An *in vitro* study on the anti-angiogenic effect of

Majorana hortensis leaves

By

Sowndharyalatha.B

(17PBT018)

A Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore-641 043

In Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

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Signature of the
Head of the Department

24/4/19

Signature of the
Supervisor

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CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

Cancer is the uncontrolled abnormal growth of cells in the body. It results when cellular changes cause the abnormal growth and division of cells (Nall, 2018). Cancer is a leading health problem in both developing and developed countries. Recently, the major treatments for cancer are chemotherapy, radiotherapy and surgery (Hosseini and Ghorbani, 2015).

Cancer has the capability to spread to the distant or adjacent organs, that makes it life threatening. Tumor cells can penetrate blood or lymphatic vessels and circulate through the intravascular stream and proliferate at another site, which is termed as metastasis. Growth of vascular network is inevitable for the metastatic propagation of cancer tissue (Rajabi and Mousa, 2017).

Cancer has progressively been a leading cause for lethality worldwide for several decades. New statistics disclose that nearly half of people diagnosed with cancer have received treatment for invasive cancer and died from cancer or its treatment. The data infer that cancer-related deaths are highly associated with metastasis. Metastasis symptoms are due to spread of cancer from a primary neoplasm to other locations in the body and the formation of secondary tumors. Unexpected and undetectable tumor translocations cause treatments against cancer to become insignificant, eventually leading to death (Yen *et al*., 2018).

The most frequently diagnosed cancer worldwide is the Breast cancer (BC) and is the cause of cancer death among women. There are more than 20 distinct subtypes that comprise breast cancer. It differs genetically, morphologically and clinically. Functionally testing breast cancer remedies *in vitro* could predict patient outcome even without molecular knowledge. The most prevalent human-derived breast cancer models to date are cell lines (Sachs *et al*., 2018).

The term angiogenesis was invented two centuries ago and meant tissue expansion in adult animals which required new blood vessel formation. Scientists referred that extensive formation of new blood vessels also occurred during tumor growth, and speculated that “the impetus which gives rise to the proliferation of blood vessels emanated from the invading cells” (Ye, 2016).

Angiogenesis is the outgrowth of new blood vessels. Cancer cells cannot grow more than the size of a pinhead (1mm – 2mm). In order to acquire vascular expansion, they require oxygen supply and nutrients which they consume from the

adjacent blood cells. Consequently, tumor gets bigger in size and metastases occur. In order to inhibit the growth of the cells, angiogenesis has to be restrained using anti-angiogenic factors. The anti-angiogenic agents seem to effectively inhibit the factors that continue to influence the angiogenic process (Zirklik and Duyster, 2018).

Anti-angiogenic therapies from the other side of the coin states that it can result in transitory improvement, in the form of tumor standstill or constriction, in some cases increasing survival (Lupo *et al*., 2017).

Anti-angiogenic therapy can essentially normalize the tumor vasculature and rarefy vessel growth for a period of time. Still and all, multitargeted approach will greatly increase health care costs and may increase the side effect profile to unacceptable levels in anti-angiogenic therapy (Whipple, 2010). Anti-angiogenesis is one of the standard-of-care therapies for several types of solid tumors (Yang *et al*., 2017).

Angiogenic and anti-angiogenic activities can be evaluated using both *in vitro* and *in vivo* assays (Goodwin, 2007). The formation of new blood vessels involves a series of coordinated biological processes such as cell proliferation, guided migration, differentiation and cell-cell communications. There are three ways of forming new capillaries: sprouting growth, longitudinal division and intussuscepted growth (Ye and Jiang, 2016).

Previous researches have uncovered the fact that various traditional medicinal plants have helped the people in acting upon uncontrolled angiogenesis. Plants with anti-angiogenic properties are of considerable significance for diseases like cancer, diabetic retinopathy, etc. (Seo *et al*., 2013).

Majorana hortensis is one such candidate plant that has been selected for the present study. In ancient systems of drugs, it is revered for developing antiseptic, carminative, stimulant and medicinal drug and nerve tonic rheumatic habits, stimulates pressure level and has different therapeutic uses (Kumar *et al*., 2011). Earlier studies in our laboratory have shown that the leaf extract serves as a potential source of antioxidant and can be used in preparation for combating free radical mediated damage to the body (Palaniswamy and Padma, 2011a). Several reports suggest that *Majorana hortensis* possesses high antioxidant and anti-cancerous properties (Gharib *et al*., 2013).

The leaves of *Majorana hortensis* are rich sources of enzymic antioxidants like superoxide dismutase, catalase, peroxidase, glutathione S-transferase and polyphenol oxidases. It was also found that it has considerable level of non-enzymic anti-oxidants like ascorbate, tocopherol, total carotenoids, lycopene, reduced glutathione, phenols and chlorophyll (Palaniswamy and Padma, 2011b).

It has also been found to exhibit significant anticancer activity against cancer cell lines by inducing apoptosis and inhibiting cell viability (Palaniswamy, 2012).

In accordance with these observations, the present study was formulated to study the effect of *Majorana hortensis* leaf extract on angiogenesis *in vitro* using chick embryo fibroblasts and breast cancer (MDA-MB-231) cell lines.

CHAPTER TWO

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Globally, cancer is one of the frequently diagnosed diseases and is a major cause of loss of human life (Singh *et al*., 2015). Cancer is a multifactorial disease and the assisting factors of such disease include various environmental stresses, specific background and improper food habits (Vijayapadma, 2015). It remains a major cause of mortality around the globe (Hu and Fu, 2012).

Cancer incidence is on the increased rate; perhaps with the advances in technology, cancer is diagnosed more frequently, and the myths associated with cancer are vanishing and people are more open to accepting and discussing cancer more openly. It is found that India contributes about 7.8% of the global cancer burden (Saranath and Khanna, 2014).

Cancer is characterized by absolute and unlimited cell proliferation induced through the dysfunction of numerous important genes. There is an indefinite number of abnormalities in place of a single aberration from which cancer arises. The current clinical therapies however show fewer success rates and exhibit serious deliberating side effects and complications of the standard treatment regimens (Islam *et al*., 2019).

Triple-negative breast cancer (TNBC) is described as a type of breast carcinoma that I negative for oestrogen and hormone receptor (ER, PR) and HER2 expression. This form of breast cancer is marked by its aggressiveness, low survival rate and a lack of specific therapies. Recently, significantly molecular characteristics of TNBC have been emphasized and led to the identification od some biomarkers which could be used in diagnosis, as therapeutic targets or to assess the prognosis (Mouh *et al*., 2016).

The therapies that are being used for the treatment of cancer showcases many limitations which add in toxicity, serious side effects and development of resistance to therapeutic agents by cancer cells (Chauhan *et al*., 2016).

The limitations of these modern therapies have paved the way to the usage of plant-derived natural products as sources of cancer chemotherapeutics (Kuete *et al*., 2011). Recent researches in cancer claim that natural compounds effectively kill the progression of the cancer cells, while exerting significantly low toxicity

towards the normal cells (Lv *et al*., 2014). Many anticancer drugs from plants have been developed and are currently in use. For example: Vinca alkaloids, taxanes, camothecin derivatives, cephalotaxus, colchicine, ellipticine, berberine, combretastatins, capsaicin, etc. (Iqbal *et al*., 2017).

Many anti-angiogenic agents that inhibit the different stages of angiogenesis in tumor growth processes have been identified and developed from plant sources. Such anti-angiogenic agents are more effective when used in combination with chemotherapy. Anti-angiogenic therapy thus has become an imperative strategy to treat cancer (Ribeiro *et al*., 2018).

Plants and their active compounds have physiologic effects on the body. Different phytochemicals obtained from plants such as polyphenols, reservaratrol, catechin, curcumin, etc., have been identified and proved that they have anti-angiogenic property and effectively could control tumor growth (Lu *et al*., 2016). These therapeutic potential of the plants are attributed by the phytochemicals present in them. Plants with significant antioxidant activity have been explored for their anticancer potential using various *in vitro* and *in vivo* studies. *Majorana hortensis* is one such plant that has been extensively studied in our laboratory.

2.1 CANCER ASSOCIATED MOLECULAR EVENTS

The following are major molecular events involved in carcinogenesis that helps in providing the targets, the effective strategies in killing cancer cells.

2.1.1 ANGIOGENESIS

Angiogenesis is the formation of new blood vessels from the neighbouring blood vessels. Angiogenesis is a normal physiological process that involves proliferation, migration and morphogenesis of endothelial cells from existing vessels to new blood vessels**.** It has been hypothesized that endothelial cells may limit tumor expansion and that for every tumor diameter increase; there should be an increase in tumor vascularization. Primary carcinomas rarely cause patient death. Rather, most carcinoma-related morbidity is due to the difficulties associated with metastasis (Bielenberg and Zetter, 2015).

The blood angiogenesis and lymph angiogenesis are triggered by some of the chemical signals from tumour cells in a phase of rapid growth that always relies on metastasis. The formation of the vascular network is very important which involves supply of adequate oxygen and nutrients and removal waste product during proliferation as well as metastatic spread. The angiogenic and lymph angiogenic factors are increasingly receiving attention, particularly in the field of neoplastic vascularization. The study of tumour angiogenesis has become a major thrust area of cancer research. The exclusive way of tumor vascularization is the sprouting of new blood vessels (Hillen and Griggioen, 2007).

2.1.2 INVASION AND METASTASIS

The principal importance in the prognosis of cancer patients is the sequence of events leading to the development of tumor cells invasion and metastasis. Metastasis is the spread of cancer cells to tissues, organs and beyond where the tumor originated to the formation of new tumors. This process finally results in the complexity of cancer as a multiplex disease. This chain of events leading to a malignant transformation of cells, either through genetic or epigenetic alternations is complex (Jiang *et al*., 2015)

Angiogenesis and invasion are the two major mechanisms that promote survival and progression of tumors. Aberrant angiogenesis plays a central role to various angiogenic diseases such as age-related macular degeneration, rheumatoid arthritis and also endometriosis. Aberrant angiogenesis is critical for cancer metastasis. Vascular, avascular and metastatic stages are the central three stages of cancer. Angiogenesis is crucial for uncontrolled growth of tumors. At the beginning, pro- and anti-angiogenic factors are balanced in the tumor microenvironment. In reaction to the hypoxic environment, cancer cells endure an angiogenic switch. Therefore, the production of pro-angiogenic factors is attenuated. An increased production of the pro-angiogenic factors will end up in the activation of endothelial cell proliferation, differentiation, and migration. Ultimately, a capillary network is efficiently set up (Lou *et al*., 2017).

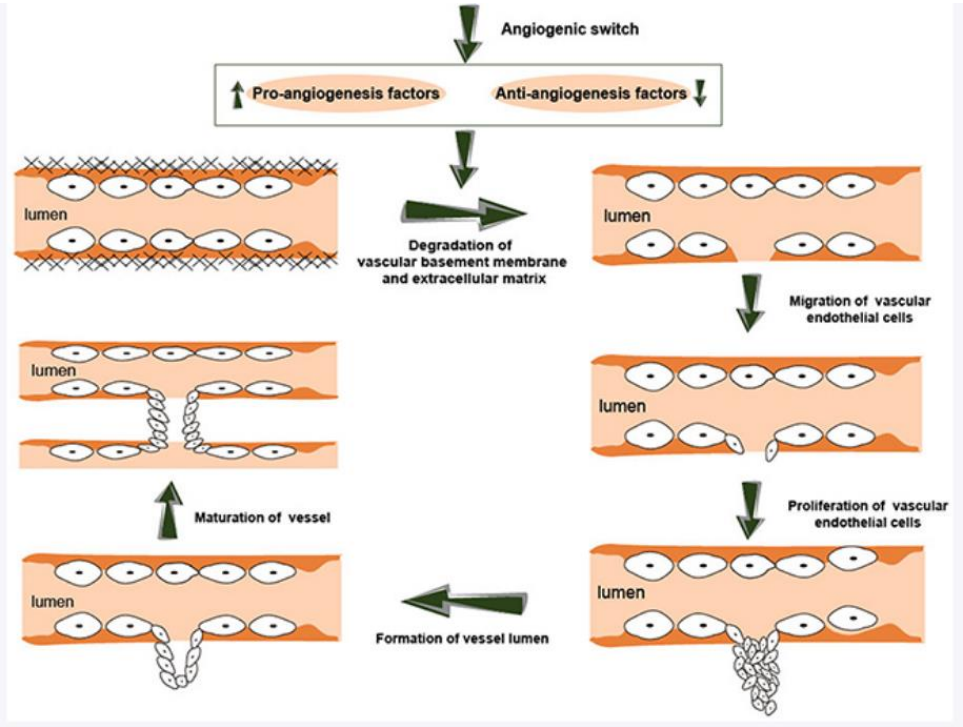


Plate 2.1: Progression of cancer angiogenesis (Lou *et al*., 2017)

2.1.3 ADHESION

Cell adhesion molecules play a key role in cancer progression and metastasis. The tumor cells in the circulation interact with platelets, leukocytes and contributes to tumour cell adhesion, extravasation and finally to metastatic lesions. Selectins and integrins are the two cell adhesion molecule families, which have been identified to participate in metastasis (Bendas and Borsig, 2012). Cancer development and progression are aided by the environmental factors, diet, the individuals' genetic makeup as well as epigenetic alterations. The knowledge that not all cancer cells within tumors have equal tumor growth-supporting potential [the concept of cancer stem cells (CSCs) and tumor cell heterogeneity] is about to revolutionize the way we develop new anticancer drugs and treat cancer patients. The focus of this review is on the role of cell adhesion molecules (CAMs) in the interaction between cancer (stem) cell and extracellular matrix (ECM) as well as changes in the expression profile of adhesion molecules as cancer cells leave the primary tumor and travel to form metastases (Farahani *et al*., 2014).

A variety of evidence says the involvement of specific adhesion molecules in metastasis.

1. For example, some cancer cells metastasize to distinctive organs, regardless of the first organ encountered by the circulating cancer cells. This ability to colonize a specific organ has been correlated with the preferential adhesion of the cancer cells to endothelial cells derived from the target organ. This suggests that cancer cell/endothelial cell adhesion is involved in cancer cell metastasis and that adhesion molecules are expressed on the endothelium in an organ-specific manner.

2. Moreover, inclusion of peptides that inhibit cell adhesion, such as the YIGSR- or RGD-containing peptides has the sufficient capability to inhibit experimental metastasis.

3. Metastasis can be augmented by acute or chronic inflammation of target vessels, or by treatment of animals with inflammatory cytokines, such as interleukin-1. *In vitro*, cancer cell/endothelial cell adhesion can be amplified by pretreating the endothelial cell monolayer with cytokines, such as interleukin-1 or tumor necrosis factor-alpha. This implies that, besides organ-specific adhesion molecules, a population of inducible endothelial adhesion molecules is associated and is relevant to metastasis.

4. Further evidence for this model is found in the comparison to leukocyte/endothelial adhesion during leukocyte trafficking. Convincing evidence exists, both *in vivo* and *in vitro*, to demonstrate an absolute vital requirement for leukocyte/endothelial adhesion before leukocyte extravasation can occur. The materiality of this comparison to metastasis is reinforced by the observation that some of the adhesion molecules involved in leukocyte/endothelial adhesion are also implicated in cancer cell/endothelial adhesion (Lafrenie and Buchana, 1993).

The development of *in vitro* models of endothelial assembly is extremely worthwhile. In addition to studying the mechanisms of endothelial assembly fully developed models would enable the testing of anti-angiogenic drugs for cancer therapy (Morin and Tranquillo, 2013).

2.2 ESTABLISHMENT OF PRIMARY CULTURE

Chick embryo is a well-established model system in developmental biology to study cell migration pathways and differentiation. Chick embryos have become a powerful animal model for studying early developmental events (Yukinori, 2013).

The chick embryo model is a practicable, economical *in vivo* system to study invasion by cancer cells in an embryonic environment (Busch *et al*., 2013)

In the present study, the culture was established to obtain fibroblasts and respective parameters were carried out using them as an *in vitro* system.

2.3 CHORIOALLANTOIC MEMBRANE

The chorioallantoic membrane (chorioallantois, CAM) (Plate 2.3) found in embryonated eggs of some amniotes is a vascular membrane which results from a fusion of mesodermal layers of two developmental structures: the allantois and the chorion. The CAM, the subject of the present study formed of three layers, which comprises the chorionic epithelium, the mesenchyme and the allantoic epithelium. In the course of the growth, blood capillaries and sinuses invade the chorionic layer and become wedged in between the epithelial cells and in so doing they acquire close proximity with the ambient air. Moreover, the CAM furthermore plays a significant role in osteogenesis by transporting calcium into the embryo from the eggshell (Makanya, *et al*., 2016).

In the CAM assay, the extraembryonal chorioallantoic membrane is used to study angiogenesis. The CAM comprises a very dense capillary network, which serves as an optimal surface for the application of test substances and also tumor cells (Jedelska *et al*., 2013).

The Chick Chorioallantoic Membrane (CAM) is a broadly used model for the study of angiogenesis, cancer growth and drug efficacy. Irrespective of this, little is known about the developmental alteration from its appearance to the time of hatching (Makanya, *et al,* 2016). In this study, the CAM has been used to study the angiogenic response to the plant *Majorana hortensis*.

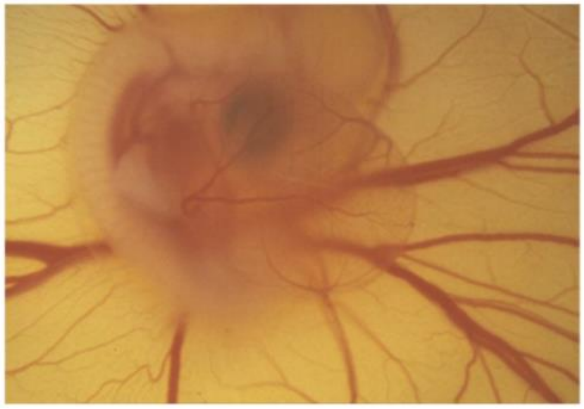


Plate 2.2: Macroscopic *in ovo* features of the chick Chorioallantoic membrane (CAM) at day 5 of incubation (Ribatti, 2016)

2.4 RELATIONSHIP BETWEEN ANGIOGENESIS AND CANCER

Angiogenesis is a normal and not a straightforward process controlled by certain biomolecules produced in the body. The new blood vessel generations from a pre-existing blood cell via the sprouting of endothelial cells, thus expanding the vascular tree, also, the steps of angiogenesis are depicted below:

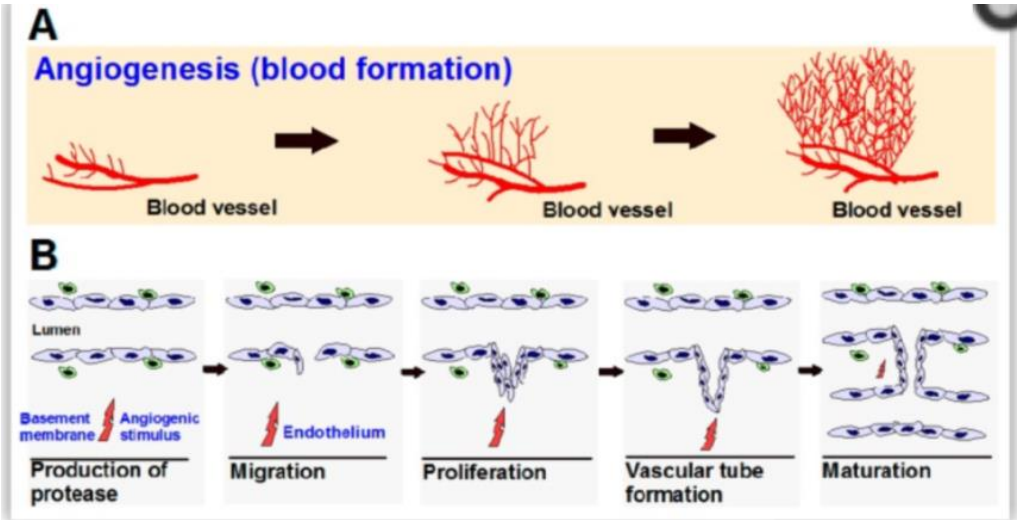


Plate 2.3: (A) Formation of blood vessels from pre-existing blood vessels (B) Steps in angiogenesis (Rajabi and Mousa, 2017)

Endothelial cells proliferate and migrate into the perivascular space, shaping into primary sprouts and also the resultant lumenation of those sprouts results in capillary loops formation, which is followed by

de novo of a new basement membrane and blood vessel maturation to complete tube-like structure through which blood can flow (Rajabi and Mousa, 2017).

The salient step concerned in angiogenic cascade includes the reactivation of endothelium cells, rupture of basement membrane, adhesion, migration, proliferation, tube formation and sprouting off new capillary blood vessels of pre-existing cells.

Tumor progression and metastasis square measure classically conditioned wherever the cells escape traditional growth and adhesion controls and invade, migrate, attach and grow at inappropriate sites. To control many of the above the above events, certain angiogenic factors come into play: growth factors, cytokines and cell adhesion molecules. Tumor cells hire new blood vessels via various angiogenic factors and are further amplified by the cytokine release which attract and activate macrophages, mast cells and neutrophils. On the basis of this newfound understanding, innovative and novel therapeutic approaches targeting various steps in this process as well as the neovasculature on its own may be developed (Mousa, 2000).

2.5 PLANT-DERIVED ANTICANCER AGENTS

Cancer chemoprevention, which is the long term intervention with natural or synthetic molecules, to prevent, inhibit or reverse carcinogenesis, is a growing field that is gaining greatest importance. Interest in the use of complementary and alternative medicine and natural health products is on the rise, predominantly to the toxicity exhibited by the conventional chemotherapy (George and Abrahamse, 2016).

Traditional treatments have been followed using medicinal plants for numerous human diseases for ages. They also have an important role as a source of anticancer agents. Over 60% of the currently used anticancer agents are derived from natural sources such as plants (Juarez, 2014).

Certain agents like curcumin, artemisinin, EGCG, resveratrol, emodin, celastrol, thymoquinone and tocotrienols have reported prominent anti-angiogenic effects in the pre-clinical models of tumor angiogenesis. Various semi-synthetic

derivatives and novel nano-formulations of these natural compounds have also exhibited outstanding anti-angiogenic activity by increasing bioavailability and delivering the drugs to the sites of tumor angiogenesis (Shanmugam *et al*., 2017).

Plant-derived compounds have been extensively studied. Many plant-derived compounds with potential anticancer activities have already entered clinical studies.

Plant name	Family	Part used	Phytochemicals	Specific cancer suppressed
Hibiscus mutabilis	Malvaceae	Pepper	Lectin	Liver, breast cancer (*In vitro*)
Plumbago zeylanica	Plumbaginaceae	Roots	Plumbagin	Blood cancer, skin cancers (*In vitro*)
Saffron crocus	Iridaceae	Dry stigmas	Saffron	Liver, lung cancer and pancreatic cancer (*In vitro*)
Taxus brevifolia	Taxaceae	Bark	nab-Paclitaxel	Ovarian and breast cancer (Both *in vitro* and *in vivo*)
Vitis vinifera	Vitaceae	Fruit	Cyanidin	Colon cancer (*In vivo*)
Actaea racemosa	Ranunculaceae	Rhizomes and roots	Actein	Liver and breast cancer (*In vivo*)
Pyrus malus	Rosaceae	Bark, fruit	Quercetin, procyanidin	Colon cancer (Both *in vitro* and *in vivo*)
Betula Sp.	Betulaceae	Leaves	Betulinic acid	Human melanoma xenografts and leukemia (*In vitro*)
Tabernaemontana divaricata	Apocynaceae	Leaves	Cononitarine B, Conophylline	Liver, lung, breast and colon cancer (*In vitro*)
Smilax chinensis	Liliaceae	Rhizomes	Tannin, saponins and flavonoid	Sarcoma-180 and ascites sarcoma (Both *in vitro* and *in vivo*)
Allium sativum	Liliaceae	Whole plant	Allin	Carcinoma of human (mammary) gland (Both *in vitro* and *in vivo*)
Aloe vera	Liliaceae	Whole plant	Aloesin, emodin	Anti-angiogenic activity (*In vitro*)
Curcuma longa	Zinziberaceae	Roots	Curcumin	Stomach cancer (*In vitro*)
Emblica officinalis	Euphorbiaceae		Polyphenols, tannins	Lymphoma and melanoma (*In vitro*)
Momordica charantia	Cucurbitaceae	Leaves, Roots	Charantin, cucurbitane-type triterpene	Colon cancer and breast cancer (*In vitro*)
Stevia rebaudiana	Asteraceae	Leaves	Labdane sclareol properties	Anti-tumorous and cytotoxic (*In vitro*)
Camellia sinensis	Theaceae	Leaves	Epigallocatechin gallate	Brain, prostate, cervical and bladder cancer (*In vivo*)
Nelumbo nucifera	Nelumbonaceae	Embryos	Neferine	Liver cancer (*In vitro*)
Ocimum sanctum	Lamiaceae	Leaves	Caryophyllene, camphor	Ssarcoma-180 solid tumor (*In vitro*)
Calvatia caelata	Agaricaceae	Fruiting bodies	Calcaelin	Breast and spleen cancer cells (*In vivo*)
Pleurotus sajor-caju	Agaricaceae	Fruiting bodies	Ribonucleases	Leukemia and liver cancer (*in vivo*)
Lentinus edodes	Marasmiaceae	Fruiting bodies	Lentinan	Sarcoma-180 in mice (*In vivo*)
Schizophyllum commune	Schizophyllaceae	Fruiting bodies	Schizophyllan	Head and neck cancer (*In vivo*)
Matricaria chamomilla	Asteraceae	Whole plant	Apigenin	Colorectal cancer (*in vivo*)
Fagopyrum sculentum	Polygonaceae	Seeds	Buckwheat inhibitor-1 protein	T-acute lymphoblastic leukemia (T-ALL) cells (*in vitro*)
Glycine max	Fabaceae	Seeds	Soybean trypsin inhibitor	Human ovarian cancer (*in vivo*)
Ipomoea batata	Convolvulaceae	Roots	Trypsin inhibitor protein	Promyelocytic leukemia cells (*In vitro* and *in vivo*)
Lavatera cashmeriana	Malvaceae	Seeds	Lavatera cashmeriana protease inhibitors (LC-pi I, II,III)	Leukemia, lung, colon cancer (*In vitro*)
Lens culinaris	Fabaceae	Seeds	Lentil (Lens culinaris trypsin inhibitor)	Human colon cancer (Both *in vitro* and *in vivo*)
Medicago scutellata	Fabaceae	Seeds	Medicago scutellata trypsin inhibitor	Human breast and cervical cancer (*In vitro*)
Phaseolus acutifolius	Fabaceae	Seeds	Tepary bean protease inhibitor	Leukemia L1210 and lymphoma MBL2 (*In vitro*)
Pisum sativum	Fabaceae	Pea	Protease inhibitors, rTI1B, rTI2B	Human colorectal and colon cancer (*In vitro*)
Phaseolus vulgaris	Fabaceae	Seeds	Tepary bean protease inhibitor	Leukemia L1210 and lymphoma MBL2 (*In vitro*)
Coccinia grandis	Cucurbitaceae	Leaves	(CG) protease inhibitors	Colon cancer (*In vitro*)
Ginkgo biloba	Ginkgoaceae	Leaves	EGb and bilobalide	Colon cancer (*In vivo*)
Curcuma zedoaria	Zingiberaceae	Whole plant	Curcumin	Colorectal cancer and B-16 melanoma cells (*In vitro*)
Clematis manshrica	Ranunculaceae	Flower, Leaves	1,4-benzoquinone,5-*o*-ethyl- embelin, 15-carbon isoprenoid	Liver cancer and blood cancer (*In vivo*)

Plate 2.4: Some important medicinal plants and their important phytochemicals against specific type of cancer (Iqbal *et al*., 2017).

2.6 *Majorana hortensis:* AN UPCOMING PRODIGY IN CANCER RESEARCH

Majorana hortensis is a perennial herb commonly known as 'majoram' belonging to the *Laminaceae* family. It is an aromatic plant and is used for culinary purposes due to its aroma. The plant extract has several therapeutic uses like curing digestive disorders, treating fever, also, used as an expectorant. It is also found to have free radical scavenging activity (Palaniswamy and Padma, 2011a).

The use of *Majorana hortensis* leaf extracts enables as an effective candidate to be employed in all medicinal preparations used to combat lipid peroxidation which in turn reduces the risks of chronic diseases and prevents generation of free radicals (Palaniswamy and Padma, 2011a).

The methanolic extract of *Majorana hortensis* is effective in protecting the liver tissue from oxidative stress and damage (Palaniswamy and Padma, 2018).

Majorana hortensis proposes a greater challenge in exhibiting anticancerous activities in the field of cancer research and is expected to have anti-angiogenic property that aids metastasis.

Plate 2.5: Leaves of *Majorana hortensis*

CHAPTER THREE

EXPERIMENTAL PROCEDURE

3. EXPERIMENTAL PROCEDURE

The present study deals with the effect of *Majorana hortensis* on cell viability, motility and angiogenesis using Chick Embryo Fibroblasts (CEF) and triple negative breast cancer cells (MDA-MB-231).

PREPARATION OF *Majorana hortensis* LEAF EXTRACT

The plant sample was collected from the local areas of Coimbatore and was authenticated by BSI.

About 5g of leaves of *Majorana hortensis* was weighed and cut into fine coarse bits. It was then added with 50 ml of methanol in a clean dry beaker. It was kept at 4ºC for 72 hours with occasional shaking. The extract was kept in boiling water bath at 60ºC and the methanol was allowed to evaporate. The dried beaker with the residue is dissolved in DMSO.

Plate 3.1: Methanolic extract of *Majorana hortensis*

ESTABLISHMENT OF PRIMARY CULTURE – CHICK EMBRYO FIBROBLASTS

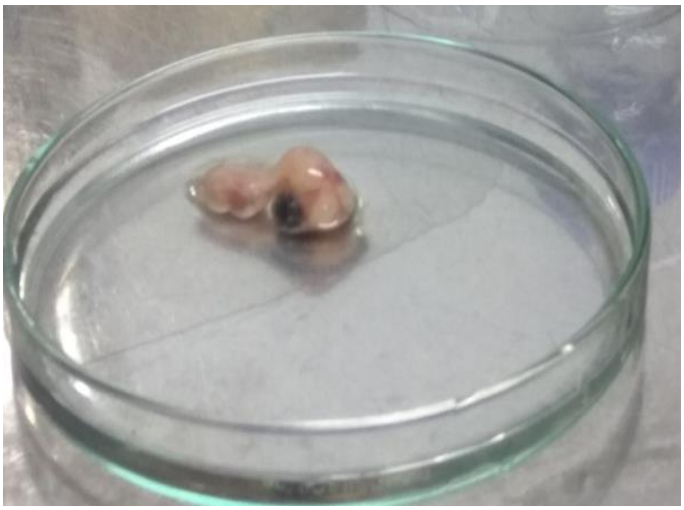
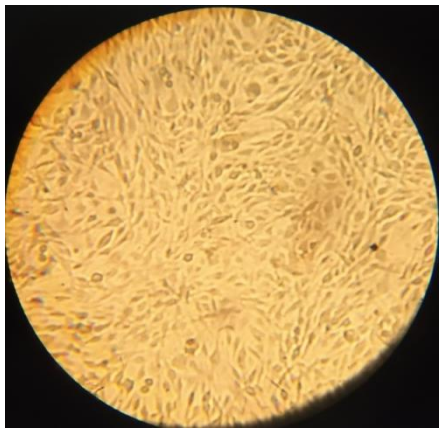


Plate 3.2: Chick Embryo

Primary cells, comprised of cells that are derived from embryonic tissue, play a major role in the preclinical drug development process. A primary culture is that stage of the culture after isolation of the cells but before the first subculture. The culture of primary CEF was established as described in Appendix I.

CULTURING OF BREAST CANCER CELLS

The triple negative breast cancer (MDA-MB-231) cells cultured under aseptic conditions (Appendix II) is used for the study



. **Plate 3.3: Breast cancer cells (MDA-MB-231)**

A wound is scratched on the cell line and the treatment was given as described in Table 3.1.

SL. NO.	TREATMENT GROUPS	
	CHICK EMBRYO FIBROBLASTS (CEF)	**MDA-MB-231**
1.	CEF alone	Cells alone
2.	CEF + Etoposide	Cells + Etoposide
3.	CEF + *Majorana hortensis*	Cells + *Majorana hortensis*
4.	CEF + *Majorana hortensis* + Etoposide	Cells + *Majorana hortensis* + Etoposide

Table 3.1: SET UP OF TREATMENT GROUPS

Etoposide is a topoisomerase II inhibitor and a chemotherapeutic agent widely used for cancer therapy. Low-dose etoposide has potent anti-tumor activity in patients (Panigrahy *et al*., 2010).

3.1 CELL VIABILITY

3.1.1 MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay

MTT assay was performed to assess the viability of the Chick Embryo Fibroblasts of the different treatment groups as described by Igarashi and Miyazawa (2001). The protocol was followed as explained in Appendix III.

3.1.2 SRB (Sulforhodamine B) assay

The SRB assay was employed to determine the cell viability of the CEFs as explained by Shekan *et al*., 1990. Cell survival was measured in all the different treatment groups employed. The assay was performed as carried out in Appendix IV.

3.2 Effect of *Majorana hortensis* leaf extract on angiogenesis as determined by Chorioallantoic Membrane (CAM) assay

CAM assay was carried out as described by Staton *et al*., 2004 and the progression of angiogenesis was observed. The process was carried out as described in Appendix V.

3.3 Effect of *Majorana hortensis o*n cell migration as determination by scratch assay using breast cancer cells

The anti-apoptotic potential of the methanolic extract of *Majorana hortensis* leaves was studied under *in vitro* condition by scratch assay according to the procedure of Liang *et al*., 2007 which is described in Appendix VI.

CHAPTER FOUR

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Cancer is defined as a major health issue all over the world, which leads to progressive erosion of interactions between multiple activating and deactivating biological processes (both immune and non-immune) of host tissue, resulting in progressive loss of integrity of susceptible tissues (Rahal *et al*., 2014). Cancer is an enormous burden on the society, afflicting all countries alike. According to the reports of the International Agency for Research in Cancer, the incidence of cancer will increase from 1 million in 2012 to more than 1.7 million in 2035 (Bray *et al*., 2013).

The formation of neovasculature from pre-existing cells is called angiogenesis which is a crucial event in during malignant tumor progression. It provides oxygen and nutritional requirements to the growing tumor and thereby creating a pathway for tumor cell metastasis to distant organs. Tumor growth and angiogenesis take place within a complex host microenvironment which comprise vascular endothelium, immune cells, fibroblasts and other cell types (Asano *et al*., 2017).

The chick embryo has served a workhorse for experimental embryological studies designed to elucidate various mechanism. Early chick embryos developing in whole-embryo culture can be readily manipulated and it makes it a better model system (Schoenwolf, 2018).

Cancer cell lines differ greatly differ in their sensitivity to anticancer drugs as a result of different oncogenic drivers and drug resistance mechanisms have been discovered, it remains a challenge to understand how they interact to render an individual cell line sensitive or resistant to a particular drug. Breast cancer cell lines have been used for various purposes such as mutations, copy number aberrations, mRNA, protein expression and protein phosphorylation in the presence and absence of the drug (Jastrzebski *et al*., 2018).

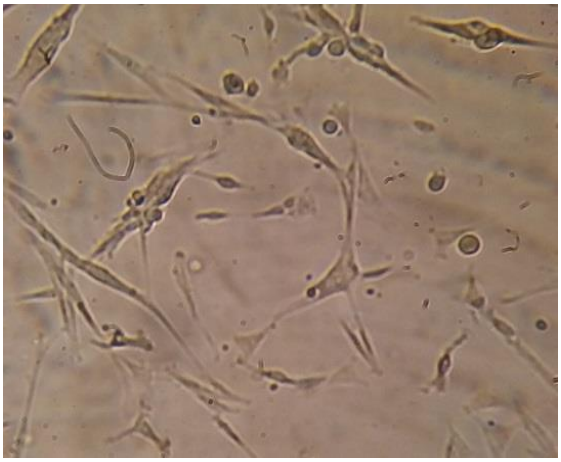


Plate 4.1: Chick Embryo Fibroblasts (CEF)

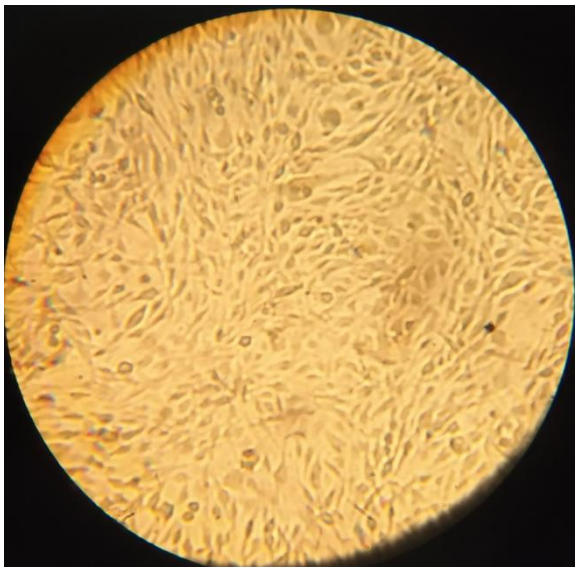
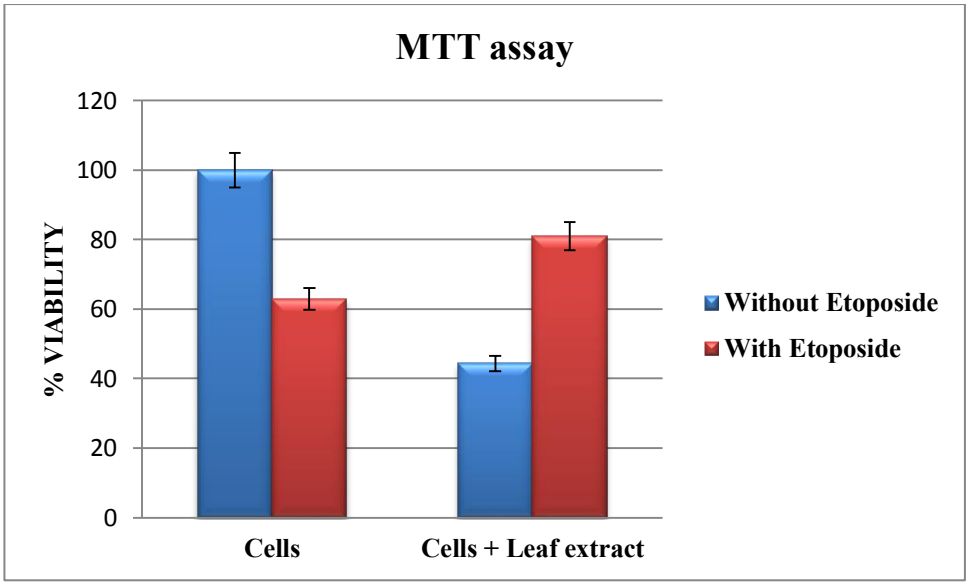


Plate 4.2 Breast cancer (MDA-MB-231) cells

In the present study, the effect of the leaf extract of *Majorana hortensis* on cell viability and angiogenesis was accessed using Chick Embryo Fibroblasts and the TNBC cell lines in the presence and absence of etoposide. The results obtained are as discussed below.

4.1 EFFECTS OF *Majorana hortensis* LEAVES ON CELL VIABILITY IN CEF:

The influences of the methanolic leaf extract of the plant *Majorana hortensis* on Chick Embryo Fibroblasts' survival were determined by quantifying the percent cell viability using MTT and SRB assays. The extent of viability in the different treatment groups are presented in the Figure 1 and Figure 2.



Values are expressed as Mean ± S.D of triplicates

The values of the untreated control group were fixed as 100% and the percent viabilities in the other groups were calculated relative to this.

Figure 4.1: Effect of *Majorana hortensis* on the viability of CEFs as determined by MTT assay.

The values of the untreated control group were fixed as 100% and the percent viabilities in the other groups were calculated relative to this.

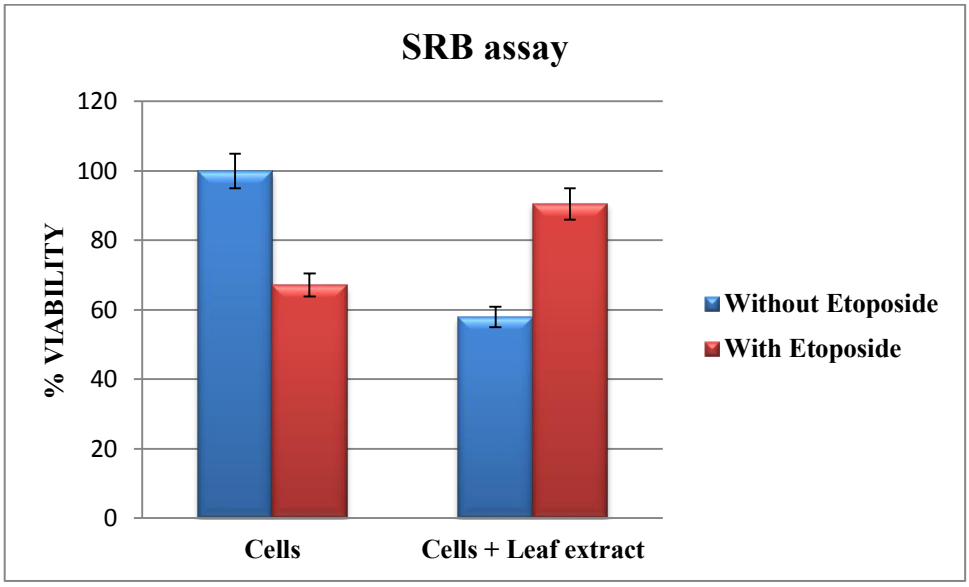
Exposure to the treatment groups had significant effect on the cell viability of CEFs. The cells showed 62% viability when exposed to the methanolic extract of *Majorana hortensis* alone. In the presence of etoposide alone, the cell viability was significantly decreased, which was improved on co-treatment with methanolic extract of *Majorana hortensis*. Thus, the results obtained shows that *Majorana hortensis* works satisfactorily in combination with etoposide and the cell cytotoxicity was tremendously reduced.

Using MTT assay, Badgujar *et al*., 2018 determined the anti-proliferative effect of methanol extract of *Butea monosperma* leaves on A-549 human lung carcinoma cells and chick embryo fibroblasts.

Kiruthika *et al*., (2013) confirmed the protective effect of the methanolic extract of *Zea mays* leaves against H_2O_2-induced oxidative stress in primary cultured chick embryo fibroblasts.

Using MTT assay, Suprapto Ma'at (2012) examined the cytotoxicity of Betel leaf extract (BLE) against primary culture of chicken embryo fibroblasts.

Yasin and Ahmet (2014) determined the mutagenic and cytotoxic effects of aqueous extracts of the roots, stems and leaves of *Limonium globuliferum Kuntze*, *Plumbaginaceae*, using MTT assay in MDBK (Madin-Darby Bovine Kidney) cells.



Values are expressed as Mean ± S.D of triplicates

The values of the untreated control group were fixed as 100% and the percent viabilities in the other groups were calculated relative to this.

Figure 4.2: Effect of *Majorana hortensis* on the viability of CEFs as determined by SRB assay.

The results obtained using SRB assay showed a similar trend as the MTT assay. Treatment with etoposide significantly decreased the cell viability, wherein co-treatment with both *Majorana hortensis* and etoposide improved the cell viability. This implicates that the leaf extract of *Majorana hortensis* has protective effect against etoposide-induced cytotoxicity.

Thus, from the results obtained from both the MTT and SRB assays infers that the etoposide-induced cytotoxicity of the non-transformed cells was remarkably reduced and the viability was improved in the presence of *Majorana hortensis* leaf extract.

Similarly, previous studies have showed that different extracts of *Majorana hortensis* leaves rendered good protection against H_2O_2-induced apoptosis in *Saccharomyces cerevisiae* cells, among which methanolic extract showed maximum protective effect (Palaniswamy and Padma, 2011).

In a similar study by Decleer *et al* (2018), MTT and SRB assays were used for characterization of the cell viability and cellular protein content of Caco-2 and HepG2 cells after exposed to CER. Meanwhile, SRB assay was used for the measurement of cellular protein content.

Vajrabhaya and Korsuwannawong (2018) evaluated the cytotoxic effect of Thai herb on cultured cells. The main aim of the study was to evaluate the cytotoxicity of a Thai herb by comparing MTT and SRB assay results. The assays were performed similarly, exhibiting moderate to excellent correlation in the evaluation of the cytotoxicity of a Thai herb.

4.2 EFFECT OF *Majorana hortensis* LEAF EXTRACT ON ANGIOGENESIS AS DETERMINED BY CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

The CAM assay is a robust technique that can be used to monitor invasion of cancer cell lines and to assess the role of noel molecules and potential therapeutic targets (Lokman *et al*., 2012).

The outermost extra-embryonic membrane refers to the chick embryo Chorioallantoic membrane which is highly vascularized for gaseous exchange calcium transportation between the embryo and its microenvironment. It provides a technically simple way of studying complex biological systems with well-developed vascular tissues. The CAM model is recognized as an intermediate model that can bridge the gap between cell-based and animal-based assays; other than showing similar patterns of cellular toxicity as *in vitro* models (Kue *et al*., 2015).

The angiogenic material is usually introduced in the form of small disks soaked in angiogenic factors and impregnated with tumor cells. Another traumatic way to introduce angiogenic material onto CAM involves the use of shell-less embryos grown *ex ovo,* which makes the CAM more accessible for repetitive

manipulations, for quantification of angiogenesis, and for direct visualization of the angiogenesis process (Deryugina and Quigley, 2009).

A window was made on the eggshell after the blood vessels are clearly visible through candling. The sample was loaded on the Whatman filter paper and placed through the window on the blood vessels. Angiogenesis was measured after 72 hours of treatment. The formation of the blood vessels determines the angiogenesis. The inhibition the blood vessels formation determines the anti-angiogenic activity of the methanolic extract of *Majorana hortensis*.

The extent of blood vessels formation in chick embryo manually by counting the number of primary vessels and vessels formed from the primary branches manually and compared between the treatment groups. The results obtained were depicted in plate 4.2 to 4.5.

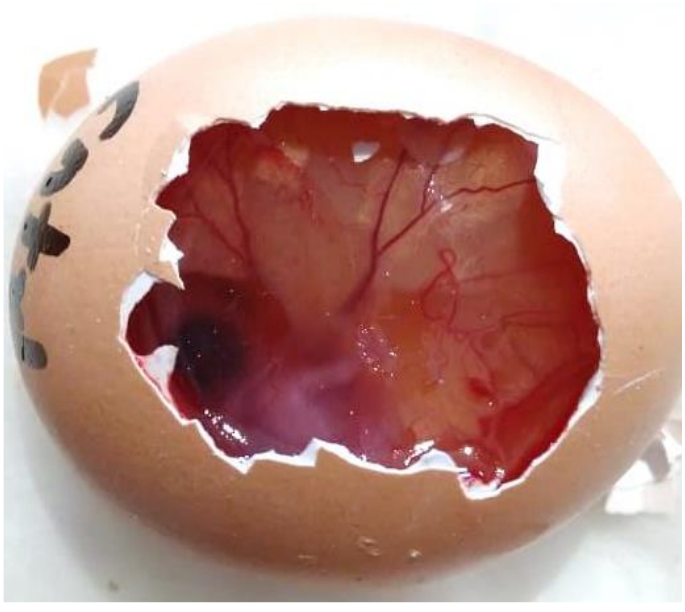


Plate 4.3: Blood vessels in chick embryo in control group

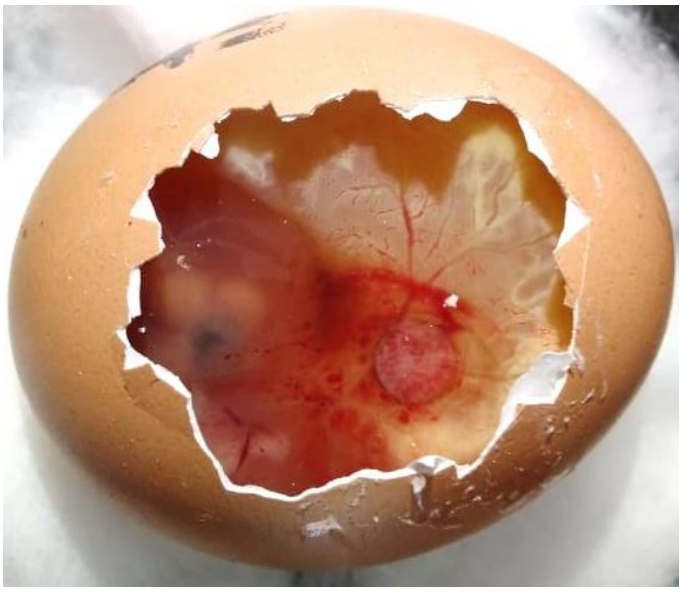


Plate 4.4: Blood vessels in chick embryo when exposed to Etoposide

Plate 4.5: Blood vessels in chick embryo when exposed to *Majorana hortensis*

Plate 4.6: Blood vessels in chick embryo when exposed to *Majorana hortensis* + Etoposide

Many studies have used CAM assay to study angiogenesis. The extent of blood vessel formation in untreated chick embryo exhibited a normal pattern of angiogenesis whereas the blood vessel formation of chick embryo treated with etoposide and *Majorana hortensis* was significantly reduced, indicating the anti-angiogenic potential of the *Majorana hortensis* leaf extract.

Using chick CAM model, Kota *et al*., 2018 reported that the new pharmacological effects of *Nigella sativa* have been confirmed by the proven inhibition of angiogenesis. The authors showed that *Nigella sativa* seed extract had significant anti-angiogenic activity at all doses of the treatment studied.

Bashir and Qadir, (2017) had showed that different doses of ginger extract exhibited anti-angiogenic effect on eggs with developing embryo as indicated by the reduction in primary and secondary blood vessels. Tertiary blood vessels were also found to be decreased in the CAM area.

Eva *et al*., 2014 had found that ethanolic leaf extract of *Antidesma bunis* has anti-angiogenic activity when tested on duck embryo using CAM assay. They had reported that 40% concentration had a greater anti-angiogenic activity in terms of

the decrease number of blood vessels developed against positive control, Bevacizumab.

Shah *et al*., 2018 showed that the effect of D-Limonene on angiogenesis using the CAM assay. They evaluated that there was a reduction in the number of blood vessels on CAM when D-Limonene was applied at 10µg per implant.

Thus, the CAM assay was found to be a very powerful tool to study the angiogenic factors and their role in angiogenesis. The method provides an overall highly efficient and cost-effective way to study the phenomenon of the angiogenesis regarding the basic mechanism and drug for angiogenesis-based therapies (Naik *et al*., 2018).

4.3 EFFECT OF *Majorana hortensis* on CELL MIGRATION AS DETERMINATION BY SCRATCH ASSAY USING BREAST CANCER CELLS

In the present study, triple negative breast cancer (MDA-MB-231) cells were used to determine the effect of *Majorana hortensis* leaf extract on cell motility using scratch assay. Using microscopic image analyzer, the extent of closure of the gap in the confluent cell monolayer was studied after 24 hour treatment period with *Majorana hortensis* both in the presence and absence of etoposide.

The experimental results obtained showed that in untreated control group, a significant mobilization of breast cancer cells was observed as evident from the closure of the gap (plate 4.6 and 4.7), whereas in *Majorana hortensis* treated group, a significant inhibition of cell motility was found both in the presence and absence of etoposide (plate 4.8 - 4.14). Thus, the results showed that the methanolic extract of *Majorana hortensis* extract exhibit anti-angiogenic effect on the breast cancer cells.

Plate title: Photomicrograph of MDA-MB-231 cells before and after treatment to determine the mobilization of cancer cells

Before treatment **After treatment**

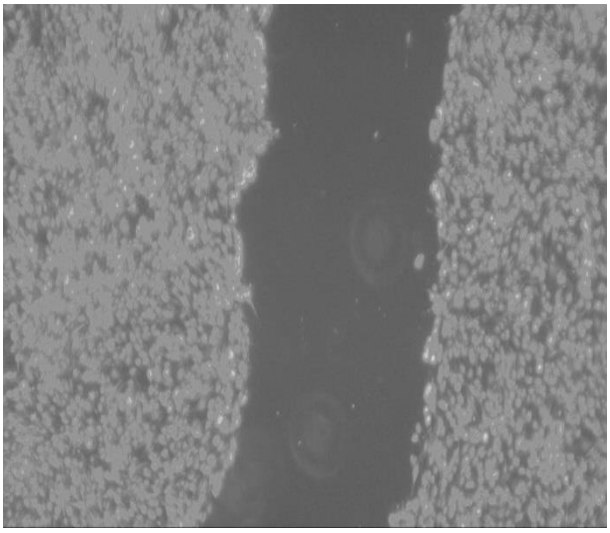


Plate 4.7: Cells alone

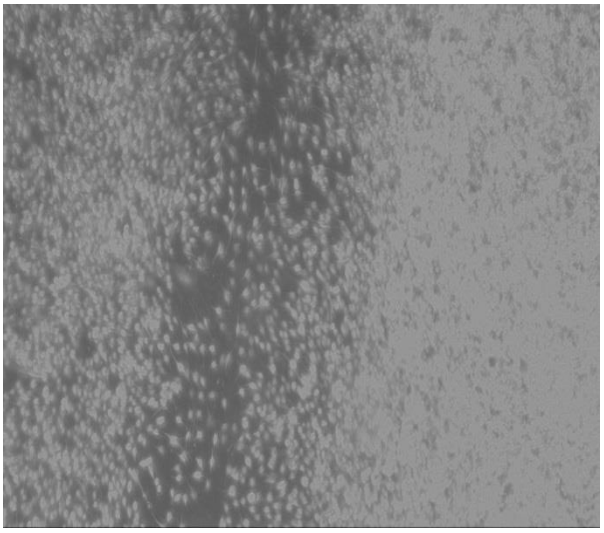


Plate 4.8: Cells alone

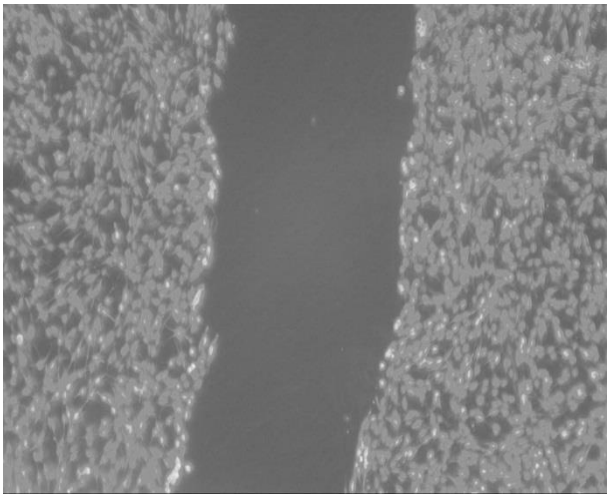


Plate 4.9: Cells + Etoposide

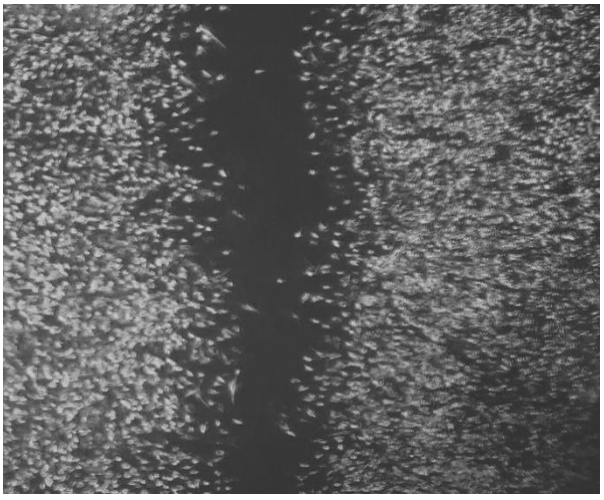


Plate 4.10: Cells + Etoposide

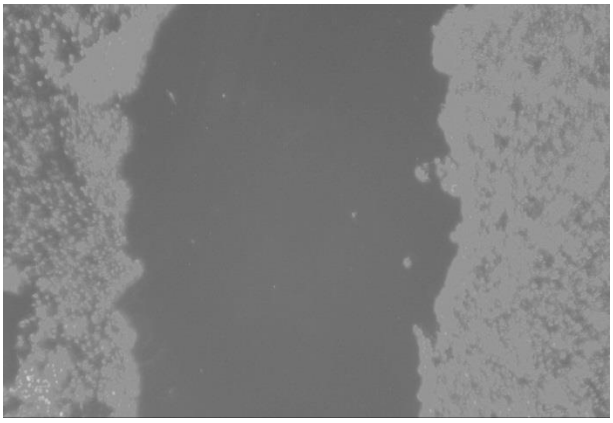


Plate 4.11: Cells + *Majorana hortensis*

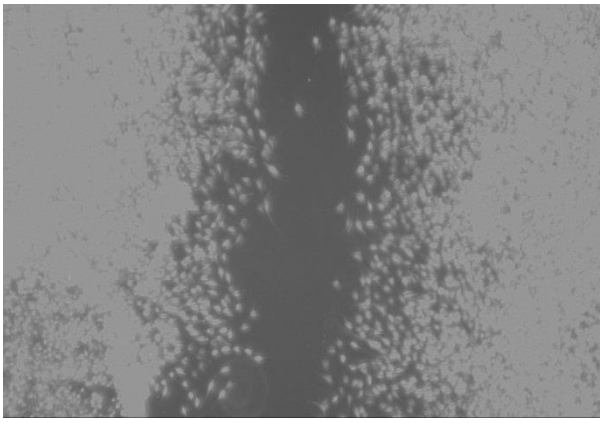


Plate 4.12: Cells + *Majorana hortensis*

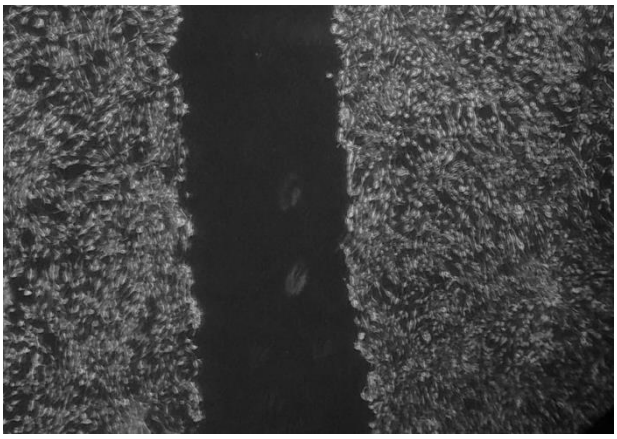


Plate 4.13: Cells + *Majorana hortensis* + Etoposide

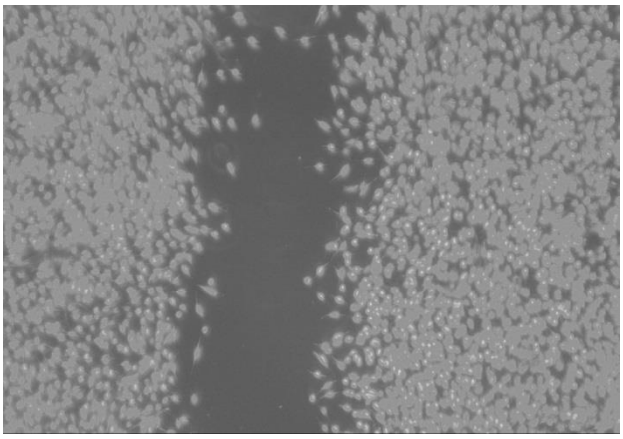


Plate 4.14: Cells + *Majorana hortensis* + Etoposide

Mansoori *et al*., 2017 demonstrated that the extract of *Urtica diocia* could exhibit cytotoxic effect and also inhibit breast cancer cell migration using MTT and scratch assay.

Using MTT and scratch assays, Chowdhury *et al*., 2017 indicated that the ethanol and acetne ectract od colocynth fruit pulp reduced cell viability and inhibited cell migration of breast cancer (MCF-7, MDA-MB-231) and cervical cancer (SiHa) cells.

Similarly, Ranjbarnejad *et al*., 2015 also reported the cytotoxic and anti-angiogenic potential of the methanolic extract of *Boswellia serrate* in human colon cancer (HT-29) cell using MTT and scratch assays.

Similarly, Zubair *et al*., 2012 reported that the wound healing property of aqueous and polyphenol-rich ethanol-based extracts of *Plantago* mIajor leaves using scratch assay on oral epithelial cells.

According to Balekar *et al*., 2012, *Wedelia trilobata* leaves are used in the treatment of wounds. WEA promoted fibroblast L929 survivability up to more than 90% before and more than 85% after hydrogen peroxide induced oxidative stress. WEA induced a 70% migration rate in the *in vitro* scratch assay.

Using the scratch assay, Fronza *et al*., 2009 reported that the *Calendula officinalis* extracts stimulated proliferation and migration of fibroblasts at low concentrations. Inhibition of proliferation showed that this effect is mainly due to stimulation of migration. Faradiol myristate and palmitate gave comparable stimulation rates indicating that they contribute partially, but not most significantly to the wound healing effects of *Calendula* preparations.

In accordance with these observations, the results obtained from the present study confirmed the anti-angiogenic potential of the *Majorana hortensis* leaf extract on breast cancer (MDA-MB-231) cells as infered from the inhibition of cell migration.

CHAPTER FIVE

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Angiogenesis is an inevitable process of tumor growth, invasion and metastasis. Upregulation of angiogenesis has been reported in various pathological conditions such as chronic inflammation, rheumatoid arthritis, atherosclerosis. Hence, understanding the process of new blood vessel formation have gained prime importance recently and anti-angiogenic therapy has now been considered as an important strategy for cancer therapy.

In recent years, various bioactive compounds especially of plant origin have been explored for their anti-angiogenic potential. As a part of this search for natural product-based anti-angiogenic agents, the present study was formulated to evaluate the effect of *Majorana hortensis* leaf extract on angiogenesis, in an effort to examine its influence against cancer cell proliferation, invasion and metastasis, using CEFs and breast cancer cells.

Cell viability analysis in chick embryo fibroblasts showed that *Majorana hortensis* leaf extract alone exhibited slight cytotoxicity as assessed by MTT and SRB assay. However, in combination with etoposide, the leaf extract exhibited a synergistic effect as evident from the improved cell viability.

Using CAM assay, the anti-angiogenic potential of the *Majorana hortensis* leaf extract was demonstrated from by the significant reduction in blood vessels formation. This observation was further substantiated by scratch assay using breast cancer (MDA-MB-231) cells, wherein the leaf extract inhibited the migration of tumor cells. Thus, these findings showed that the leaves of *Majorana hortensis* possess significant anti-angiogenic potential. Further research need to be carried out to validate the anti-angiogenic effect of the leaves by studying their effect on cell-tube formation, cell proliferation and invasion in transformed cells.

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BIBLIOGRAPHY

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LIST OF APPENDICES

APPENDIX I

ESTABLISHMENT OF PRIMARY CULTURE – CHICK EMBRYO FIBROBLASTS

In order to establish fibroblasts, chick embryo has to be dissected and the cells have to be cultured. The following protocol was followed to establish chick embryo fibroblasts.

Materials

1. 70% (v/v) ethanol
2. Phosphate Buffered Saline (PBS)
3. Trypsin (1X) - commercially available
4. Minimum essential medium (DMEM)
5. Fetal calf serum
6. Pen-strep (100x) - commercially available

Procedure

The eggs were pre-cleaned and wiped with ethanol. The egg was held with broad side up and tapped the shell gently with scalpel holder all around the broad side of the egg to allow the shell to crack uniformly, thus exposing the underlying membrane. With a second pair of sterile scissors, carefully cut away and removed the chorioallantoic membrane, exposing the embryo. The embryo was identified and carefully removed using a sterile metal hook or a bent glass rod, and placed the embryo in a 100mm Petri dish containing phosphate buffered saline (PBS). It was then washed several times with PBS by transferring the embryo to fresh petriplates. After removal of all yolk and/or blood, moved the embryo to a clean dish with PBS. By using two sterile forceps, the head, limbs and viscera were removed. The entire limb was removed by pulling at the proximal end. The remaining tissues of the embryo was moved to yet another dish and washed with PBS. The embryo was minced finely with scissors and transferred the minced tissue to a flask containing PBS. The tissue pieces were allowed to settle and removed the PBS with a sterile pipette and added 25ml of trypsin, a proteolytic enzyme. The solution was stirred gently at 37ºC for 15-20 minutes.

The larger, undigested tissue pieces were allowed to settle and decanted the supernatant into an equal volume of Minimal Essential Medium (MEM) +

10% Fetal Calf Serum (FCS). FCS contains protease inhibitors which will inactivate the trypsin. The cells were centrifuged in DMEM at 1000rpm for 10 minutes in a standard clinical centrifuge. The supernatant was removed and resuspended the pellet in 25ml of fresh MEM + 10% FCS and removed 2µl of the culture to determine the viability. Two 25cm^2 plastic culture flasks were seeded with 5ml of MEM + 10% FCS to a final concentration of 105 cells / ml. The cultures were labelled and placed in the tissue culture incubator at 37ºC and examined daily for cell density and morphology. The cultures were examined using an inverted phase contrast microscope (Gurney .T and Jr., 1969).

APPENDIX II

CULTURING OF TRIPLE NEGATIVE BREAST CANCER CELL LINE (MDA-MB-231)

Materials

1. DMEM
2. 10% FBS
3. Trypsin-EDTA

Procedure

The cell line was procured from National Centre for Cell Science, Pune, India. The cells were maintained in a CO_2 incubator with 5% $CO_2$and 95% humidity, supplemented with DMEM and 10% FBS. Penicillin and streptomycin (PAA) were also added to the medium to 1X final concentration from a 100X stock. Once the cells had attained confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA) and 10^5 cells required for various assays was seeded into sterile 6-well and 96-well plates. A sterile coverslip was placed in each well of the 6-well plate before the cells were seeded. The plates were then incubated in a CO_2 incubator with 5% CO_2 and 95% humidity atmosphere.

The cells were treated with the oxidant, in the presence and the absence of the flower extract. The exposure of etoposide was given for 24 hours at 37ºC. The time point was arrived at by conducting a time related response analysis of each cell type. After treatment, the medium in the 96-well plates was replaced with fresh medium and were used for cell viability assays. For staining analysis, the coverslips from the 6-well plates were removed and placed on a glass slide, sealed with Vaseline and were used for various staining techniques.

CELL VIABILITY

APPENDIX III

MTT dye reduction assay

The MTT cell viability assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, there is reduction in the cell viability. This colorimetric assay that determines the reduction of yellow [3-(4,5-dimethyl-thiazol-2-yl)-2,5-biphenyltetrazolium bromide] by mitochondrial succinate dehydrogenase in actively growing cells to produce a purple formazan derivative. This derivative is dissolved in a suitable solvent like isopropanol and measured in an ELISA reader.

Reagents:

1. PBS
2. MTT
3. Acid-propanol (isopropanol containing 0.4% 0.04N HCl
4. HCl (0.04N)

Procedure:

To 100µl of cells, added 50µl of MTT and incubated at 37°C with mild shaking for 3 hours. Later the incubation period, 200µl of PBS was added to all the samples and the liquid was gently aspirated. 200µl of acid-propanol was added and left overnight in the dark. The absorbance was read at 650nm in ELISA reader. The viability of the untreated cells was fixed at 100% and the percent viability of the other treatment groups were measured accordingly.

APPENDIX IV

SRB assay

The assay is based on the ability of the protein dye sulforhodamine B to bind electrostatically and pH dependent on protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions, it binds to and in mild basic conditions it can be extracted from cells and solubilized for measurement. The absorbance was measured at 564nm in an ELISA reader.

Reagents:

1. Ice cold 40% TCA
2. SRB
3. 1% acetic acid
4. 10mM Tris

Procedure:

The treated cells were layered with 350µl of ice cold 40% TCA and incubated at 4°C for 1 hour. At the end of incubation, it is washed 5 times with cold water. 350µl of SRB stain was added to all the samples and incubated at room temperature for 30 minutes. The cells were then washed with 1ml portions of 1% acetic acid. Further, 350µl of 10mM Tris (pH10.5) was added to solubilize the dye and the samples were shaken gently for 20 minutes. The absorbance was measured in a 96 well plate in a microtitre plate reader at 492nm. Cell survival was measured as the percentage absorbance.

APPENDIX V

EFFECT OF *Majorana hortensis* LEAF EXTRACT ON ANGIOGENESIS AS DETERMINED BY CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

The progression of angiogenesis can be observed using CAM assay. The eggs were candled and checked for the blood vessels and the window was carved on the shell and the treatment groups were established. The angiogenesis progression on the eggs was observed.

Reagents:

1. 70% ethanol
2. Saline
3. Methanol

Procedure:

Fertilized chicken embryos were cleaned with ethanol and preincubated at 37.5 °C in 85% humidity for 2 days. Egg morphology feels like a meta-ellipse, with a relatively larger side and a smaller one and the air sac is normally located on the larger side right behind the shell. After medical aid of the shell centre outside the air sac with alcohol, a hole highlighted with marker pen was buffed and drilled gently over the air sac with a nipper to not break the shell, and the vascular zone was easy to be identified on the CAM. Two drops of normal saline were then added to moisten the inner shell membrane adjacent to the CAM so the membrane was straightforward to be separated from CAM.

After being clamped and raised by ophthalmic forceps, the membrane and the CAM separated unforcedly, and then a 1 x 1 cm window on the membrane was sectioned to expose the vascular zone. A 5mm x 5mm sterilized Whatman filter-paper disks that were used as a carrier for directly loading the indicated concentrations of chemicals, were then directly applied and cling to the vascular zone. Upon sealing the openings with sterile flexible packing film, the eggs were further incubated for indicated periods. Finally, a mixture of methanol and acetone (1:1 in volume) was directly added to immerse and fix the blood vessels of the experiment zone. After being clamped and raised by ophthalmic forceps, the CAM was easy to be separated from the embryo, and it was cut and spread on

glass slide, and the blood vessels were viewed, photographed and quantified by investigating the amount of vas branch points.

APPENDIX VI

EFFECT OF *Majorana hortensis* on CELL MIGRATION AS DETERMINATION BY SCRATCH ASSAY USING BREAST CANCER CELLS

A scratch was made on the confluent breast cancer (MDA-MB-231) cells. The treatment was given with the desired treatment groups. The wound healing was observed in regular time intervals.

Reagents:

1. DMEM
2. FBS
3. Antibiotics (Penicillin and Streptomycin)

Procedure:

The scratch assay was performed using Chick Embryo Fibroblasts (CEFs). They were maintained under standard laboratory conditions in DMEM supplemented with 10% FBS and penicillin-streptomycin and grown in a 5% CO_2 and 95% humid atmosphere. A scratch was made in the confluent culture in a straight line with a sterile micropipette tip. The debris was removed and the edge of the scratch was made smooth by washing the culture with 1.0 ml of DMEM. A volume of 5.0ml of DMEM supplemented with serum and antibiotics was then added. The methanolic extract was then added to the medium and dispersed gently. Markings were made close to the scratch using an ultrafine tip marker, to use a reference point to obtain the same field during image acquisition. Images of the scratch were taken by placing the culture dish under a phase-contrast microscope, followed by incubation of the dish in the CO_2 incubator at 37°C for 12 hours. At regular intervals, the culture dish was taken out and the images were captured for the control and the treated groups under a phase contrast microscope matching the reference points.