cells of immune system (Duke,1985; Kuttan and Kuttan, 1992; Mathew and Kuttan, 1999; Sunila and Kuttan, 2004).The immunostimulatory effects of potentized preparations are not well studied or yet been proved experimentally. The present study evaluated the effect of homeopathic preparations of *Thuja*, *Carcinosinum* and *Ruta* at their different potencies on the immune system in normal conditions through verification of various haematological factors and other significant parameters which can assess the response status of immune system.

4.2. Materials and methods

4.2.1. Homeopathic medicines and method of administration

The potentiated preparations of *Thuja*, *Carcinosinum* and *Ruta* (1M, 200c and 30c potencies) were administered orally in a final volume of 100 µL (10 µL drug /dose, diluted to 100 µL with sterile water). Potentiated ethanol was used as the vehicle control.

4.2.2. Chemicals

The chemicals, Con A, PHA, PWM, LPS, para-rosaniline, α-naphthyl acetate and radioactive (^{3}H) thymidine were specifically used in the experiments. All other reagents were of analytical grade.

4.2.3. Animals

Inbred Balb/c mice (male, 4-6 weeks old) were used for the experiments.

4.2.4. Determination of the effect of homeopathic drugs on haematological parameters

Eleven groups of Balb/c mice (20–25 g body weight, n=8/group) were used in this study. Group I was kept as untreated normal control. Groups II, III and IV

were treated with *Thuja* 1M, 200c and 30c respectively. Groups V, VI and VII were treated with *Carcinosinum* 1M, 200c and 30c respectively. Group VIII, IX and X were treated with *Ruta* 1M, 200c and 30c respectively. Group XI was kept as vehicle control, administered with same dose of potentized alcohol. All the potencies of the drugs were administered for ten consecutive days. Blood was collected from the caudal vein prior to the administration of the drug and also every third day after commencement of drug administration and various parameters such as total White Blood Cell (WBC) count, differential count and haemoglobin content were recorded which was continued for one month. Body weight, food consumption, water consumption and behaviour of the animals were also noted simultaneously.

4.2.5. Determination of the effect of homeopathic drugs on lymphoid organ weight

Balb/c mice were grouped (n=8/group) that includes normal control, vehicle control and three treatment groups for *Thuja* 1M, *Carcinosinum* 1M and *Ruta* 200c respectively. Homeopathic medicines and vehicle were administered orally for ten consecutive days. Animals were sacrificed after 24 h of the last dose and weight of the lymphoid organs such as spleen and thymus were recorded and expressed as relative organ weight with respect to the body weight.

4.2.6. Determination of the effect of homeopathic drugs on bone marrow cellularity and α-esterase activity

Balb/c mice (n=8/group) were divided into various groups as described above. The animals were sacrificed 24 h after the last dose of treatments and bone marrow cells from the femur was collected in to the medium containing 2% goat serum. The bone marrow cell number was determined using a hemocytometer and expressed as total number of live cells/femur. The number of α-esterase positive cells was also determined by the azodye coupling method and was expressed out of a total of 4000 cells.

4.2.7. Determination of the effect of homeopathic drugs on circulating antibody titre

Balb/c mice (n=8/group) were divided into various groups as described above. Control group animals were immunized with Sheep Red Blood Cells (SRBC) (2.5×10^8 cells/animal, i.p.). Other groups of animals were treated with respective homeopathic preparations for ten consecutive days and after one hour of the 10^{th} dose, SRBC was administered. Blood was collected from the caudal vein, prior to treatment and continued every third day after commencement of treatment for a period of 30 days. Serum samples were used for the estimation of antibody titre using SRBC as antigen.

4.2.8. Determination of effect of homeopathic drugs on the number of antibody-producing cells

Balb/c mice were divided into various groups (n=21/group) as described earlier. Control group animals were immunized with SRBC (2.5×10^8 cells/animal, i.p.). Other groups were treated with respective homeopathic preparations for ten consecutive days and after one hour of 10^{th} dose, all the animals were immunized with SRBC. Three animals from each group were sacrificed on different days starting from the third day after immunization up to the 9th day. Spleen cells were used to perform Jerne's Plaque assay.

4.2.9. Determination of the effect of homeopathic medicines on blastogenesis in lymphoid organs

Balb/c mice were divided into various groups (n=8/group) as described. Animals were sacrificed after 24 h of the last dose of treatment and spleen, thymus, and bone marrow were collected under aseptic conditions. Spleen and thymus were processed into single cell suspension. Bone marrow cells were also collected from the femur and made into single cell suspension. Cells were separately plated in 96-well round bottomed titre plates with a cell density of $5x10^4$ cells/well in RPMI medium. This is followed by incubation in the presence and absence of various mitogens such as PHA, Con A, PWM and LPS in a humidified 5% CO_2 atmosphere at 37°C for 48 h. Cells were further labelled with ^3H thymidine and incubated for 18 h. DNA was precipitated,

pellets were dissolved and transferred to scintillation fluid. Radioactivity was measured using a Rack Beta fluid scintillation counter.

4.3. Results

4.3.1 Effect of homeopathic medicines on haematological parameters

It was observed that, among the three potencies of *Thuja*, 1M and 200c potencies showed a gradual increase in the Total WBC Count which reached a desirable maximum value of 14850±137.84 cells/mm³ (P<0.001) on the 12th day and 12175 ± 2266 cells/mm³ (P<0.001) on the 9th day respectively. Meanwhile, the 30c potency did not show any significant change (Figure 4.1a). In the case of *Carcinosinum*, only the 1M potency showed an increase in WBC count to a maximum value of 14541±2032 cells/mm³ (P<0.001) on the 6th day but the 200c and 30c potencies could not cause a significant change (Figure 4.1b). *Ruta* also showed a similar increase in WBC count at its 1M and 200c potencies, with the 200c potency having a relatively prolonged effect (Figure 4.1c). The animals of vehicle control group and the untreated normal control group maintained the normal WBC count throughout the study (Figure 4.1d). There was no significant difference in the ratio of lymphocytes to neutrophils (data not shown) as well as hemoglobin level (data not shown) after treatment with the homeopathic medicines. There were no observable undesirable changes in body weight, food or water consumption (data not shown) and behavior of animals. Thus, those potencies of the homeopathic medicines which have shown maximum and prolonged effect, presently, *Thuja* 1M, *Carcinosinum* 1M and *Ruta* 200c, were selected for further immunological experiments.

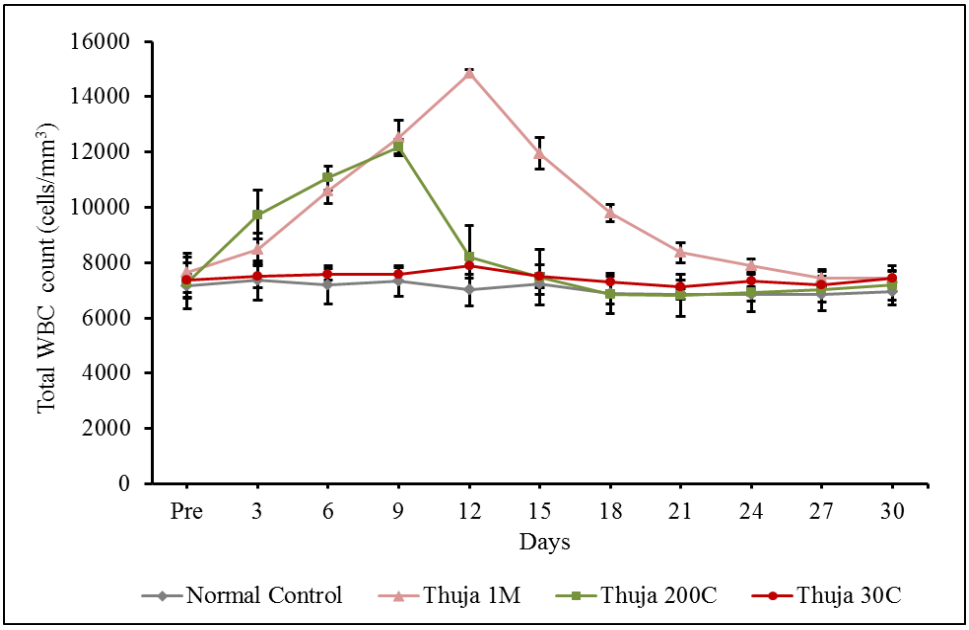
4.3.2 Effect of homeopathic medicines on lymphoid organ weight

Effect of homeopathic medicines on lymphoid organ weight is given in table 4.1. It was observed that certain treated groups showed significant increase in the weight of spleen and thymus compared to control group. *Thuja* 1M treated group showed an average thymus weight of 0.17±0.04 g (P<0.05) and spleen weight of 044±0.04 g (P<0.001). Meanwhile, *Carcinosinum* 1M treated group had an average thymus weight of 0.16 ± 0.03 g (P>0.05) and spleen weight of

0.37±0.04 g (P<0.05). Meanwhile, *Ruta* 200c showed an average thymus weight of 0.14 ± 0.04 g (P>0.05) and spleen weight of 0.4 ± 0.02 g (P<0.001). The

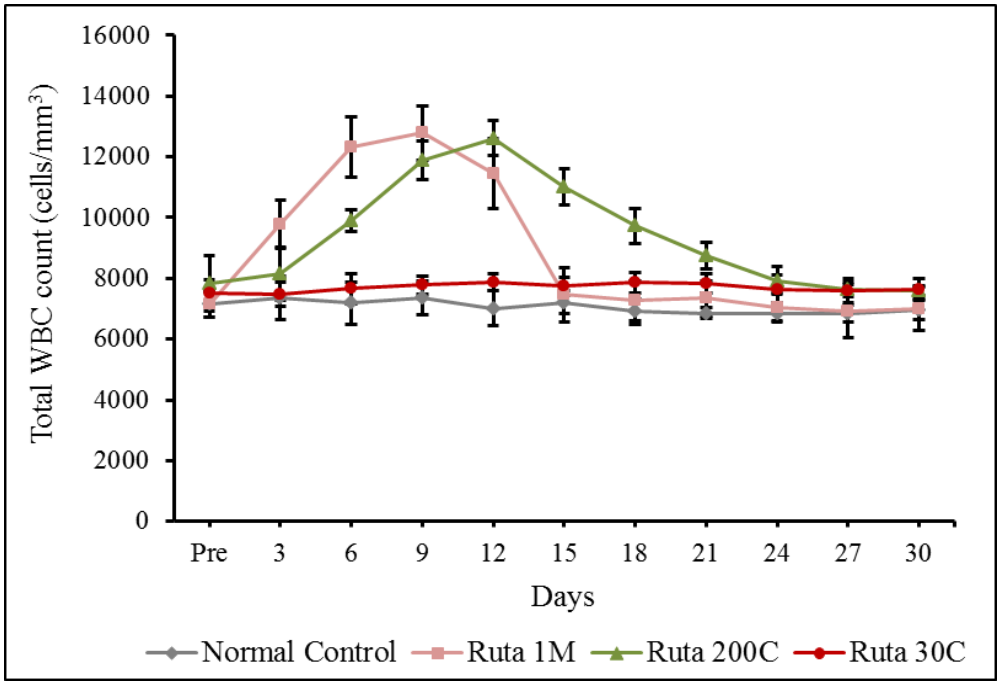
Figure 4.1

a. Effect of different potencies of Thuja on total WBC count



Values are mean ± standard deviation.

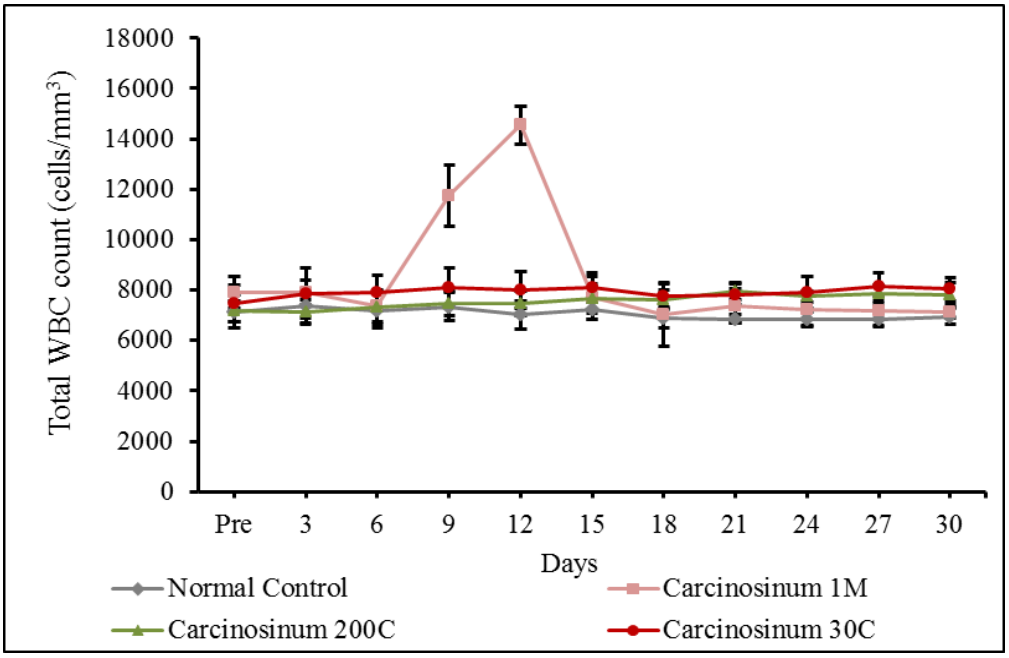
b. Effect of different potencies of Ruta on total WBC count



Values are mean ± standard deviation.

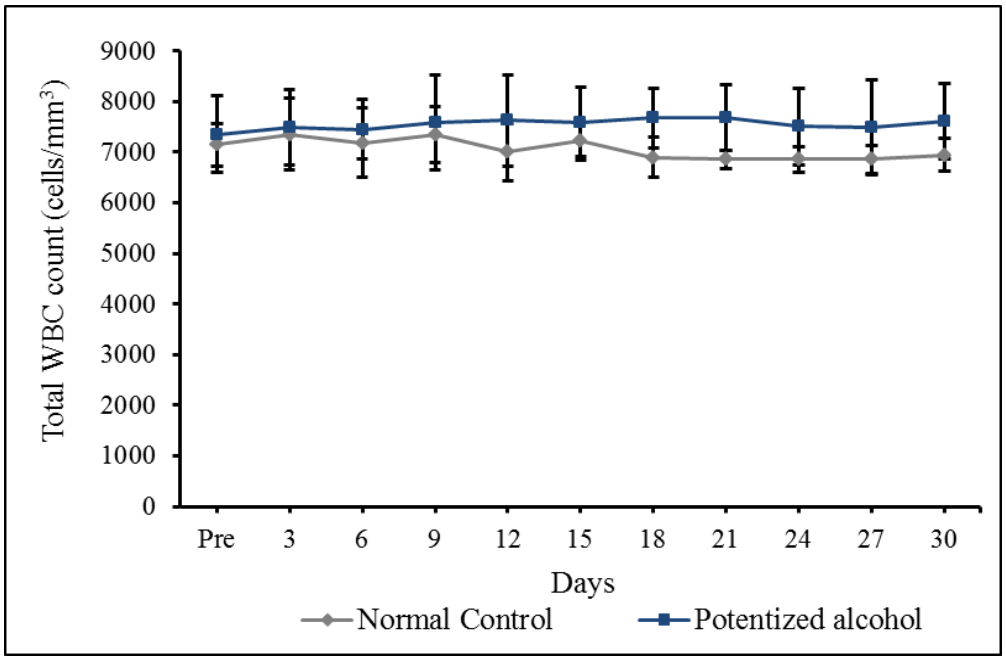
Figure 4.1 continued…

c. Effect of different potencies of Carcinosinum on total WBC count



Values are mean ± standard deviation.

d. Effect of potentiated ethanol on total WBC count



Values are mean ± standard deviation.

statistical significance of these values were evaluated by comparing to the control and vehicle control groups which showed average thymus weights of 0.12 ± 0.02 g and 0.12 ± 0.02 g and spleen weights of 0.32±0.03 g and 0.29 ± 0.03 g respectively.

Table 4.1

Effect of homeopathic medicines on lymphoid organ weights

Group	Relative organ weight (g/100g body weight)	
	Thymus	**Spleen**
Normal Control	0.12 ± 0 .02	0.32 ± 0.03
Potentiated alcohol	0.12 ± 0.02	0.29 ± 0.03
Thuja 1M	0.17 ± 0.04[c]	0.44 ± 0.04[a]
Carcinosinum 1M	0.16 ± 0.03[d]	0.37 ± 0.04[c]
Ruta 200C	0.14 ± 0.04[d]	0.4 ± 0.02[a]

Values are mean ± standard deviation. [a]$P < 0.001$; [c]$P < 0.05$; [d] not significant, compared to the normal control group

4.3.3 Effect of homeopathic medicines on the bone marrow cellularity and α-esterase activity

As shown in Table 4.2, administration of *Thuja* 1M, *Carcinosinum* 1M and *Ruta* 200c significantly increased the bone marrow cellularity and the number of α-esterase positive cells. Bone marrow cellularity was increased to 22.25±2.65x10^6 cells/ femur (P<0.001), 18.75±1.21x10^6 cells/ femur (P<0.05) and 19.6 ± 1.69x10^6 cells/ femur (P<0.001) for *Thuja* 1M, *Carcinosinum* 1M and *Ruta* 200c respectively compared to normal control group which showed an average cellularity of 15.83±1.12 65x10^6 cells/ femur. Similarly, the number of α-esterase positive cells was also increased to 1020±63.24/4000 cells (P<0.001) for *Thuja* 1M and 936 ± 52.78/4000 cells (P<0.001) for *Ruta* 200c when

compared to the value 650±56.21/4000 cells for the normal control group. Meanwhile, *Carcinosinum* 1M did not show a significant enhancement. The vehicle control group showed no significant change in bone marrow cellularity and α-esterase activity compared to the normal control group.

Table 4.2

Effect of homeopathic medicines on bone marrow cellularity and α –esterase activity

Treatment	Bone marrow cellularity ($x10^6$ cells/femur)	No: of α-esterase positive cells (per 4000 cells)
Normal Control	15.83 ± 1.12	650 ± 56.21
Potentiated alcohol	16.25 ± 1.44	643 ±42.73
Thuja 1M	22.25 ± 2.65[a]	1020 ±63.24[a]
Carcinosinum 1M	18.75 ± 1.21[c]	728 ± 67.82[d]
Ruta 200C	19.6 ± 1.69[a]	936 ± 52.78[a]

Values are mean ± standard deviation. [a]$P < 0.001$; [c]$P < 0.05$; [d] not significant, compared to the normal control group

4.3.4 Effect of homeopathic medicines on the circulating antibody titre

As shown in Figure 4.2, there was a significant increase in the production of SRBC-specific antibodies in all the drug treated groups compared to the control group. The maximum titre value of 1024 was found on 15th and 18th day in *Thuja* 1M and *Ruta* 200c treated groups respectively which remained up to 256 on the 27th day. *Carcinosinum* 1M treated group showed the maximum value of 512 on the15th and 18th day itself and remained up to 128 on the 21st day. Meanwhile, the vehicle control group showed a maximum titre of only 64 from 12th to 21st day which is similar to the normal control group.

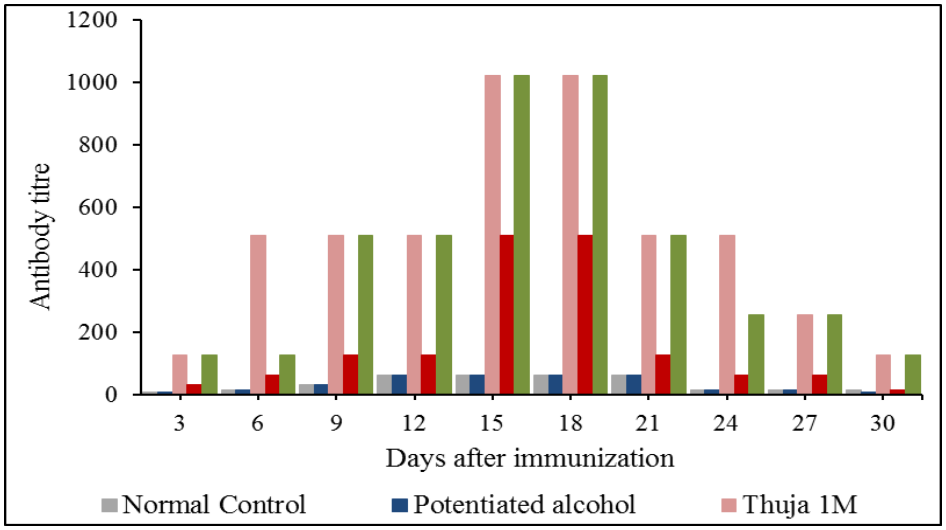
4.3.5 Effect of homeopathic medicines on the antibody-producing cells

Administration of the selected potencies of all the three drugs significantly enhanced the number of antibody producing cells in the spleen (Figure 4.3). The maximum number of plaque forming cells was observed on the 6th day after

immunization in all the treated groups such as 195.83±4.04 PFC/10^6 spleen cells, 183.33±4.16 PFC/10^6 spleen cells and 210.83±5.03 PFC/10^6 spleen cells for *Thuja* 1M, *Carcinosinum* 1M and *Ruta* 200c groups respectively. The vehicle control group showed only an average of 157.5±6.5 PFC/10^6 spleen cells on the same day which is similar to the normal control group.

Figure 4.2

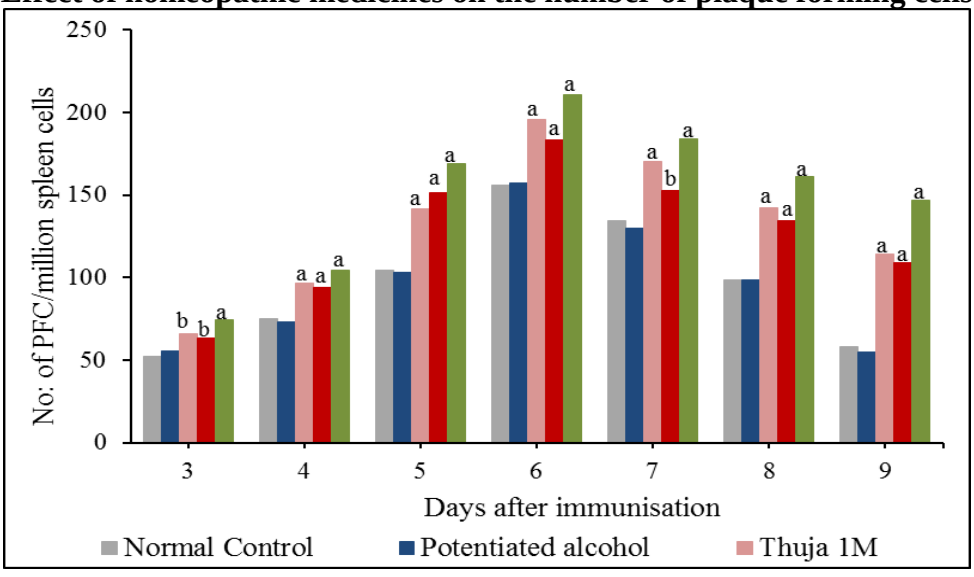
Effect of homeopathic medicines on antibody titre



Values are antibody titres against SRBC antigen

Figure 4.3

Effect of homeopathic medicines on the number of plaque forming cells



Values are mean ± standard deviation. [a]$P < 0.001$; [b]$P < 0.01$, compared to the normal control group.

4.3.6 Effect of homeopathic medicines on blastogenesis of the cells in lymphoid organs

A significant enhancement was observed in the proliferation rate of spleen, thymus and bone marrow cells of drug treated animals compared to normal animals. Besides, when the treated cells were incubated with the mitogens such as PHA, Con A, PWM and LPS which are having mitogenic potential towards specific cells, there observed a further increase in the rate of proliferation when compared to the untreated mitogen control as well as the controls treated with respective drugs without mitogen (Table 4.3).

4.4 Discussion

Hahnemann described that a homeopathic cure imparts its effect not directly but creates a 'primary action' in an organism which then compel the organism itself to cause a 'secondary action' that ultimately results in the remedial action. This was described in paragraphs 63-69 of *Organon*, quoted as follows. 'Every agent that acts upon the vitality, every medicine, deranges more or less the vital force, and causes a certain alteration in the health of the individual for a longer or a shorter period. This is termed primary action. Although a product of the medicinal and vital powers conjointly, it is principally due to the former power. To its action our vital force endeavors to oppose its own energy. This resistant action is a property, is indeed an automatic action of our life-preserving power, which goes by the name of secondary action or counteraction'. The basic theories on homeopathy thus pave light to thoughts about how these ultra-diluted medicines make prepare the organism to cause this secondary action. It could be majorly attributed to the defense system of the body, which has got unique mechanisms of effector functions that protect the body form a vast majority of pathogens to which the organism is being exposed. However theories are not sufficient to prove the surprising healing effects of homeopathic preparations, but scientific proofs should do the needful.

Starting materials of many plant-based homeopathic preparations were reported to possess multiple pharmacological activities. Our study revealed that ultra-

Table 4.3

Effect of homeopathic medicines on proliferation of lymphoid organs

Group	Control	Potentiated alcohol	Thuja 1M	Carcinosinum 1M	Ruta 200C
Spleen					
No mitogen	1758±133	1729±117	1974±124[b]	1949±96[c]	2006±137[b]
Con A	4297±147	4273±134	5265±138[a]	4728±123[a]	4965±143[a]
PHA	3552±158	3514±138	4722±149[a]	4167±137[a]	4218±153[a]
LPS	3784±123	3792±124	5148±136[a]	4136±146[a]	4529±167[a]
PWM	3816±115	3835±121	4837±148[a]	4253±124[a]	4567±159[a]
Thymus					
No mitogen	1685±138	1672±94	2039±135[a]	1918±135[b]	2014±148[a]
Con A	4424±129	4431±125	5182±123[a]	4929±129[a]	5080±132[a]
PHA	3964±154	3971±138	4372±121[a]	4117±137[d]	4228±118[b]
PWM	4123±108	4110±129	4294±135[d]	4127±127[d]	4359±157[b]
Bone marrow					
No mitogen	1256±138	1239±127	1594±133[a]	1337±137[d]	1514±134[b]
Con A	1382±144	1263±151	1572±142[d]	1535±122[d]	1573±124[c]
PHA	1334±139	2075±157	1428±153[d]	1550±136[c]	1558±118[c]
LPS	3246±161	3175±143	4867±128[a]	3826±124[a]	4294±133[a]
PWM	1989±128	2034±123	3451±116[a]	2671±117[a]	3183±120[a]

Values are mean ± standard deviation. [a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.05$; [d] not significant, compared to the specific mitogen control group.

diluted preparations of these starting materials still retain their biological properties significantly. As described earlier, all the homeopathic preparations selected for this study possess proven pharmacoactive effects in the *in vivo* system but their mechanism of action is still unclear. In the present study, preliminary results from the analysis of haematology itself demonstrated that the higher potencies are more effective than the lower ones. Among the three potencies studied, 1M potency of *Thuja* and *Carcinosinum* as well as 200c potency of *Ruta* showed maximum peak values for total WBC count at particular time points. The gradual increase in WBC count and the reach of peak value during or immediately after the course of drug treatment followed by its gradual drop clearly indicated the drug mediated stimulated production of WBCs which will make the immune system more competent. This effect was further confirmed by the increase in the proliferation of bone marrow cells and more specifically the number of differentiating stem cells, reflecting the stimulation of haematopoietic system. Bone marrow is the primary site of haematopoiesis where the diverse lineages of blood cells derive, which include the principal immune effector cells also. Hence the drug induced increase in the number as well as differentiation of bone marrow cells signifies the primary step for the preparation to make up a boosted defense system. We also observed that the selected potencies showing maximum effect on haematological parameters could also cause a relatively significant increase in circulating antibody titre against SRBC antigen when compared to the control group. This is a clear indication of increased production of SRBC specific antibodies in order to result efficient and rapid clearance of the antigen.

To further confirm whether this increase in antibody titre is due to the increased proliferation of antibody producing cells or the increased production of antibodies by the antigen encountered cells, Jern's plaque assay was carried out against SRBC antigen. It was found that the peak values for the number of antibody producing cells was observed after 6 days of immunization with SRBC, while the antibody titre showed gradual increase from 6^{th} day of immunization but reached peak values after 15 days for all the drugs. This indicates that antigen specific B cells are induced to get proliferated only at the

early stages of immunization after which these proliferated cells keep on producing increasing number of antibodies and at the later stages, the cell number as well as antibody response is declining. These results confirmed the specific immunostimulatory effects of the drugs through the humoral wing of immune system. Moreover, lymphoid organs such as thymus and spleen also showed a little increase in their weight which may be due to increased proliferation of lymphoid cells. This is again substantiated by the drug induced increase in the proliferation of the lymphoid organ cells which was determined by blastogenesis assays. The assay confirmed the enhanced proliferation of not only B cells but also T cells when compared with the mitogen treated controls which revealed the further involvement of cell mediated wing of the immune system also. The immunomodulatory effects of *Thuja* containing Canova medication is well documented (Cesar et al., 2008) and the present results provide a substantiated proof for considering *Thuja* as an immunostimulating constituent.

Furthermore, there is an important point to clear out how the immunostimulatory effects are correlated to the anticancer effects. Preparations of *Thuja*, *Carcinosinum* and *Ruta* were reported to possess ameliorative effects in various cancer cell lines *in vitro* and tumour models in mice (Preethi et al., 2006; Sunila et al., 2007; Sunila et al., 2009; Preethi et al., 2011). *In vitro* cytotoxic effects, where the cells are directly exposed to the drug, has been accredited by the induction of apoptotic pathways mediated through the upregulation of proapoptotic genes like p53 and caspases with simultaneous downregulation of antiapoptotic genes like Bcl-2 (Sunila et al., 2009). Meanwhile, the *in vivo* effects of orally administered drugs could not be proved as specifically directed to cancer cells. This effect can be partly explained by the specific inhibition in the proliferation of rapidly dividing cells but drug induced effects should also be definitely influenced by the different physiological systems of the individual. Among them, the most susceptible one must be the immune system, whose possible effects and mechanisms are not well studied. However, a study regarding the gene expression profiling after Canova treatment in mice have shown the upregulation, downregulation and fold change

of a vast number of genes involved in the regulation of cell cycle, metabolism, transport and immune response. Those involved in immune response included the up-regulation of certain genes like histocompatibility and HLA genes along with down-regulation of chemokine receptors and ligands, interferon activated and inducible genes etc. (de Oliveira et al., 2008). Thus the immune modulatory effects might be influenced at the genetic level by regulated changes in the expression of cytokines, chemokines and other cross talking molecules such as histocompatibility antigens. However, all the observations of the present study clearly demonstrate that *Thuja* 1M, *Carcinosinum* 1M and *Ruta* 200c could effectively stimulate the immune system effectors, which will be mediated through the combined and regulated activation of humoral and cell mediated immune responses, whose molecular targets should be further studied. Thus it can be concluded that these preparations are safe to use as non-toxic immunomodulators and the immunomodulatory property might be playing a key role in the mechanism of antineoplastic action of these preparations *in vivo*. These findings will definitely assist and encourage further preclinical and clinical studies regarding the applications and mechanism of action of these homeopathic remedies. Double blinded, randomized controlled clinical trials should be further recommended whose outcomes, if successful, will benefit the attention and acceptance of homeopathic branch of alternative medicine and help to spread the treatment among common people.

However, the actual constituents of potentized preparations are still not clear. Many studies were reported which tried to explain the biophysical properties of ultra-diluted remedies even though it is a controversy that whatever percentage of the starting material responsible for the activity is still remaining in these highly diluted potencies (Benveniste, 1993; Jonas et al., 2003). Recently, it has been published that the process of potentization creates a nanoparticle top layer which will be retained even after several steps of dilution (Chikramane et al., 2010). These proofs should be more clarified by subjection to advanced studies. This will certainly support those scientific studies which are vibrantly ongoing and updating the ‘world of ultra- dilutions’ which is hopefully capable to create sensational therapeutic approaches.

The present study focused to check whether the homeopathic preparations of *Thuja*, *Carcinosinum* and *Ruta* have any modulatory effects through the immune system. The results indicate that particular higher potencies of these homeopathic preparations possess significant effect on boosting the immune system under normal conditions. These effects are stimulatory at a desirable level and hence can render an enhanced and regulated mode of therapeutic action through the immune system during the pathological symptoms for which the drugs are being presently used. Studies should be elaborated to find out the key molecular targets and their mechanisms through which these immune modulatory effects become correlated to the antineoplastic action.

Chapter 5
Evaluation of anti-metastatic and anti-angiogenic effects of the potentiated preparation of Thuja occidentalis

5.1. Introduction

Cancer remains one of the most dreadful chronic diseases of the time with spontaneous prognosis at the metastatic stages and poor response to conventional therapies which are further associated with severe side effects too. Early stage diagnosis will help to manage the malignancy with the therapeutic strategies of surgery, chemotherapy and radiotherapy at minimum toxic doses. But there are always chances of recurrence at multiple sites due to the presence of dormant metastases or cancer stem cells which could not be detected clinically at single cellular level by any of the known medical procedures or techniques. Among the various steps of oncogenesis, starting from initiation and promotion followed by establishment of primary tumour and its further invasive development and metastasis leading to secondary tumours triggered by angiogenesis, there are multiple targets having therapeutic scope (Steeg, 2006; Colak and Medema, 2014; Morgan et al., 2009; Talmadge and Fidler, 2010). In fact, metastasis and angiogenesis are the major processes which increase the aggressiveness and treatment limitations of a tumour after its development (Zetter, 1998). The scientific community is yet eager to develop such a therapeutics targeting multiple pathways which could beneficially block the exponential spread of malignancy and make the pathological condition manageable with current therapies and novel curing agents.

A handful of promising research outputs proved the efficacy of several synthetic and natural compounds alone or in combination to inhibit different kinds of cancer cells in the *in vitro* system as well as tumour development in the *in vivo* system. The mechanisms of action of many of them are also proved which is by inhibiting specific pathways of cell survival and proliferation at various stages of tumour development (Singh et al., 2015; Roleira et al., 2015; DiMarco-Crook and Xiao, 2015; Gupta et al., 2010; Kunnumakkara et al., 2008; Miura et al., 2015; Pistollato et al., 2015; Sagar et al., 2006). Now a days, alternative medicines including homeopathy is being well authenticated to be an excellent source of antineoplastic remedies with their major advantage of least or null side effects (Yance and Sagar,

2006; Rausch Osian et al., 2015; Ben-Arye and Samuels, 2015; Frass et al., 2015; Frenkel, 2015; Rossi et al., 2015). However, an integrative treatment strategy is still an unexplored platform which might lead to healing wonders if we could scientifically study and successfully implement the trials. For that, we should shed even more light towards the way of scientific validation of alternative and complementary therapies to make them widely acceptable and eligible for clinical trials synergistically with modern medicine.

Thuja occidentalis extract was reported to possess diverse pharmacological actions due to the bioactive component Thujone (Naser, 2005). The homeopathic preparations of the plant also retained many of these properties without any toxicity. We have already reported the cytotoxic effect of different potencies of *Thuja* on DLA cells mediated through the intrinsic pathway of apoptosis (Preethi et al., 2011). Later, our preliminary study on the antimetastatic potential of *Thuja* was also proved effective at its 1M potency in B16F10 induced lung metastatic model (Sunila and Kuttan, 2006). Moreover we have recently reported the immune stimulatory property of the preparation in Balb/c mice, out of which the 1M potency was observed to be highly active (Remya and Kuttan, 2015). Hence the present included detailed studies to prove the mechanism of antimetastatic activity of *Thuja* 1M using B16F10 melanoma model and a further evaluation of its antiangiogenic activity against the tumour burden. This will provide a solid evidence for the mechanism through which it is blocking the progressive development of tumour in the *in vivo* system.

5.2. Materials and methods

5.2.1. Homeopathic preparations and dosage

Homeopathic preparation of *Thuja* 1M was orally administered to the animals for ten consecutive days. 100 µL of the drug was diluted to 1mL using sterile water and 100 µL of it is administered to each animal.

5.2.2. Cell lines

B16F10 cells were used to study pulmonary metastasis and tumour directed angiogenesis in C57BL/6 mice.

5.2.3. Animals

C57BL/6 mice (6-8 weeks old) were used as the animal model for B16F10 melanoma induced pulmonary metastasis and also for inducing subcutaneous solid tumour for angiogenesis studies.

5.2.4. Kits, Chemicals and Reagents

ELISA kits, TNP-470, hydroxyproline, oligonucleotide primer sequences, N-acetyl neuraminic acid and glucuronic acid lactone were specifically used for the experiments. All other reagents used were of analytical grade.

5.2.5. Determination of anti-metastatic parameters

5.2.5.1. Determination of lung tumour nodules and biochemical parameters

C57BL/6 mice (6-8 weeks old, 25-30g body weight) were divided into three groups (N=14/group). Group I was kept as untreated control without tumour induction. The other two groups were injected with B16F10 cell suspension ($1x10^6$ cells/animal) through the lateral tail vein in order to induce spontaneous lung metastasis. Group II was kept as untreated metastatic tumour control. Group III was treated with *Thuja* 1M, simultaneously with tumour cell injection. Treatment was started just after 24^{th} hr of tumour inoculation and continued for ten consecutive days. On the 21^{st} day of tumour inoculation, animals were sacrificed, blood was collected by heart puncture and lungs were excised out.

Lungs were observed to count the developed tumour nodules and the tissue was then used for the estimation of collagen hydroxyproline, hexosamine, and uronic acid content. For the estimation of hydroxyproline, lung hydrolysate was dried, reconstituted in water and neutralized. Estimation of hydroxyproline was done

using chloramine-T method, as described by Bergman and Loxley, and the concentration of hydroxyproline was determined with respect to a reference graph of hydroxyproline standard (Bergman and Loxely, 1970). Hexosamine content was estimated as per the method of Elson and Morgan. The lyophilized tissue that has undergone acid hydrolysis was dried and the residue is dissolved in water and treated with 2% acetyl acetone, followed by addition of Ehrlich reagent. The concentration of hexosamine was determined using a standard graph of glucosamine (Elson and Morgan, 1993; Burton, 1962). The standard method for the estimation of uronic acid, as described by Bitter and Mair, involves the preparation of the hydrolysate of the tissue that have undergone papain digestion, followed by the addition of sulfuric acid. After final treatment with carbazole reagent, optical density was measured at 530 nm. The concentration was determined using a standard graph of glucuronic acid lactone (Bitter and Muir, 1962; Schiller et al., 1961). Serum was separated from the blood and used for the estimation of sialic acid content as well as γ–glutamyl transpeptidase (GGT) levels. Serum level of sialic acid was determined by Thiobarbituric acid assay and the concentration was estimated using a standard graph of N-acetyl neuraminic acid (Bhavanandhan et al., 1981). GGT level was measured by estimating the release of p-nitroaniline from γ–glutamyl p-nitroanilide in presence of glycyl glycine. Concentration of GGT was estimated using p-nitroaniline as standard (Tate and Meister, 1974). The methods were explained in detail in section 2.7.1.1.

5.2.5.2. Histopathological analysis

A portion of the tissue was fixed in 10% formalin and subjected to histopathological analysis. Briefly, the fixed tissue was sectioned after paraffin embedment to obtain sections approximately 4-5µm thickness. Paraffin embedded sections were dehydrated through an alcohol series, washed and stained with hematoxylin and eosin for microscopic analysis.

5.2.5.3. Survival rate

Two groups of animals were induced with metastatic tumour as described above (N=14/group). Group I was kept as untreated metastatic tumour control, whereas group II was treated with *Thuja* 1M. All the animals were observed for their survival period and percentage increase in life span was calculated as described earlier.

5.2.5.4. Estimation of serum cytokine levels

The above experimental set up of three sets including normal control, metastatic tumour control and *Thuja* 1M treated groups was repeated and the animals were sacrificed on the 21st day after tumour inoculation. Blood was collected by heart puncture and the serum was used for the estimation of cytokines such as IL-1β, IL-6, TNF-α and GM-CSF using ELISA kits according to the manufacturer's instructions.

5.2.5.5. Expression of metastatic marker genes

Lungs were excised out from the metastasis-induced animals of tumour control and *Thuja* 1M treated groups. Total RNA of the lung tissue was isolated to synthesize cDNA using Moloney Murine Leukemia Virus reverse transcriptase. Amplification of sequences for MMP-2, MMP-9 (Matrix Metallo Proteinases), ERK-1, ERK-2 (Extracellular signal Regulated Kinases) and VEGF were performed using specific primer sequences (section 2.2.9). Amplified products were electrophoresed in 1.8% agarose gel containing Ethidium bromide and photographed under ultraviolet light.

5.2.6. Anti-angiogenic assay

5.2.6.1. Counting of tumour directed capillaries

Three groups of C57BL/6 mice (N=14) were arranged as described above. All the animals were shaven at the ventral skin and were intradermaly inoculated with B16F10 melanoma cells ($1x10^6$cells/animal) to develop solid tumours. Treatment was started after 24 hr of tumour inoculation and continued for ten consecutive

days. On the 11th day, animals were again shaven at their ventral skin and sacrificed. The skin around the developed tumour was dissected out and the number of tumour directed capillaries was counted using a dissection microscope (Leyon and Kuttan, 2004).

5.2.6.2. Determination of VEGF and GMCSF levels

Blood was collected from the caudal vein of all the animals from the experiment, 24 hr after the commencement of treatment and the final day of sacrifice. Serum was separated and used for the estimation of VEGF and GMCSF levels by ELISA.

5.3. Results

5.3.1. Evaluation of metastatic markers

5.3.1.1. Lung tumour nodules

As shown in table 5.1, it was observed that the weight of lung tissue has increased in all of the tumour induced animals compared to the normal control. Also, those animals which were treated with *Thuja* 1M showed significant decrease in the lung weight compared to the tumour bearing control group ($p<0.001$). Moreover, *Thuja* 1M treatment significantly reduced the number of lung tumour nodules in the treated group ($p<0.001$), whereas the tumour bearing control group showed massive growth of lung nodules assigned with an arbitrary number of 250. The survival rate of the treated animals was also increased to 53.4% (Fig. 5.1).

5.3.1.2. Biochemical parameters of lung tissue and serum

The net amount of hydroxyproline, hexosamine and uronic acid in the lung tissue was found to be highly increased in tumour bearing untreated animals, which reflects prominent fibrosis of the lung tissue and advancement of metastasis. On the other hand, analysis of the lung tissues collected from the treated animals showed significantly lessened concentration of these biochemicals ($p<0.001$) (Tab. 5.1). Similarly, the serum markers of metastasis such as sialic acid and GGT levels were significantly elevated in the tumour bearing control animals, whose levels encountered a significant fall in the treated animals ($p<0.001$), as shown in table 5.2.

Table 5.1

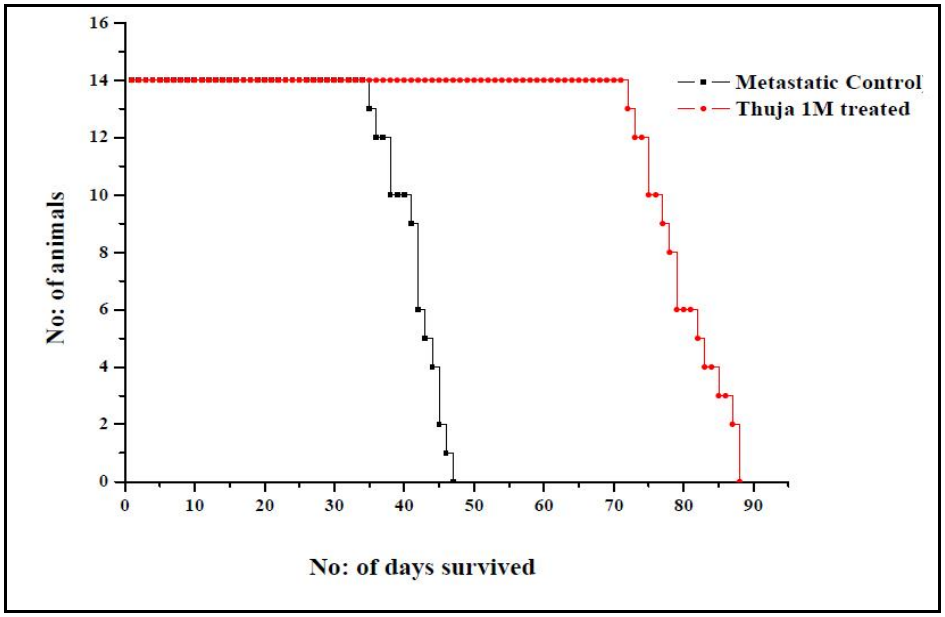
Lung tissue parameters in metastatic tumour bearing animals with or without Thuja 1M treatment

Group	Weight of lung tissue (g)	No: of lung tumour nodules	% reduction in tumour nodules	Hydroxy proline content (µg/mg protein)	Hexosamine content (mg/100 mg tissue dry weight)	Uronic acid content (µg/100mg tissue wet weight)
Normal Control	0.166 ± 0.013			1.5 ± 0.16	0.56 ± 0.11	43.54 ± 7.27
Tumour Control	0.295 ± 0.022	250*		31.47 ± 5.67	4.92 ± 0.44	357.35 ± 13.68
Thuja 1M treated	0.213 ± 0.019[a]	115.85 ± 15.62	53.65	13.93 ± 3.72[a]	2.47 ± 0.69[a]	154.21 ± 15.87[a]

Data represent mean ± standard deviation. [a]$P < 0.001$, compared to the tumour control group. * Represents arbitrary number.

Figure 5.1

Survival rate of metastatic tumour bearing animals with and without Thuja 1M treatment



Grouped animals were induced with metastatic tumour and treated with or without the drug. Death pattern of the animals were noted and expressed graphically. Values are individual observations of the death pattern of animals.

Table 5.2

Serum parameters in metastatic tumour-bearing mice with or without Thuja 1M treatment

Group	Sialic acid (µg/mL serum)	GGT (nmol p-nitroaniline/ mL serum)	VEGF (pg/mL)
Normal Control	24.09 ± 3.45	25.80± 3.45	15.33 ± 3.25
Tumour Control	111.82 ± 14.84	119.34 ± 11.67	155.48 ± 16.20
Thuja 1M treated	58.33 ± 11.31[a]	57.91 ± 10.31[a]	89.42 ± 13.30[a]

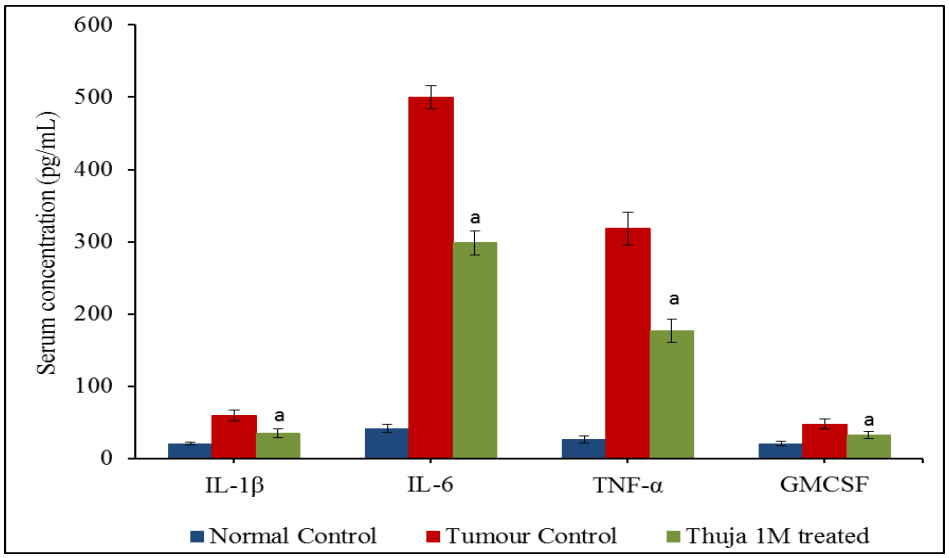
Data represent mean ± standard deviation. [a]$P < 0.001$, compared to the tumour control group.

5.3.1.3. Serum cytokine levels

Figure 5.2 shows the cytokine concentrations in the serum of treated as well as untreated groups of metastasis-induced animals. It was evident from the results that the levels of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GMCSF displayed an extensive increase correlated with the tumour burden and metastasis. While, the *Thuja* 1M treated group of animals were significantly benefitted with low levels of these cytokines ($p<0.001$) compared to the tumour control group.

Figure 5.2

Serum levels of pro-inflammatory cytokines



Data are expressed as mean ± standard deviation, [a] $P < 0.001$, compared to the tumour control group.

5.3.1.4. Histopathology of the lung tissue

Histopathological analysis of the lung tissues of normal control, tumour control and treated group of animals revealed the extent of lung fibrosis and infiltration of macrophages. From figure 5.3, it is obvious that the tumour control lung was highly fibrotic compared to the normal lung, which reflects aggressive metastasis of the tumour cells to the lungs and establishment of tumour metastases. The alveolar spaces were narrowed and thickened with necrotic lesions with infiltration of large

number of pleomorphic metastatic cells distributed all around the area. Therefore, it was a significant note that the lung of *Thuja* 1M-treated animal showed drastic reduction in fibrosis, and infiltration of metastatic cells as shown in the figure, which evinced the inhibitory effect of the drug on the metastatic promotion.

5.3.1.5. Cellular expression of the metastatic marker genes

The pro-metastatic marker genes such as MMP-2, MMP-9, VEGF, ERK-1 and ERK-2, were reported to be overexpressed in a wide variety of tumours. As shown in figure 5.4, treatment with *Thuja* 1M down-regulated the expression of these genes in comparison with their expression in untreated tumour controls.

5.3.2. Angiogenesis assays

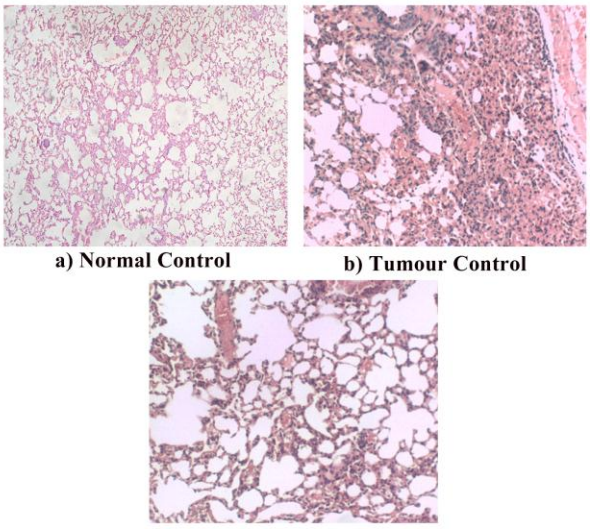
5.3.2.1. Determination of capillary formation

Tumour directed capillary formation can be a good model for assessing the angiogenic ability of the drug. The tumour control group showed numerous capillaries arising from the surrounding blood vessels and spreading around the established solid tumour (Fig. 5.5). TNP 470, which is accepted as the standard for angiogenic inhibitor (Yamaoka et al., 1993), showed highly reduced number of capillaries (89.2%) and hence the size of solid tumour also. The treated group, with *Thuja* 1M, on the other hand, should be well noted that the number of capillaries was reduced significantly (63.8%) compared to that of the tumour control animal, where the tumour size was also decreased.

5.3.2.2. Serum levels of VEGF and GMCSF

VEGF, the promoter of angiogenesis, showed an elevated increase in the untreated tumour bearing animals which is better lessened in the *Thuja* treated group (Tab. 5.3). Similarly, the level of GMCSF was also found to be reduced in the treated group when compared to that of the raised level in the untreated group ($p<0.001$).

Figure 5.3. Histopathological analysis of lungs



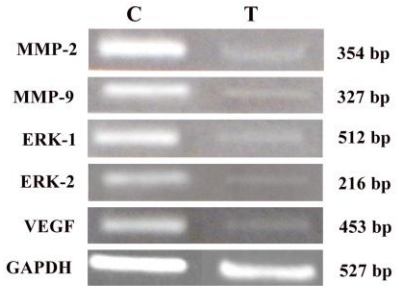
a) Normal Control

b) Tumour Control

c) Thuja 1M treated

Figure 5.4.

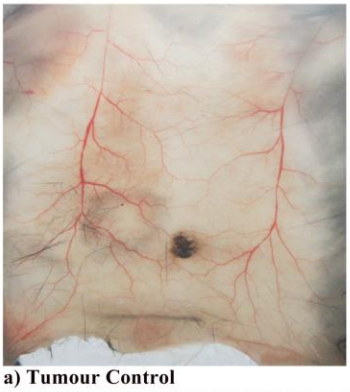
Gene expression patterns of metastatic markers



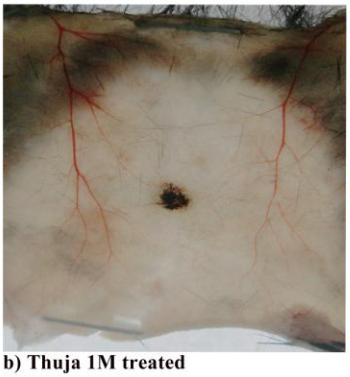
Lane 1 (C) - Metastatic tumour control
Lane 2 (T) - Thuja 1M treated.

Grouped animals were induced lung metastasis, followed by treatments with or without the drug. Lung tissues were excised out on 21st day and the expression of lung metastatic markers was analyzed by reverse transcriptase PCR using specific primers.

Figure 5.5. Effect of Thuja 1M on tumour directed angiogenesis



a) Tumour Control



b) Thuja 1M treated

c) TNP-470 treated

Grouped animals were induced with B16F10 solid tumours subcutaneously, followed by treatments with or without the drug. On the 11^{th} day, the skin along with the tumours were excised and observed for the formation of tumour directed capillaries. TNP-470 is the standard angiogenesis inhibitor.

Table 5.3

Cytokine levels of solid tumour induced animals with or without Thuja 1M treatment

Group	Cytokine levels (pg/mL)			
	VEGF		GMCSF	
	Day 1	Day 11	Day 1	Day 11
Normal Control	18.19 ± 3.69	-	19.93 ± 3.04	-
Tumour Control	71.44 ± 4.76	172.2 ± 8.21	69.43 ± 6.30	30.15 ± 5.05
Thuja 1M treated	40.91 ± 4.44[a]	79.5 ± 5.89[a]	39.66 ± 5.39[a]	23.46 ± 2.3[a]

Data represent mean ± standard deviation. [a]$P < 0.001$, compared to the tumour control group.

5.4. Discussion

Cancer progression is primarily facilitated by metastatic and neoangiogenic pathways, which remains the major hurdles of intervening therapeutic strategies. Even though a primary tumour is well confined, >90% of cancer deaths are credited to the invasion-metastatic cascade leading to systemic metastases which are further insensitive to conventional therapies. Hence a therapeutics which can target the metastatic and angiogenic pathways will be more sensible and clinically effective with fewer side effects (Gupta GP and Massague, 2006; Sawyers, 2004; Steeg, 2006). Many natural products were reported to interdict the said pathways by dowregulating the metastatic and angiogenic factors and promoting those which can cause cancer regression (Reddy et al., 2003; Da Rocha et al., 2001). The branch of alternative medicines also possesses many medications which can effectively interfere with neoplastic growth and their progression, but there is a necessity to make them evidence-based. In the present study, we have attempted a detailed investigation of the effect of the homeopathic preparation of *Thuja occidentalis* (1M potency), which has already reported to have antineoplastic properties and also with

a preliminary document on its antimetastatic effects, in interfering with the metastatic and angiogenic pathways.

B16F10 melanoma cells induced metastatic lung colonization in C57BL/6 mice represents a convenient model to study metastasis. Similar to dissemination from the primary tumour site, these cells when injected intravenously will have a homing tropical tendency to colonize at lungs, which is their metastatic site. While invading the lung tissue, they form numerous colonizing metastases in the form of tumour nodules in the lungs which will be characterized by lung fibrosis and decreased function, where the key marker is the increased levels of hydroxyproline, associated with collagen metabolism. It was observed that administration of *Thuja* 1M significantly reduced the number of lung tumour nodules and hydroxyproline content ($P<0.001$). Besides, increased oxidation of sugar derivatives in tumour cells result in increased level of uronic acid. It will be esterified to form glucuronic acid lactone which will accelerate hydroxyproline formation and thereby collagen deposition. Hexosamine is also a significant marker of tumour cells which will promote the synthesis of N-acetyl neuraminic acid (sialic acid), which is essential for the formation of glycolipids present on cell surface (Voet and Voet, 1995). The overexpression of these marker molecules are involved in various pathways associated with tumour progression. γ-glutamyl transferase (GGT) is an enzyme that regulates GSH homeostasis and overexpressed in tumour cells, provide amino acid residues for intracellular GSH synthesis, thereby protecting the cells from oxidative stress damage (Zhang et al., 2005). Similar to the previous literature, the above said key parameters that represent cancer progression and aggressiveness were found to alleviate significantly in the lung metastasis-induced animals, by treatment with *Thuja* 1M ($P<0.001$). Moreover, the life span of the treated group was also improved profoundly when compared to the tumour control group ($P<0.001$). Histopathological analysis of the lung tissue of the treated group showed better architecture with significant reduction in metastatic tumour nodules and fibrosis of the alveolar walls, when compared with the control group, which

correlates with the effect of *Thuja* to inhibit lung fibrosis and metastatic colonization.

To account for a detailed analysis, we have further checked the levels of pro inflammatory cytokines such as IL-1β, IL-6, TNF-α and GMCSF, which will be released by the tumour cells or sequestered at the ECM to mediate inflammatory processes, recruitment of various cells and molecules to ECM, cancer cell proliferation etc. They are soluble proteins which are the major regulatory molecules of different wings of immune system and hence also have specific roles in immune response to tumours (Voronov et al., 2003; Grivennikov and Karin, 2008; Szlosarek and Balkwill, 2003; Langowski et al., 2006; Andela et al., 2000; Zerbini et al., 2003). The experiment revealed that treatment with *Thuja* 1M significantly reduced the serum levels of these cytokines, which were showed drastic increase in tumour control animals ($P<0.001$).

To further explore the mechanism of metastasis inhibition, we have checked the gene expression patterns of metastatic marker genes such as matrix metalloproteinases (MMPs), extracellular signal regulated kinases (ERKs) and vascular endothelial growth factor (VEGF). It was well-evidenced that MMPs are zinc dependent endopeptidases which can degrade the ECM components, out of which MMP-2 and MMP-9 are well-studied which can degrade type IV collagen matrix, the major component of basement membrane. Upon proteolytic activation from pro-MMPs, they will promote the invasive phenotype of cancer cells along with liberation various growth factors tethered to the ECM, which will initiate cell proliferation and metastasis (Gupta et al., 2007; Padua et al., 2008; Psaila and Lyden, 2009). The gene expression patterns clearly indicate that *Thuja* 1M could down-regulate the production of MMPs and hence inhibit the metastatic progression of B16F10 cells.

ERKs are major proteins of MAPK pathway, which is upregulated in most cancers and have major roles in providing survival and proliferating signals to cancer cells (Lewis et al., 1998; Pearson et al., 2001). *Thuja* 1M treatment was found to down-

regulate the expression of ERK-1 and ERK-2, which is again an evidence for that the drug could inhibit metastasis.

VEGF is an important growth factor that can mediate metastatic and angiogenic pathways. Angiogenesis is an inevitable component of metastatic pathways even from primary tumour site, where tumour expansion >1-2mm requires efficient supply of oxygen and nutrients through sprouting blood vessels to the tumour mass, followed by the transport to secondary metastatic sites as well as for the establishment of macroscopic metastases, which requires disorganized capillary networks to grow inside the tumours. Thus the density of vascularization is an important prognostic marker of tumour status. Fibroblast growth factor (FGF) and Vascular endothelial growth factor (VEGF) are the major molecules that are reported to facilitate angiogenesis, out of which VEGF levels are reported to be a pro-angiogenic factor which is correlated directly to microvessel density (Hoeben et al., 2004; Coultas et al., 2005). Moreover, overexpression of VEGF levels can induce MMP production and promote invasion and metastasis (Gupta GP and Massague, 2006). VEGF expression of the lung tissue was also found to be demoted by *Thuja* 1M treatment when compared to the tumour control, which is a significant result to demonstrate not only the anti-metastatic effect, but supposedly the anti-angiogenic effect also.

More substantiation of the anti-angiogenic activity was obtained by the observation of tumour directed capillary formation, where the control group showed a spread capillary network arising from the adjacent blood vessels towards the growing tumour. Meanwhile, treated group showed a significant reduction in the number of capillaries as well as their scattered growth towards the tumour. Along with VEGF, GMCSF is also a cytokine with pro-metastatic and pro-angiogenic roles resulting in invasion and proliferation of cancer cells (Bussolino et al., 1991). Analysis of the VEGF levels and GMCSF levels in the serum of same animals also seemed to be decreased significantly when compared to the tumour control group, signifying the anti-angiogenic property of *Thuja*.

All the above results definitely substantiate the capability of the homeopathic preparation of *Thuja* to effectively combat metastatic progression of B16F10 metastatic tumour along with inhibiting angiogenesis by concurrent down-regulation of MMPs, ERKs and VEGF. Thus, the study provides a scientific validation for the conventional use of *Thuja* in many homeopathic medications for advanced cancers emphasizing its anti-metastatic activity.

Chapter 6
Study on the effect of homeopathic medicines on breast cancer metastasis

6.1. Introduction

Breast cancer is the most common type of cancer in women and the leading cause of cancer related death in women of developed as well as developing countries. It was estimated that worldwide over 5, 08,000 women died in 2011 due to breast cancer (Global Health Estimates, WHO 2013). Although breast cancer is thought to be a disease of the developed world, almost 50% of breast cancer cases and 58% of deaths occur in less developed countries (GLOBOCAN 2008). However, it is a significant point to note that not the primary tumour but their metastasis to distant sites becomes the major reason for 90% of breast cancer related deaths (Clarke et al., 2005). Hence breast cancer is defined as a heterogeneous disease where 10-15% of the patients have the chance to develop aggressive metastasis within 3 years after the diagnosis of the primary tumour or late metastasis during their life time (Harris et al., 2012). The disseminated breast carcinoma cells from the primary tumour will form metastases at their common sites such as lungs, liver, bone etc (Lee, 1983). The presence of lymph node metastasis, large sized primary tumour and the lack of differentiation of tumour tissue (evaluated by grading) are the common prognostic markers that determines the advancement of breast cancer (Koscielny et al., 1984; Rosen et al., 1989; Carter et al., 1989). The modern techniques for early diagnosis such as screening by mammography and adjuvant chemotherapy helped to decrease the mortality rates significantly (Clarke et al., 2005). However, chemotherapy has a wide range of acute and long term unavoidable side effects (Eifel et al., 2001). Since the possibility of metastasis cannot be predicted during the diagnosis of a primary tumour, 80% of the patients will receive chemotherapy even though only 40% of them may sometimes develop metastasis. Hence most patients will be over treated with chemotherapy which will adversely affect the quality of their life (Weigelt et al., 2005). Various reports approved that alternative medicines contribute some good sources of medications which are being used by a significant population in different parts of the world against cancer and other diseases, apart or along with conventional medicines (Ernst E, 1998; Begbie et al., 1996; Gotay et al., 1999; Burstein et al., 1999). For instance, reports regarding the use of CAM in Canadian breast cancer survivors mentioned that

the treatment is very prevalent in the population and concluded that it may play a role in the positive benefits associated with support group attendance (Boon et al., 2000). Another study on the use of herbal medicines used by breast cancer patients in Palestine revealed that the use of herbal remedies is prevalent in breast cancer patients in Palestine and majority of users were satisfied with herbal remedies (Jaradat et al., 2016). Likewise, many studies were reported which suggests that CAM therapies may have beneficial effects in breast cancer patients (Cui et al., 2004; Helyer et al., 2006). While relating to homeopathy and its use in cancer, a few clinical reports support their use to treat several cases of cancer or to lessen the side effects of chemotherapy along with the evidences from various laboratory experiments (Banerji et al., 2008; Oberbaum et al., 2001; Jacobs et al., 2005; Balzarini et al., 2000; Ullman et al., 2008). But some of the literatures still claim that homeopathic medications could not provide any definitive proofs in clinical studies (Ernst, 2002; Cucherat et al., 2000; Cassileth and Deng, 2004). In this context, it is a scientific need to effectively validate the efficacy of homeopathic medicines against any medical condition including cancer. 4T1 mammary carcinoma induced breast tumour is a convenient mice model to study metastasis which resembles advanced human mammary cancer (Pulaski and Ostrand-Rosenberg, 2000). The present study is designed to evaluate the efficacy of *Thuja* occidentalis, *Ruta* graveolens and *Carcinosinum* on breast cancer metastasis induced by 4T1 mammary carcinoma cells in experimental animals.

6.2. Materials and Methods

6.2.1. Homeopathic medicines and dosage

Homeopathic preparations (1M, 200c and 30c potencies) of *Thuja* occidentalis, *Ruta* graveolens and *Carcinosinum* were orally administered to the experimental animals for ten consecutive days at a dose of 10 µL/ animal in a final volume of 100 µL, diluted with sterile water. Potentiated ethanol was used as the vehicle control.

6.2.2. Cell line and animals

4T1 mouse mammary carcinoma cell line was used to induce spontaneous metastasis in female BALB/c mice (8 weeks old).

6.2.3. Chemicals

The chemicals such as 6-thioguanine, collagenase type IV/elastase cocktail, collagenase type I cocktail, hyaluronidase cocktail, hydroxy proline, N-acetyl neuraminic acid (NANA), glucosamine hydrochloride and glucuronic acid lactone, glycyl glycine, γ-glutamyl-4-nitroanilide were specifically used for the experiments in the study. All other reagents were of analytical reagent grade.

6.2.4. Determination of 4T1 induced mammary tumour development and survival rate in BALB/c mice

Mice were divided into eleven groups (15 animals/ group). The animals were injected with 4T1 mammary carcinoma cells ($7x10^4$/ 0.1 mL) subcutaneously into the fourth mammary gland by gently piercing the skin. Group I was kept as untreated tumour control. Group II was treated with the vehicle control. The groups III, IV and V were treated with 1M, 200c and 30c potencies of *Thuja* respectively. The groups VI, VII and VIII were treated with *Ruta* 1M, 200c and 30 C respectively. The groups, IX, X and XI were treated with *Carcinosinum* 1M, 200c and 30c potency respectively. All the drugs as well as vehicle control were administered for ten consecutive days, starting from the 7^{th} day of tumour induction. The tumour diameters were measured using vernier calipers, every week starting from the 7^{th} day up to the 42^{nd} day of tumour induction. The tumour volume was calculated using the parabolic equation,

Tumour volume (cc) = $(LW^2)/2$,

where L is the length and W is the width, where L >W.

Afterwards, all the animals were observed to notice their death pattern and the mean survival days of each experimental group was calculated, as described in section 2.2.10.

6.2.5. Determination of 4T1 mammary carcinoma-induced spontaneous metastasis to multiple organs (lymph nodes, lungs and liver) in BALB/c mice

Mice were divided into two groups (32 animals/ group). The animals were injected with 4T1 mammary carcinoma cells ($7x10^4$/ 0.1 mL) subcutaneously into the fourth mammary gland. Group I was kept as untreated tumour control. Group II was treated with *Thuja* 1M for ten consecutive days, starting from the 7^{th} day of tumour induction. The animals were sacrificed and the metastatic organs such as draining lymph nodes, lungs and liver were respectively collected at different time points aseptically.

6.2.5.1. Determination of lymph node metastasis

The animals were sacrificed (8 animals from each group) on the 18^{th} day of tumour induction and the draining lymph nodes were aseptically collected and washed in 1X HBSS. The lymph node was then transferred culture media supplemented with 60 µM 6-thioguanine. The lymph node was teased well; the cells were dispersed and the resulting suspension was then incubated for 48 hrs at 37ºC provided with 5% CO_2 atmosphere. After incubation, the attached cells were photographed. The cell density was also estimated. The change in the number of metastasized cells was expressed in terms of fold change with respect to the control. This will give easy interpretation of the effect of drug on the metastasis of cells when compared to the control. Fold change is calculated by the formula, fold decrease = (x/y), where x is the number of metastasized cells in the treated group and y is the number of metastasized cells in the control group. A sample of lymph node tissue was fixed in formalin and used for histopathological analysis. The methods were explained in detail in section 2.2.10.

6.2.5.2. Determination of lung metastasis

The animals were sacrificed (16 animals from each group) and the lung tissue was aseptically collected on the 25^{th} day of tumour induction. The tissue was washed in 1X HBSS and transferred it to another culture dish. Half number of the samples (8 no:s) was used for screening of metastatic cells and the remaining

samples were used for the determination of biochemical parameters and gene expression studies. The tissue was processed to cell suspension which was then centrifuged to obtain the cell pellet. The pellet was suspended in culture medium containing 60 µM 6-thioguanine and incubated for 48 hr at 37ºC provided with 5% CO2 atmosphere. After incubation, the attached cells were photographed and the cell density was estimated and expressed as fold change. The lung sample was also fixed in formalin and used for histopathological analysis. The methods were explained in detail in section 2.2.10.

6.2.5.2.1. Determination of biochemical parameters of lung tissue

The various biochemical parameters of the lung tissue such as hydroxyproline (Bergman and Loxley, 1970), hexosamine (Elson and Morgan, 1933), uronic acid (Bitter and Muir, 1962) were estimated by standard methods as described in section 2.2.7.

6.2.5.2.2. Estimation of biochemical parameters of in the serum

Blood was collected by heart puncture; serum was separated and used for the estimation of sialic acid content as well as γ–glutamyl transpeptidase (GGT) levels. Serum level of sialic acid was determined by Thiobarbituric acid assay (Skoza and Mohos, 1976) and GGT level was measured by estimating the release of p-nitroaniline from γ–glutamyl p-nitroanilide (Szasz, 1969) as described in section 2.2.7.

6.2.5.3. Determination of liver metastasis

Animals were sacrificed (8 animals from each group) and the liver was aseptically collected on the 32nd day of tumour induction and washed in 1X HBSS. The tissue was minced well and processed into cell suspension which was then incubated in culture medium containing 60 µM 6-thioguanine for 48 hrs at 37ºC provided with 5% CO_2 atmosphere. After incubation, the attached cells were photographed; cell density was estimated and expressed as fold change. A sample of liver tissue was fixed in formalin and used for histopathological analysis. The methods were explained in detail in section 2.2.10.

6.2.5.4. Analysis of gene expression of metastatic markers

The primary tumour tissue was collected on the 32nd day from the experimental group described in section 6.2.5.3., RNA was isolated, cDNA was prepared and the amplification of genes such as MMP-2, MMP-9, TIMP-1, TIMP-2, VEGF and GAPDH were performed using specific primers as per methods described in section 2.2.9. The amplified products were then resolved in 1.5% agarose gels and viewed under UV using the gel documentation system.

6.2.6. Determination of serum cytokines

A similar set of experimental groups were designed (N=8/group) and treated with the test material as described in section 6.4. Blood was collected from the caudal vein of all the animals on the 7th and 42nd day after tumour induction. Serum was separated and used for the estimation of cytokines such as VEGF, IL-1β, TNF-α and GM-CSF using highly specific ELISA kits.

6.3. Results

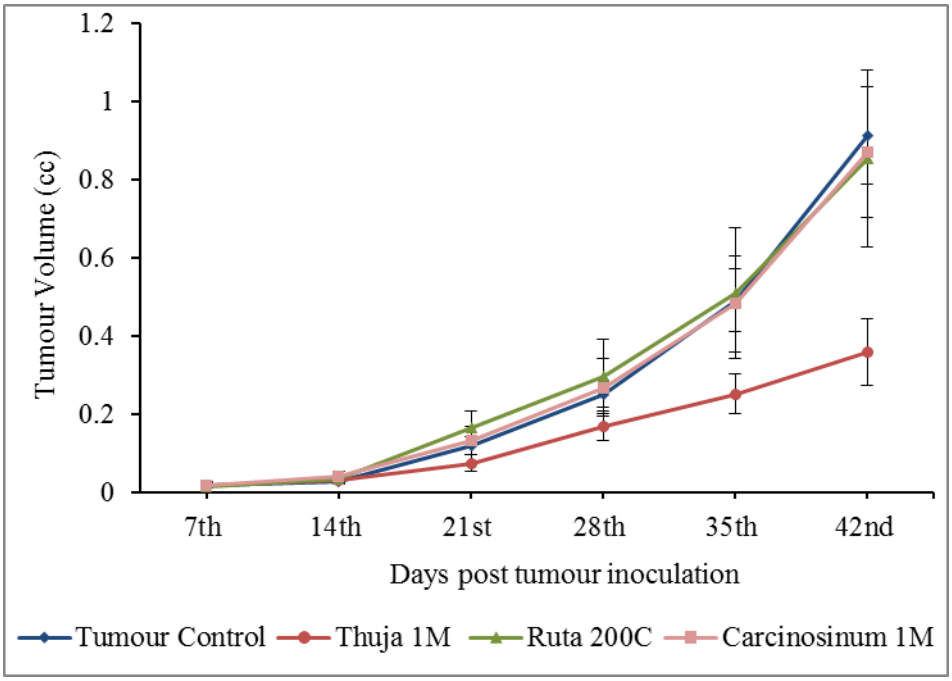
6.3.1. Determination of 4T1 induced mammary tumour development and survival rate in BALB/c mice

The effect of Thuja 1M, Ruta 200c and *Carcinosinum* 1M on 4T1 induced solid tumour development is represented in figure 6.1 (other potencies did not show any significant effect, hence data not shown). It is evident from the results that the tumour volume of *Thuja* 1M treated group was significantly reduced even from the 21st day after tumour induction, while the other drugs did not show any effect on the tumour development. On the 42nd day, the tumour volume of *Thuja* treated group was found to be 0.34 ± 0.09 cc, which seemed significantly reduced when compared to the tumour control group ($P<0.001$), which showed a tumour volume of 0.96 ± 0.08 cc.

As shown in figure 6.2, the survival of *Thuja* 1M treated animals were also significantly improved when compared to the tumour control group.

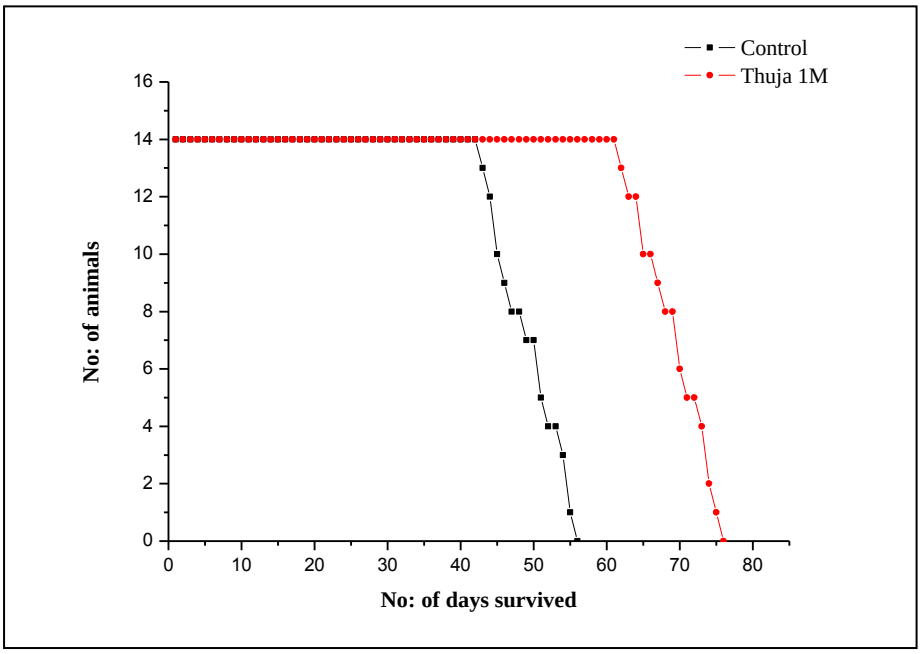
Figure 6.1

Effect of homeopathic medicines on 4T1 induced solid tumour development



Data are mean ± standard deviation.

Figure 6.2 Effect of Thuja 1M on the survival of 4T1 tumour bearing mice



Grouped animals were induced with solid tumour and treated with or without the drug. Death pattern of the animals were noted and expressed graphically. Data are individual observations of the death pattern of animals.

6.3.2. Effect of *Thuja* 1M on 4T1 mammary carcinoma-induced spontaneous metastasis to multiple organs (lymph nodes, lungs and liver) in BALB/c mice

6.3.2.1. Effect of *Thuja* 1M on lymph node metastasis

Figure 6.3 depicts the effect of *Thuja* 1M treatment on the metastasis of 4T1 mammary carcinoma cells to the lymph nodes. It is evident from the figures 6.3a. and 6.3b. that *Thuja* 1M treatment significantly reduced the number of metastatic cells in the lymph node compared to the tumour control. Histopathological analysis of the lymph node (Fig. 6.3c.) also correlated with the same result.

6.3.2.2. Effect of *Thuja* 1M on lung metastasis

Figure 6.4 represents the effect of *Thuja* 1M on the metastatic progression to the lung tissue. The fold decrease in the number of metastatic cells compared to the tumour control, as evident from the figures 6.4a. and 6.4b., clearly showed the efficacy of the drug to inhibit metastatic progression of 4T1 cells to the lungs. Moreover, the improved tissue architecture of the lung, as evident by the histopathological analysis (Fig. 6.4c.) also confirmed the anti-metastatic effects of *Thuja* 1M.

6.3.2.2.1. Effect of *Thuja* 1M on biochemical parameters of lung tissue and serum

As represented in Table 1, the concentration of hydroxyproline, hexosamine and uronic acid in the lung tissue of tumour control animals was significantly higher than the normal values. Meanwhile, treatment with *Thuja* 1M could significantly decrease the concentration of these biochemicals. ($p<0.001$) (Tab. 1).

Similarly, the serum markers such as sialic acid and GGT levels were significantly elevated in the tumour control group, which was found to be significantly decreased in the treated animals ($p<0.001$), as shown in table 1.

6.3.2.3. Effect of *Thuja* 1M on liver metastasis

As shown in figure 6.5, the metastasis of 4T1 cells to the liver was also significantly reduced by *Thuja* 1M which is evident from the fold decrease in the number of metastatic cells (Fig. 6.5a. and 6.5b.). Histopathological analysis of the liver tissue showed areas with infiltrating pleomorphic cells having hyperchromatic nuclei and areas of haemorrhages. The treated liver maintained significantly better architecture and infiltration of metastatic cells compared to the tumour control (Fig. 6.5c.).

6.3.2.4. Effect of *Thuja* 1M on expression of marker genes

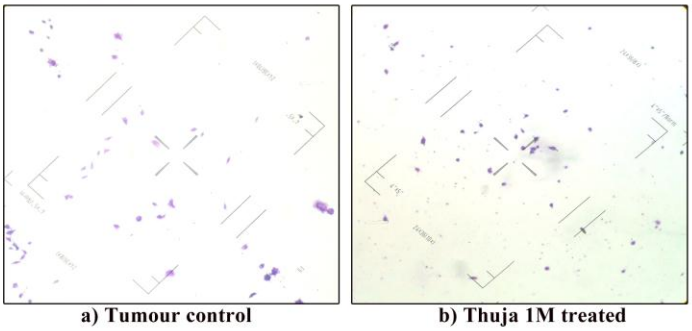
As shown in figure 6.6, treatment with *Thuja* 1M down-regulated the expression of the genes such as MMP-2, MMP-9 and VEGF, while upregulated the expression of TIMP-1 and TIMP-2 proteins.

6.3.3. Effect of *Thuja* 1M on serum cytokine profile

Figure 6.7 shows the cytokine profile of tumour control and *Thuja* 1M treated animal groups. As shown in the figure, the cytokines such as IL-1β, TNF-α, VEGF and GMCSF were drastically increased in the tumour control group which were found significantly reduced in the animal group treated with *Thuja* 1M ($p<0.001$).

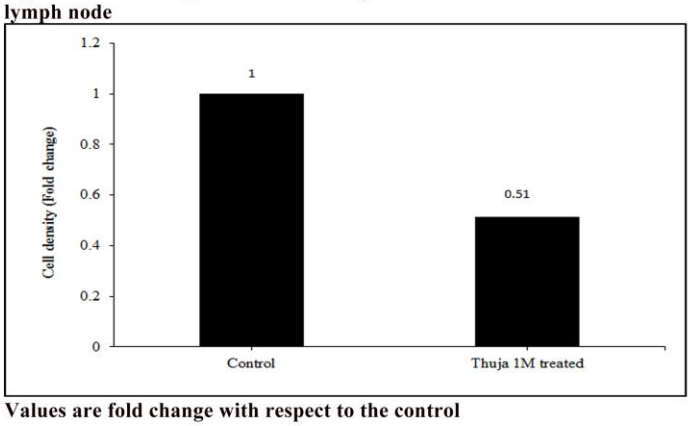
Figure 6.3. Effect of Thuja 1M on 4T1 induced lymph node metastasis

Fig. 6.3a. Screening of metastatic 4T1 cells in lymph node of metastasis induced animals



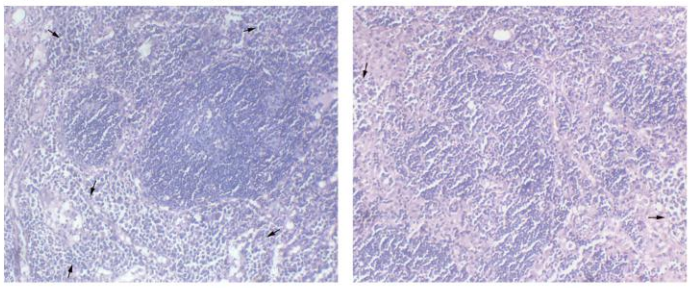
a) Tumour control **b) Thuja 1M treated**

Fig. 6.3b. Fold change in the cell density of metastatic 4T1cells in the lymph node



Values are fold change with respect to the control

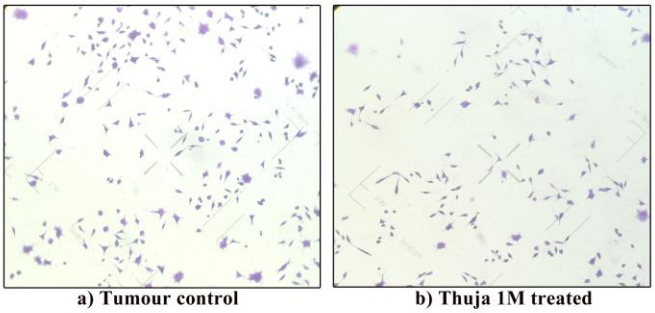
Fig. 6.3c. Histopathology of lymph node of metastasis induced animals



a) Tumour control **b) Thuja 1M treated**

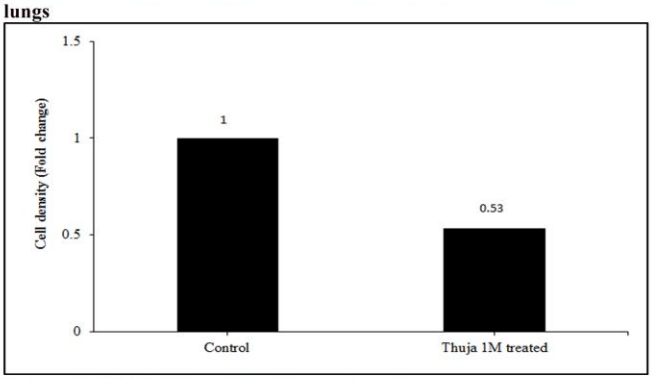
Figure 6.4. Effect of Thuja 1M on 4T1 induced lung metastasis

Fig. 6.4a. Screening of metastatic 4T1 cells in lungs of metastasis induced animals



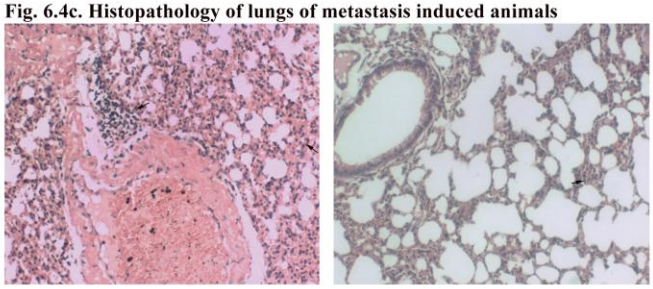
a) Tumour control **b) Thuja 1M treated**

Fig. 6.4b. Fold change in the cell density of metastatic 4T1cells in the lungs



Values are fold change with respect to the control

Fig. 6.4c. Histopathology of lungs of metastasis induced animals



a) Tumour control **b) Thuja 1M treated**

Table 6.1

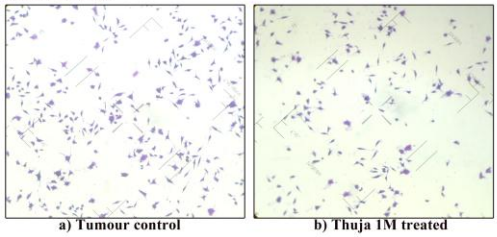
Effect of Thuja 1M on lung biochemical parameters

Group	Hydroxyproline (µg/mg proetein)	Hexosamine (mg/100mg tissue dry weight)	Uronic acid (µg/100mg tissue wet weight)	Sialic acid (µg/mL serum)	GGT (nmol p-nitroaniline/mL serum)
Normal Control	1.29 ± 0.23	0.50 ± 0.2	52.8 ± 8.97	23.62 ± 6.63	25.01 ± 5.52
Tumour Control	25.7 ± 5.43	4.20 ± 0.7	325.94 ± 21.92	136.07 ± 15.58	134.04 ± 14.37
Thuja 1M treated	11.77 ± 2.18[a]	1.96 ± 0.27[a]	118.95 ± 14.4[a]	82.15 ±9.41[a]	80.13 ± 10.46[a]

[a]$P < 0.0001$, with respect to the tumour control group.

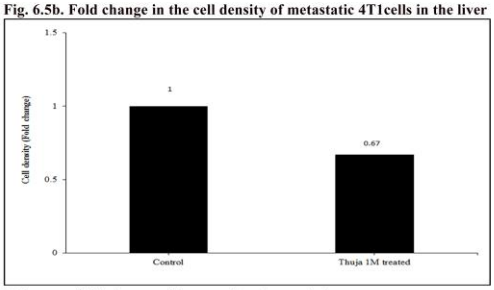
Figure 6.5. Effect of Thuja 1M on 4T1 induced liver metastasis

Fig. 6.5a. Screening of metastatic 4T1 cells in liver of metastasis induced animals



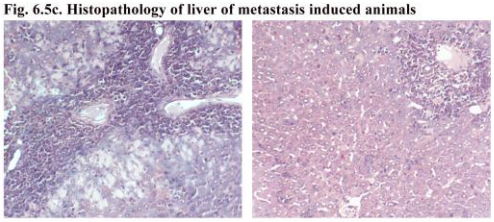
a) Tumour control **b) Thuja 1M treated**

Fig. 6.5b. Fold change in the cell density of metastatic 4T1cells in the liver



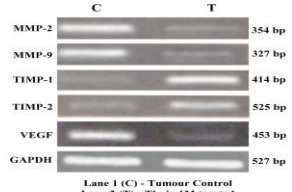
Values are fold change with respect to the control

Fig. 6.5c. Histopathology of liver of metastasis induced animals



a) Tumour control **b) Thuja 1M treated**

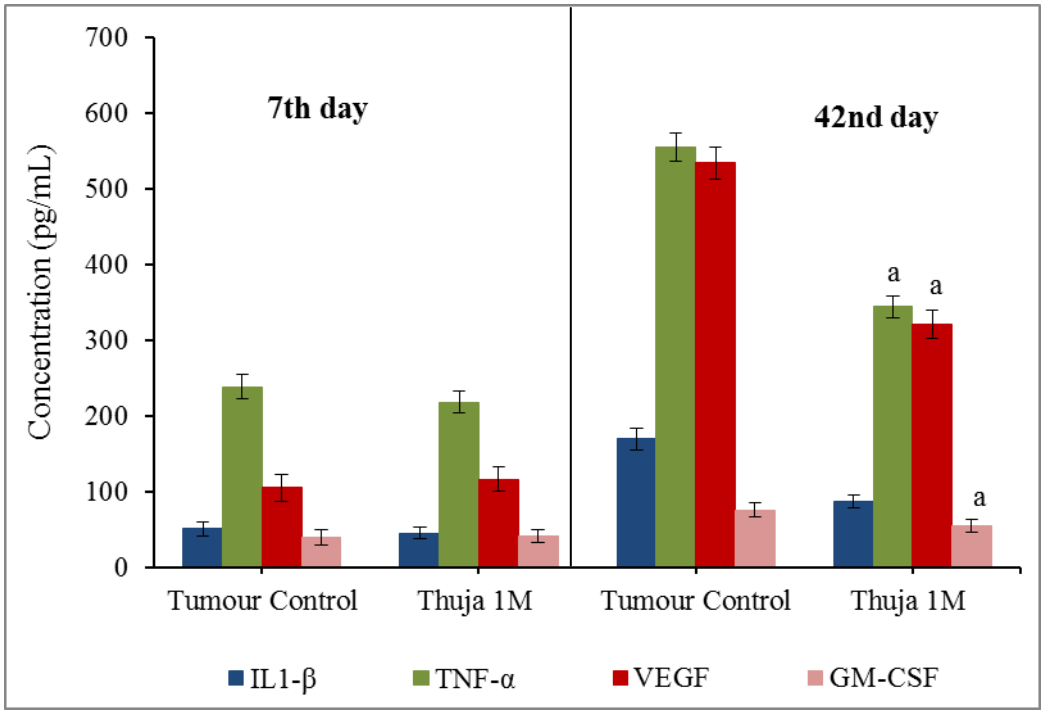
Figure 6.6.
Expression of metastatic markers of breast primary tumour



Lane 1 (C) - Tumour Control
Lane 2 (T) - Thuja 1M treated

Figure 6.7

Effect of Thuja 1M on serum cytokine profile



Values are mean standard deviation. [a]*P*<0.001, compared to the tumour control.

6.4. Discussion

The present study aimed to investigate the role of selected potencies of *Thuja* occidentalis, *Ruta* graveolens and *Carcinosinum* 1M on spontaneous metastasis induced by 4T1 mammary carcinoma cells. The preliminary results regarding the effect of these drugs on 4T1 solid tumour development revealed that out of the studied potencies of the three drugs, only the 1 M potency of *Thuja* could significantly inhibit the development of mammary solid tumour, while any of the other drugs did not show any effect (Fig. 6.1). Hence, *Thuja* 1M was further subjected to a detailed analysis for its role to inhibit the metastatic progression of the tumour cells to lymph node, lungs and liver. The increased survival rate of animals in the *Thuja* 1M treated group also correlated with the efficacy of *Thuja* 1M to interfere with the breast cancer progression.

Mouse 4T1 mammary carcinoma is a convenient model to study spontaneous breast cancer metastasis where the cells will metastasize to multiple sites such as lungs, liver etc. and since the cells are resistant to 6-thioguanine, the estimation of metastatic cells migrated to each organs can also be studied easily (Pulaski and Ostrand-Rosenberg, 2001). The effect of *Thuja* 1M on the metastasis of 4T1 cells to lymph node, lungs and liver were analysed by screening of the cells of the tissues using 6-thioguanine, Since 4T1 cells are resistant to 6-thioguanine, any effect to inhibit the metastatic progression can be correlated with the decrease in the number of resistant cells survived after screening experiment compared to that of the tumour control. The results strongly suggested that *Thuja* 1M could inhibit the metastatic progression of 4T1 cells to its secondary sites such as lymph node, lungs and liver with 0.51, 0.53 and 0.67 fold decrease in the number of metastatic cells in these tissues respectively compared to the tumour control tissue. The histopathological analysis of the tissues also showed that the infiltration of tumour cells in the lymph nodes, the colonization of tumour cells in the lungs and liver were significantly decreased by the treatment with *Thuja* 1M.

The invasion and colonization of metastatic cells to the lungs will result in fibrosis and collagen deposition in the alveoli of the lung tissue resulting in decreased function. Hence estimation of collagen is a marker of lung fibrosis. The most common method for evaluating collagen deposition is hydroxyproline quantification (Woessner, 1961; Kliment et al., 2011) since it is a major component of collagen, comprising around 15-30% of its composition (Voet and Voet, 1995). The basis of hydroxyproline quantification is that total collagen can be assessed by acid hydralization of proteins followed by measurement of hydroxyproline content (Kliment et al., 2011). As discussed in section 3., the increased oxidation of the alcohol group of sugar derivatives results in the increased formation of uronic acid in tumour cells, which will then be converted to its esterified form, glucuronic acid lactone. This product can promote the formation of hydroxyproline from prohydroxyproline with the help of the enzyme prolyl hydroxylase. Moreover, glucuronic acid lactone activates the formation of collagen fibres during fibrosis (Voet and Voet, 1995; Pradeep et

al., 2003). Hexosamine is an important ground substance of the lung tissue with its components as glucosamine and galactosamine. Hence the hexosamine content of the lung tissue is an important marker of chronically diseased lung including lung fibrosis (Saltzman et al., 1961). Moreover, hexosamines will promote the synthesis of N-acetyl neuraminic acid (sialic acid), which is essential for the formation of glycolipids present on the cell surface (Voet and Voet, 1995). The results signify that the levels of hydroxyproline, hexosamine and uronic acid were elevated in the lung tissue of tumour bearing animals and treatment with *Thuja* 1M significantly lowered their concentrations.

Serum sialic acid has been established as a non-specific and potent tumour marker, which can closely monitor the tumour burden and metastasis and is useful for the evaluation of the later course and prognosis of malignant neoplasms (Crook, 1993; Stefenelli et al., 1985; Silver et al., 1979). The increased binding of sialic acid to the glycoproteins of the cell membrane is related to an essential property of malignant cells. Thus, the high turnover of malignant cells cause an increased release of sialic acid and this may contribute to the elevated levels of sialic acid in the serum (Van Beek et al., 1973; McNeil et al., 1965; Shetlar et al., 1949; Stefenelli et al., 1985). γ-glutamyl transferase (GGT) is a cell surface enzyme that regulates GSH homeostasis and overexpressed in tumour cells. The primary role of cellular gamma glutamyl transferase (GGT) is to metabolize extracellular reduced glutathione (GSH), allowing for precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis, thereby protecting the cells from oxidative stress damage.(Zhang et al., 2005; Lee et al., 2004). Moreover, its expression is often significantly increased in human tumours, and its role in tumour progression, invasion and drug resistance has been repeatedly suggested (Pompella et al., 2006). GGT has been reported to be a sensitive marker of metastatic patients with renal cell carcinoma (Simic et al., 2007) as well as in early detection of breast carcinoma (Seth et al., 2003). The results of the present study revealed that the serum levels of sialic acid and GGT was found to be elevated in tumour control animals and these were found profoundly decreased in *Thuja* 1M treated group.

The invasion and metastasis of malignant cells involve degradation of extracellular matrix (ECM), which is catalysed by the important class of degrading enzymes called the matrix metalloproteinases (MMPs) produced by the tumour cells. The metastatic process is usually characterized by up-regulation of MMPs and hence inhibition of MMPs will directly correlate to the inhibition of invasion and metastasis (Mook et al., 2004; Deryugina and Quigley, 2006). Anti-metastatic studies on B16F10 melanoma tumour bearing animals proved the inhibitory activity of *Thuja* on MMP-2 and MMP-9 production (section). The MMPs system also includes the tissue inhibitors of metalloproteinases (TIMPs) which are MMP inhibitors and hence they are expected to inhibit cancer progression, invasion and metastasis, even though TIMPs are multifunctional in nature (Valente et al., 1998; Wurtz et al., 2005; Leung et al., 2015). However, the present study assessed that *Thuja* 1M could inhibit the over expression of MMPs and VEGF while up-regulated the expression of TIMP proteins in the primary tumour signifying its role to inhibit invasion and metastasis of 4T1 carcinoma cells.

The serum level of cytokines is yet another strategy to evaluate the anti-invasive and immunomodulatory properties of the test material and therefore we checked the concentrations of IL-1β, TNF-α, VEGF and GMCSF in the serum of tumour bearing animals. Secretory IL-1β accounts for the promotion of invasion, inflammation and tumour induced suppression as evident from studies of various tumour models and cancer patients (Apte et al., 2006). TNF-α is a known pro-inflammatory cytokine and generates an autocrine tumour promoting network in ovarian and breast cancer (Kulbe et al., 2007; Roy et al., 2009). VEGF is known to play a critical role in vasculogenesis, angiogenesis, and metastasis. Up-regulation of these cytokines may therefore account for the enhanced breast cancer-associated secondary metastasis as which will be released by the tumour cells or sequestered at the ECM to mediate inflammatory processes, recruitment of various cells and molecules to ECM, cancer cell proliferation etc. (Loges et al., 2009 Chan, 2009; Byrnes et al., 2006; Roy et al., 2009). GMCSF is reported to be produced by 4T1 cells to promote pro-metastatic inflammatory processes (Tao et al., 2008). It has already discussed in

chapter 3 that *Thuja* 1M could reduce the serum levels of these cytokines in B16F10 melanoma induced metastasis bearing animals. The results of the present study also revealed that the levels of all of these cytokines were significantly down-regulated in 4T1 tumour bearing animals by treatment of with *Thuja* 1M.

All the results revealed through this study demonstrated that the homeopathic preparation of *Thuja* occidentalis could effectively inhibit the progression of 4T1 breast carcinoma in experimental animals. Even though potentiated preparation of *Thuja* occidentalis has already reported to have antineoplastic and immunostimulatory properties, and it is well known for its use in homeopathy for malignancies, its role in advanced stages of cancer progression has not been scientifically studied in detail. The present study thus exposes a scientific supportive evidence for its use in various homeopathic formulations against advanced stages of malignancies and thus proposes the drug as a better candidate for integrative therapies of cancer.

Chapter 7
Evaluation of the effect of Thuja 1M on cell mediated immune responses during metastatic challenge

7.1. Introduction

Metastasis remains the major threat for most cancer related deaths rather than the primary neoplasias. The invasiveness and metastasis of malignant cells are affected by various oncogenes, tumour suppressor genes, metastasis suppressor genes etc. Besides, the cells of the innate and adaptive immunity, adjacent stroma cells as well as chemokines and their receptors also play a vital role in the spread of cancer cells (Leber and Efferth, 2009). Even though the immune system can implement anti-tumour responses, most cancers escape immunosurveillance because they are fundamentally "self," and autoreactive immune cells are usually deleted or anergized so that they do not attack self (Koh et al., 2006). Antitumor immune-mediated mechanisms are activated as soon as the first cancer cell is detected and operate both during primary tumor formation and during metastasis. However, when both innate and adaptive immunity becomes impaired, tumor development occurs. In this sense, compelling evidences indicate that tumor cells employ mechanisms that circumvent the immune response to enhance their own growth. These mechanisms include the secretion of immunosuppressive factors and the induction of distinct regulatory lymphoid or myeloid cells and, as occur with the immune response, they operate both during primary tumor formation and metastasis. Interestingly, cellular and molecular mechanisms of the immune response are important components of the tumor microenvironment and have the ability to promote or suppress tumor progression depending of the context of each cell interaction (O Croci and Salatino, 2011). These immune escape mechanisms in combination make the tumour a formidable foe for the immune system (Koh et al., 2006).

Cell-mediated immunity (CMI) is the major effector of the host immune response against tumours (Pratheesh kumar and Kuttan, 2011; Burleson et al., 2010). The central roles of immune effector cells, such as B, T, natural killer (NK) and natural killer T (NKT) cells, and of type I and II interferons (IFNs), and perforin (pfp) have been well elucidated in cancer immune surveillance (Kim et al., 2007; Dunn et al., 2002; Dunn et al., 2005). NK cells are primarily

viewed as cytotoxic lymphocytes which will non-specifically eliminate tumour cells and virus infected cells (Pratheesh kumar and Kuttan, 2011; Moretta et al., 2001). They have several important effector functions such as the initiation and amplification of the inflammatory response, the production of chemokines and cytokines, and the lysis of sensitive target cells (Langers et al., 2012; Vivier et al., 2008; Wu and Lanier, 2003). Antibody dependant cell mediated cytotoxicity (ADCC) and antibody dependent complement mediated cytotoxicity (ACC) have also been reported to have efficient roles in antitumour immune responses of certain therapeutics such as anti-tumour antibodies (Seidel et al., 2015; Dechant et al., 2008).Antibody-dependent cell-mediated cytotoxicity (ADCC) is the killing of an antibody-coated target cell by a cytotoxic effector cell through a nonphagocytic process, characterised by the release of the content of cytotoxic granules or by the expression of cell death-inducing molecules. ADCC is triggered through interaction of target-bound antibodies (belonging to IgG or IgA or IgE classes) with certain Fc receptors (FcRs), glycoproteins present on the effector cell surface that bind the Fc region of immunoglobulins (Ig). Effector cells that mediate ADCC include natural killer (NK) cells, monocytes, macrophages, neutrophils, eosinophils and dendritic cells (Teillaud, 2012). ACC is regarded as the most powerful mechanism of antibody mediated cell lysis which is mediated though activation of complement pathways (Dechant et al., 2008).

Apart from the escape mechanisms implemented by the growing tumour to overcome the cell-mediated immune response barriers, the clinical situation is that the conventional therapies of cancer also induce immune suppressive conditions impairing the effector roles of CTLs and NK cells (Tsavaris et al., 2002; Pratheesh kumar and Kuttan, 2011). Therefore, any therapeutic strategy which can mediate anti-tumour properties by boosting the anti-tumour immune responses of the host system itself will be a better choice in the present context.

7.2. Materials and Methods

7.2.1. Cell lines

K-562 cell line and 4T1 mammary carcinoma cell line were specifically used for the experiments.

7.2.2. Animals

Female BALB/c mice (4-6 weeks old) were used to induce 4T1 mediated spontaneous metastasis.

7.2.3. Homeopathic preparations and dosage

Homeopathic preparation of *Thuja* 1M was orally administered to respective animals for ten consecutive days. 100 µL of the drug or vehicle was diluted to 1mL using distilled water and 100 µL of it is administered to each animal.

7.2.4. Chemicals

Radiolabelled ^{51}chromium and ELISA kits for mouse IL-2 and IFN-γ were specifically used for the experiments. All other reagents were of analytical grade.

7.2.5. Determination of the effect of *Thuja* 1M on CMI response in experimental animals under metastatic challenge

BALB/c mice were divided into two groups (N = 36/group). Both the groups were induced spontaneous breast tumour metastasis using 4T1 cell line (7×10^4 cells/animal, orthotopically on the fourth mammary pad). Group I was kept as untreated control. Group II animals received *Thuja* 1M from 7th day after tumour induction for ten consecutive days. Three animals from each group were sacrificed at different time points after tumour induction such as 24 hr, 48 hr, 72 hr and continued every other day up to 21st day. Spleen cells were collected and used for the determination of NK cell activity and ADCC. Blood was collected by heart puncture; serum was separated and used for the estimation of ACC. The methods were explained in detail in section 2.2.6.

7.2.5.1. Determination of the effect of *Thuja* 1M on NK cell activity

NK cell activity was determined by 4 hr chromium release assay using chromium-labelled K-562 cells as the target cells and spleen cells as effectors. The cells were mixed to achieve an effector-target ratio of 100:1. The assay was performed and the % cell lysis was calculated as described in section 2.2.6.

7.2.5.2. Determination of the effect of *Thuja* 1M on ADCC

ADCC was determined by 4 hr chromium release assay using chromium-labelled SRBC as the target cells and spleen cells as effectors. The cells were mixed to achieve an effector-target ratio of 100:1 along with anti-SRBC antibody in presence of complement serum. The assay was performed and the % cell lysis was calculated as described in section 2.2.6.

7.2.5.3. Determination of the effect of *Thuja* 1M on ACC

Serum samples collected from the experimental animals were incubated with 4T1 target cells in presence of complement serum at 37^0C for 3 hr. Control groups were set as samples containing 1) 4T1 cells alone, 2) 4T1 cells treated with complement and 3) 4T1 cells treated with antibody. The cytotoxicity was assessed by trypan blue exclusion method. The methods were explained in detail in section 2.2.6.

7.2.6 Determination of the effect of *Thuja* 1M on serum cytokine levels

BALB/c mice were divided into two groups (N = 16/group). Both the groups were induced spontaneous breast tumour metastasis using 4T1 cell line (7×10^4 cells/animal, orthotopically on the fourth mammary pad). Group I was kept as untreated control. Group II animals received *Thuja* 1M from 7^{th} day after tumour induction for ten consecutive days. Eight animals from each group were sacrificed on 12^{th} day and 21^{st} day of tumour induction. Blood was collected by heart puncture; serum was separated and used for the estimation of cytokines using specific quantitative ELISA kits as per the manufacturer's protocol.

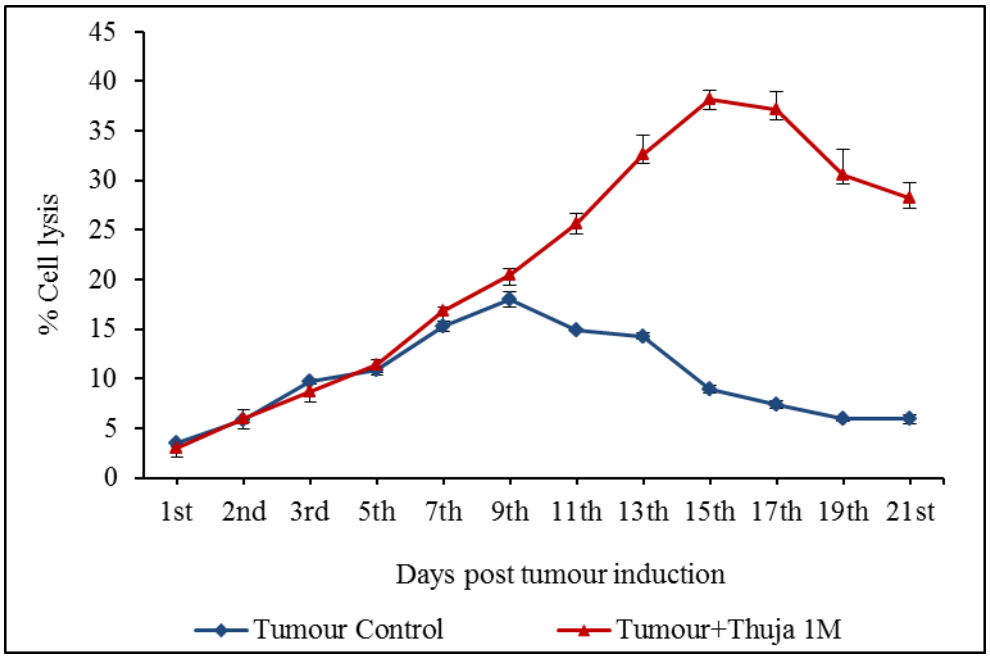
7.3. Results

7.3.1. Effect of *Thuja* 1M on NK cell activity

The effect of *Thuja* 1M on NK cell-mediated cytotoxicity of tumour cells is shown in figure 7.1. It is evident from the results that *Thuja* 1M significantly enhanced the NK cell activity in animals under 4T1 induced metastatic challenge with a peak value of 38% lysis on the 15th day of tumour induction. The tumour control group also showed a base line activity with 17.9% lysis on the 9th day of tumour induction.

Figure 7.1

Effect of *Thuja* 1M on NK cell activity in metastatic tumour bearing animals



Values are mean ± standard deviation.

7.3.2. Effect of *Thuja* 1M on ADCC

As shown in figure 7.2, administration of *Thuja* 1M significantly enhanced ADCC in metastatic tumour bearing animals compared to that of the tumour control. It was found that maximum value of cell lysis for *Thuja* 1M treated

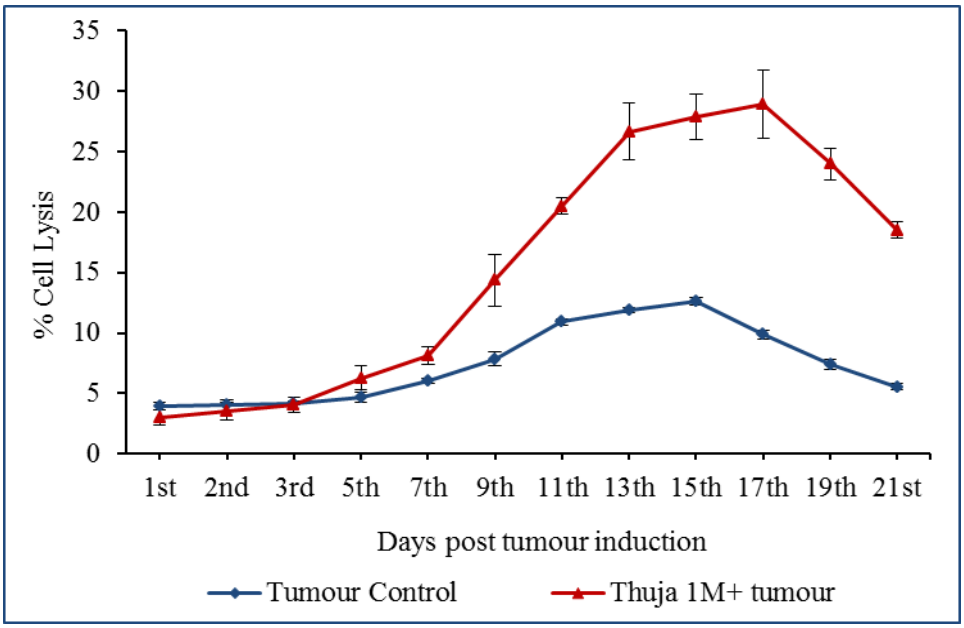
group was obtained on the 17th day after tumour induction with 28.9% cell lysis whereas the tumour control shows a peak value of 12.6% on the 15th day.

7.3.3. Effect of *Thuja* 1M on ACC

Figure 7.3 depicts the effect of *Thuja* 1M administration on ACC in animals under 4T1 induced metastatic challenge. It is evident from the result that *Thuja* 1M significantly improved ACC compared to the tumour control. The peak value for tumour control was 13.6% cell lysis on the 17th day, which was increased in *Thuja* 1M treated group which showed 22.6% cell lysis on day 15 post tumour induction.

Figure 7.2

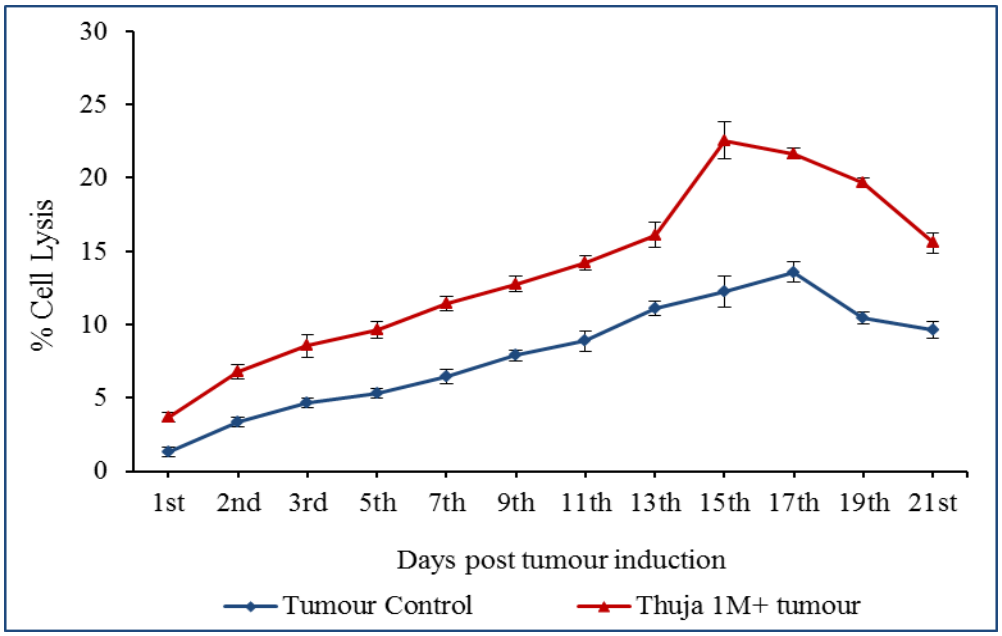
Effect of *Thuja* 1M on ADCC in metastatic tumour bearing animals



Values are mean ± standard deviation.

Figure 7.3

Effect of *Thuja* 1M on ACC in metastatic tumour bearing animals



Values are mean ± standard deviation.

7.3.4. Effect of *Thuja* 1M on IL-2 and IFN-γ production

As shown in table 7.1, *Thuja* 1M treatment significantly enhanced the levels of effector cytokines such as IL-2 and IFN-γ in the serum of metastatic tumour bearing animals compared to the tumour control group.

Table 7.1

Effect of *Thuja* 1M on serum cytokine levels in metastatic tumour bearing animals

Cytokine concentration (pg/mL)	12^{th} day		21^{st} day	
	Tumour Control	Tumour+*Thuja* 1M	Tumour Control	Tumour+*Thuja* 1M
IL-2	7.25 ± 1.12	10.53 ± 1.06	4.62 ± 0.48	15.7 ± 1.26
IFN-γ	1322.9 ± 101.9	1800.9 ± 125.9[a]	2174.3 ± 113.1	2925.2 ± 121.8[a]

[a]$P < 0.001$, compared to the tumour control group.

7.4. Discussion

Immunological approaches to the treatment of cancer have been attempted for over a century, with tantalizing but unsustainable results. Experiments in animals have, however, provided evidence for immune responses to tumours and have shown that T cells are a critical mediator of tumour immunity. More recently, advances in our understanding of antigen presentation and the molecules involved in T-cell activation have provided new immunotherapeutic strategies based on a better molecular understanding of the immune response (Janeway et al., 2005). The present study evaluated the efficacy of *Thuja* 1M preparation, which has shown anti-tumour and anti-metastatic properties in animal models (chapter 3 and chapter 5), to enhance the anti-tumour immune responses during metastatic challenge by analysing the NK cell activity, ADCC and ACC.

NK cells, up on activation, exocytose cytotoxic granules containing perforin and various granzymes, leading to the perforation of target cells and subsequent apoptotic death induced by the permeated granzymes (Lieberman, 2003; Voskoboinik et al., 2006). In addition to the perforin/granzyme pathway, the engagement of tumour necrosis factor (TNF) receptor superfamily (TNFRSF) members, such as Fas/CD95, TRAIL receptors and TNFR1, on tumour cells by the corresponding ligands (FasL, TRAIL and TNF) expressed on or secreted by NK cells contributes to NK cytotoxicity under certain circumstances (Zamai et al., 1998, 2007; Voskoboinik et al., 2006). A second, often underrated effector mechanism of NK cells is their capacity to secrete a variety of cytokines and chemokines, including interferon-γ (IFN-γ), TNF, GM-CSF (granulocyte-macrophage colony stimulating factor), MIP-1α (macrophage inflammatory protein-1α) and RANTES (regulated upon activation, normal T cell expressed and secreted) (Biron et al., 1999; Dorner et al., 2004). In fact, NK cells are considered as the major source of IFN-γ *in vivo*, and recent studies demonstrated that NK-derived IFN-γ is crucial in priming T helper 1 (Th1)-biased T-cell responses (Mocikat et al., 2003; Martin-Fontecha et al., 2004) (Waldhauer and Steinle, 2008). The results of the present study confirmed that *Thuja* 1M could

significantly augment the NK cell response during metastatic challenge, signifying the modulation of cell mediated immune system.

A substantial role of ADCC has been demonstrated *in vitro* and in mouse tumor models (Seidel et al., 2015). NK cells are thought to be the key mediators of ADCC, since only NK cells do not co-express the inhibitory FcγRIIb. In contrast, all other FcγR-expressing cells are tightly controlled by the balance between activating and inhibitory FcγRs. Efficient FcγRIIIa signaling depends on high avidity for specific binding to antibodies which ensures that NK cells are activated when antibodies have bound to a multimeric or multivalent cognate antigen only (Banks et al., 2002). These antibodies of the subclasses IgG1 and IgG3 binding to FcγRIIIa induce a potent activating signal which overcomes inhibitory signals and results in both cytotoxicity and a cytokine response (Chan et al., 2012). Co-engagement of other activating receptors has a synergistic effect and may enhance NK cell activation (Bryceson et al., 2006) (Seidel et al., 2015). The present study revealed that ADCC has also been enhanced in metastasis induced animals by the treatment with *Thuja* 1M.

ACC has also been demonstrated as one of the effector mechanisms of therapeutic antibodies for cancer. Classically, the assumption has been that these activated complement proteins play a role in tumour defense directly through complement-dependent cytotoxicity (Ostrand-Rosenberg, 2008) and indirectly through antibody-dependent cell-mediated cytotoxicity (Gelderman et al., 2004) (Rutkowski et al., 2010). It is evident from the results that ACC has also been augmented in tumour bearing animals during the period of drug treatment.

We have also analysed the cytokine levels in the serum of experimental animals. IL-2 and IFN-γ are the major effector cytokine of cell mediated immune response. IL-2 stimulates NK cell and T cell proliferation, (Misawa et al., 2000) while IFN-γ, a key cytokine secreted by activated NK cells, interferes with the recognition and killing of target cells by up-regulating class 1 major histocompatibility complex molecules and also exerts direct antitumor activity (Wodnar-Filipowicz and Kalberer, 2006) (Sheeja and Kuttan, 2007). The results signify that administration of *Thuja* 1M could increase the level of IL-2 and

IFN-γ which can be again correlated to the increased NK cell activity and ADCC.

The immunomodulatory effect of the potentiated preparation of *Thuja* 1M has been studied well which proved that the drug could modulate the humoral and cell mediated wings of the immune system in normal mice (Remya and Kuttan, 2015). The results of the present study again substantiated the anti-tumour immunomodulatory effects of the drug which could produce beneficial effects during metastatic challenge, in addition to its anti-metastatic property.

Summary and Conclusions

Cancer remains one of the leading dreadful diseases of the time with a significant statistical increase in the global incidence of new cases as well as mortality. The cause and effect mechanisms of the disease are very wide including endogenous and exogenous factors, which can trigger the initiation of carcinogenesis; its progression to a neoplastic stage and its further transformation to aggressive metastasis to the secondary sites. Even though the disease is being managed with conventional therapies as well as the advanced methods for early diagnosis of cancer, it still remains a serious threat to the world as the scenario is that most of the advanced cases cannot expect an exact cure for cancer, but an increase in the life expectancy of the patient to some more months or years. Moreover, the side effects of conventional therapies and drug resistance again intensifies the scientific urge to explore more anti-cancer drugs, either from natural sources or by synthetic methods.

Apart from conventional medicines, alternative medicines such as Homeopathy, Ayurveda, Acupuncture, Unani etc. has been gaining attention now a days, as a significant population of cancer patients will be following some of these medications with or without prescription along with conventional medicines. In spite of the disputes and controversies, CAM still paves the way of an alternate source of anti-cancer drugs and therefore scientific findings to prove their efficacy and proper validation will definitely help to create an integrative platform of treatment combining the conventional and CAM therapies.

We have designed the present study including some homeopathic medicines which are having a good literature background for their anticancer property. The study attempted to validate the efficacy of these homeopathic medicines to modulate the immune system and to defend the metastatic malignant conditions in experimental animals. The 1M, 200C and 30C potencies of the drugs were selected for the study. The plants, *Thuja occidentalis* and *Ruta graveolens*, which are rich with their bioactive components Thujone and Rutin respectively, and *Carcinosinum*, a preparation of tumour cells were proved for their immune stimulatory and anti-tumour properties, in the form of basic extracts or the potentiated preparations.

As the first step of the study, we have screened the 1M, 200C and 30C potencies of selected medicines, namely *Thuja occidentalis*, *Ruta graveolens* and *Carcinosinum*, for their anti-tumour activity using different *in vivo* tumour models in order to select the most active potency which has to be used for further studies using these tumour models. The results from the study using DLA, B16F10, EAC and EL-4 induced tumour bearing animals indicated that the 1M potency of *Thuja* could inhibit the development of tumours in all of these tumour models. *Ruta* and *Carcinosinum* showed varying anti-tumour properties in different models and out of the three potencies, *Ruta* 200c and *Carcinosinum* 1M was concluded to have comparatively higher anti-tumour property. Moreover, the anti-tumour activities of any of these drugs cannot be correlated in a concentration dependant manner, rather the diluted potencies showed higher activity.

Homeopathic medications are known to treat the whole system of a diseased individual and not the particular disease manifestations, in the sense that the immune system has an effective role in the mechanism of action of homeopathic medicines. In order to decipher the concept, we have further evaluated the effect of these medicines on the immune system in healthy animals. We have found that *Thuja* 1M, *Ruta* 200C and *Carcinosinum* 1M showed higher immunomodulatory activity compared to their other potencies as confirmed by the enhancement of haematological and hematopoietic parameters. Moreover, the enhanced blastogenesis of lymphoid organ cells also reflects the augmented or boosted status of immune system. It is an interesting fact that the results of anti-tumour and immunomodulatory studies can be well correlated positively, as the drugs were responded to the experiments in a similar pattern. This explains that immunomodulation may be one of the mechanisms by which the anti-tumour effects are accomplished in the physiological system.

The drugs selected for the study has already been reported to have anticancer properties in several laboratory experiments. Hence, further studies to analyse its effects on the progressive stages of cancer should be the next step of the study. Moreover, metastasis, being the prime cause of >90% of global cancer deaths, is a relevant threat in the present scenario of cancer. Spontaneous

metastatic mice models of B16F10 melanoma and 4T1 breast carcinoma were used for the studies. We have found that out of the three drugs, only *Thuja* 1M could inhibit the development of primary solid tumours in both the models and hence *Thuja* 1M was selected to further evaluate its anti-metastatic potential in these models.

B16F10 is a convenient model to study lung metastasis and we have observed that *Thuja* 1M could effectively inhibit the metastatic colonization of B16F10 cells in the lung tissue, which was assessed by the analysis of various biochemical parameters of the lungs and serum, survival rate of the animals, expression of metastatic marker genes and analysis of various inflammatory cytokines. Moreover, the capillary formation assay clearly indicated the anti-angiogenic activity toward the tumour directed capillaries, imposed by *Thuja* 1M, along with the down-regulation of pro-angiogenic cytokines.

Thuja 1M was subjected to the analysis of its effect on 4T1 mammary carcinoma induced spontaneous metastasis. The 4T1 breast tumour model closely resembles the advanced stages of human breast cancer and hence the drug can be evaluated for its effects on advanced malignant conditions. Moreover, such a study will provide solid scientific validation for its use in various homeopathic medications prescribed and practised in homeopathic treatment of late-stage malignancies of breast cancer. The results revealed that *Thuja* 1M could significantly inhibit the proliferation of primary tumour as well the metastasis of tumour cells to its secondary sites such as lymph node, lungs and liver. Moreover, the expression of metastatic marker genes was significantly down-regulated in the primary tumour, lungs and liver, which further confirmed the beneficial effects of *Thuja* 1M. Histopathology of the tissues and the serum levels of inflammatory cytokines are well correlated with the above results.

Since our studies proved the immunomodulatory effects of the drugs including *Thuja* 1M, and it might be correlated to its anti-tumour properties, it is sensible to evaluate the immune modulatory role of *Thuja* 1M during metastatic progression of 4T1 breast carcinoma. The cell mediated wing of the immune system is majorly contributing to the anti-tumour immune defences and it is

known that the tumour cells display an array of escape mechanisms to overcome the immune surveillance implemented by the immune system. We have evaluated the major effectors of cell mediated immune responses such as NK cell activity, ADCC and ACC up on treatment with *Thuja* 1M under metastatic challenge and found that the drug could significantly augment the cell mediated immune defences by boosting the tumour cell lysis mechanisms along with the up-regulation of effector cytokines needed for the activation of cell mediated immune responses. The results provide a substantiated support and evidence for the immunological effects of these ultra-diluted homeopathic medications in order to combat tumour development and progression.

In conclusion, the results of the study altogether demonstrate that out of the three homeopathic medicines, *Thuja occidentalis* possess potent anti-tumour and anti-metastatic potential and it is to be emphasized that the pharmacological effects are exactly evident at its higher potency compared to the lower ones. The immune modulatory and anti-metastatic potential at this ultra-diluted potency highlights that the drug can serve as a potent non-toxic, immunomodulatory, anti-cancer drug which could be recommended in homeopathic medications for malignancy at the early and advanced stages. Thus the results of the present study, when correlated with the good number of supporting scientific evidences during the last decade, provide a strong background to promote the use of homeopathic preparation of *Thuja occidentalis* in an improved and integrative platform of cancer therapy. Further scientific evaluations to check the efficacy and reproducibility in clinical scenario needs to be initiated to further enhance the promotion and critically analyse the limitations on the therapeutic side.

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