Molecular Insights into the Metastasis of Human Breast Cancer Cells and its Intervention by Embelin

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Nupur NIGAM

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Nupur NIGAM

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1. Abstract

Human Breast Cancer, despite improved treatment and surgical resection stakes millions of lives. Chemotherapy drugs provide an option for cancer treatment, but the complication arises when these drugs target nearly every quickly multiplying cells in the body. This, in turn also targets the non- carcinoma that is normal cells in the body of the patient, leading to various side-effects such as bleeding, low-immune response, hair loss, fatigue, nausea, hot flushes, mood swings, infertility, hormone- imbalance, to name a few. Moreover, very few of the chemotherapy drugs target metastatic cancer cells, making the relapse of cancer more accountable, and also difficult to treat metastatic cancer through chemotherapy drugs. Natural chemicals are gaining increased limelight in the field of cancer research, due to their increased bioavailability and non-harmful nature. Embelin is a type of quinone derivative anti - cancer drug. It occurs commonly in Embelia ribes Burm. f. (Primulaceae) fruit. The therapeutic properties of Embelin include Anti – Diabetic, Anti – Inflammation, Anti – Bacterial, Anthelmintic, and Anti – Tumor. As immune – related body reaction, inflammation leads to generation of multiple regulatory cytokines, a key cellular cytokine TNFα. TNFα generation is reported to perform pivotal functions in human pathologies such as inflammation, neuro-degeneration and cancer development. Various studies have reported Embelin showing its anti- inflammatory activity by reducing TNFα. TNFα is present as a cell-membrane bound protein, proactive form TNFα (inactive form); the active unit of inactive proenzyme -TNFα is then secreted by the cell, followed by cleavage by enzyme termed as TACE (TNF – alpha Converting Enzyme). Therefore, TACE is considered a key biomolecules for targeting inflammatory responses and progression of cancer. In this study, molecular dynamic studies and in – vitro experimental techniques were used to understand the efficiency of docking and biochemical effects on TACE of Embelin, its effect on breast cancer cell line characteristics. I illustrate here that Embelin can prove as a promising negative regulator of TACE. Moreover, in vitro experiments explained that it hinders invasive potential of cancer cells by down-regulation of metastasis targets including MMPs, VEGF, mortalin, TGF-β and hnRNP-K in human breast cancer cell lines.

2. Abbreviations

BSA	:	Bovine Serum Albumin
BRCA1	:	Breast Cancer 1
BRCA2	:	Breast Cancer 2
DMEM	:	Dulbecco's Modified Eagle Cell culture Medium
DNA	:	Deoxyribonucleic Acid
DMSO	:	Dimethyl sulfoxide
ECL	:	Enhanced Chemiluminescence reagent
FBS	:	Fetal Bovine Serum
FGF-10	:	Fibroblast growth factor -10
HSP	:	Heat Shock Proteins
hnRNP-K	:	HNRNPK heterogeneous nuclear ribonucleoprotein K
MMP	:	Matrix Metalloproteinase
μL	:	Micro – liter
mL	:	Milli – liter
mM	:	Milli – Molar
μM	:	Micro – Molar
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
PVDF	:	Poly Vinylidene Fluoride
RNA	:	Ribonucleic acid
SDS	:	Sodium dodecyl sulfate
TGF-β	:	Transforming growth factor beta
TACE	:	Tumor necrosis factor-alpha converting enzyme
TNF-α	:	Tumor necrosis factor-alpha

3. CHAPTER I

General Introduction

Cancer

Cancer defines a pathology where defected cells in the body multiply uncontrollably, which also capably form tumors in other body parts. Cancerous cells metastasize to distinct regions of patients' body by circulating system and lymphatic systems. Cancer begins to harm the host's health when the unhealthy cells multiply without control thus developing primary tumors (exceptionally, during leukemia where cancer hinders functioning of normal blood tissue due to development of large population of defected cells in blood).

Tumors that generate and grow may cause trouble with functioning of multiple systems in the body, such as nervous, circulatory and digestive system by releasing hormones. Tumors that are confined to an area and exhibit limited growth are generally called as benign. More invasive or malignant tumors form when two requirements are fulfilled:

1. A cancer cell manages to move to distant body locations using the circulatory or lymphatic systems, by damaging tissues that are healthy in a phenomenon termed invasion.
2. The cancer cell survives, multiplies and grows, developing new blood vessels to meet the energy requirements by a process called angiogenesis.

Metastasis can be explained as the process in which cancer cells transport through the circulatory or lymphatic system. Cancer cells possess an enhanced tendency to multiply extensively and the multiplication rate is independent to telomere length on DNA. (a division controlling mechanism in normal cells). The disease affects people at all ages, but the risk factor increases with age, due to the fact that DNA damage becomes more apparent in aging DNA.

Current Cancer Statistics

Cancer accounts for majority of deaths worldwide. In the year 2012, the world witnessed approximately 14.1 million new cancer cases: out of which 7.4 million (53%) in males and 6.7 million (47%) in females, giving a female: male ratio of 9:10. The World age – standardized incidence rate showed that there occurs 205 new cases for every 100,000 men in the world, and 165 for every 100,000 females. About 585,720 people, in the year 2014, were estimated to fall prey of cancer. Among men, prostate, lung, and colon cancers sum up to about half of all newly diagnosed cancers, with prostate cancer alone marking for about one in four cases. Women are mainly reported for breast, lung, and colon cancer, which accounts for most common among them. Cancer of the breast alone is expected to marks for 29% of all new cancers cases among women. (Figure 1-1)

Causes of Cancer

Cancer occurs as a result of cells that multiply without control and accumulate in patients' body. In general cases, healthy normal cells maintain a balanced path of growth, division, and programmed death. Apoptosis or Programmed cell death, is an important process and when this stops occurring in normal cells, cancer begins to form.

Due to Mutations, cancer cells undergo unbalanced growth and division, if there is DNA damage which result in mutations to the genes involved in control of cell division. Key families of genes that regulate cellular division are oncogenes (which guide the cells to multiply) and tumor suppressor genes (which function to stop cells from multiplication).

Due to exposure to Carcinogens or Cancer inducing agents, are a type of substances that are superiorly involved in DNA damage, promoting and enhancing cancer. Examples of carcinogens range from Asbestos, Arsenic

Tobacco, and radiations such as x-rays and gamma-rays, UV radiation from the sun, and also, compounds in car exhaust fumes.[1] Carcinogens exposure to human bodies release free radicals. These free radicals damage cellular functioning in the cells, hindering their ability to function normally.

Family history can be one more reason for cancer; Cancer can result from inherited genetic faults from members of same family. Chemical or toxic compound exposures can also lead to cancer occurrence. Chemicals such as Benzene, Asbestos, Nickel, Cadmium, Vinyl chloride, N-nitrosamines, tobacco or cigarette smoke contains multiple known potential carcinogenic chemicals and toxins.

Mechanism of Cancer

Cancer begins when cells in the host's body start to multiply uncontrollably. There are various kinds of cancers, but they all begin because of uncontrolled development of abnormal cells. Cancer cells grow differently than the normal cells. Instead of undergoing death, cancer cells continue division to form new, abnormal cells. Cancer cells are also capable of invading other tissues, a trait normal cell don't possess. Cells become cancerous due to presence of damaged DNA. In normal cases, when DNA is damaged in the cell, it is mainly rapidly repaired or the cell undergoes death. In cancer cells, the damaged DNA is not repaired and they don't die. Instead, the damaged cell continues making more damaged cells. This happens due to 2 major mutations occurring in the two very important classes of gene, the oncogenes and the tumor-suppressor genes.[2]

Tumor – suppressor genes and Oncogenes

Tumor – suppressor genes control cellular division and inhibit tumor growth and development. In most cancers, these genes lose their fuctioning, therefore thus enhancing cancer cellular proliferation as the regulators of cell division are not functional any more. During year 1971, Scientist Alfred Knudson explained the mechanism behind retinoblastoma development which requires 2 essential mutations in the Rb tumor – suppressor gene. Very prominent deletions of chromosome 13q14 were discovered in some retinoblastoma, bringing highlight that Rb gene inactivation initiates tumor growth and progression.

The 2nd major tumor – suppressor gene that discovered: p53, also frequently inactivated in a wide range of human cancers, including lymphomas, sarcomas, carcinomas of many tissues, including colon, lung, and breast and leukemia. Overall, mutations of p53 play a major role in development of more than 50% of cancers, exhibiting it as the most vulnerable target of genetic alterations in human malignancies.[3]

Like p53, *PTEN* and *INK4* tumor suppressor gene are also very commonly exposed to mutations in most cancers, such as, melanoma, prostate and lung cancer. Inherited mutations of 2 more tumor suppressor genes, *BRCA1* and *BRCA2*, holds capable in inherited cases of breast cancer. This marks for about 10% of total breast cancer cases. Opposite to proto-oncogene, proteins coded by tumor suppressor genes prohibit cellular growth and promote apoptosis of cancerous cells. Deactivation of these genes results in growth of tumor by ruling out inhibitory proteins.[4]

Studies on tumor viruses explained that certain genes (termed oncogenes) hold potential of stirring up cell transformation, thus bringing 1st facts into molecular basis of cancer. But, most of cancers of human origin are not caused due to viruses and are mainly arises from different factors, eg. Chemical carcinogens and radiation. The main bridge between cellular oncogenes and viruses was 1st

obtained by analysis of highly oncogenic retroviruses. DNA obtained from human bladder carcinoma could effectively cause tumorigenic transformation in recipient mouse cells by in – vitro tissue culture, highlighting that human tumors possess a functionally active oncogene. These assays of gene transfer along with other techniques have resulted in detection of active oncogenes in human tumors of multiple types. The 1st human oncogene identified was the human homolog of *ras*H oncogene of Harvey sarcome virus. 3 closely linked members of the ras gene family (*ras*H, *ras*K, and *ras*N) are oncogenes that are most commonly encountered in human tumors. These genes are found in average of 20% of all human malignancies, including 25% of lung carcinomas and 50% of colon cancer.

Oncogenes such as *Ras* do not occur normally in cells; however, they are developed in cancerous cells due to alterations that happen while tumor development. *Ras* oncogene is different than proto – oncogenes by single amino acid substitutions that occur as point mutations at significant positions. 1st such mutation found was a change of Valine for Glycine at position 12. Other amino acid changes at position 12, 61 and 13, are also discovered in ras oncogene of tumors of human origin. Study in animal systems depicted that mutations which convert ras proto-oncogenes to oncogenes are caused by chemical carcinogens, bringing a direct link between the mutagenic action of carcinogens and cell transformation.[5]

The gain-of-function of oncogenes and loss-of-function of tumor-suppressor genes are major steps in tumor initiation and progression. Collective damage to multiple genes eventually leads to enhanced cell division, invasiveness, and metastatic potential that are main features of cancer cells.

Treatments for Cancer

4 general methods for treatment of cancer include: surgery, chemotherapy, radiation therapy, and immunotherapy/biologic therapy. **Surgery: mainly performed** to remove cancerous tissue or as much of the as possible tumors. It is usually done together with chemotherapy or radiation therapy. **Chemotherapy:** utilizes anti-cancer drugs to eliminate cancer cells. Unlike surgery, chemotherapy effects the whole body of the patient, and not just a specific part. Its mechanisms involve killing fast rate diving cells. As other types of cells in human bodies also multiply at high rates, therefore, leading to occurrence of multiple side effects like fatigue, hair loss, and an ulcers. This kind of therapy is mainly given in the form of tablets or by intravenous injections (IV). **Radiation Therapy:** used to shrink the size of tumors and/or eliminate cancer cells. It functions by destroying cancer cell's DNA, making it unable to divide. Cancer cells are very sensitive to radiation and typically die when exposed. Nearby healthy cells are usually damaged as well in the process, but in most cases recover completely. **Biological or Targeted Therapy:** used for drugs whose mechanism of action focus on targeting unique characteristics of cancer cells. Few targeted therapies work by hindering the survival processes of tumors that allow tumors to multiply and develop. Other kinds cut out the blood – oxygen supply to cancerous tissue, causing it to starve and die because of a lack of nutrients. **Immunotherapy:** works to develop immune system to fight against the tumor. Local immunotherapy involves injecting a treatment into a specific area, for example, a mechanism to cause inflammation that makes tumor shrink. Systemic immunotherapy involves administering an agent into the whole body that can target shrink tumors throughout the body. **Autologous stem Cell Transplantation or Bone Marrow Transplant:** a most common method to treat leukemia and multiple myeloma. Bone marrow, which is made up of hematopoietic stem cells, is a spongy substance found inside large bones in the body, such as the femur, hips and ribs.

Breast Cancer

A large number of breast cancer cases are detected each year, making it stake multiple lives. (Figure 1-2)

2 medicines for the women with very high breast cancer risk are – raloxifene and tamoxifen. Raloxifene is seen to lesser side effects, such as blood clots and uterine cancer. Moreover, it used only in post – menopausal women. Tamoxifen, on the other hand is a drug used for several past, but it may lead to development of unwanted side – effects. Some common side – effects caused by tamoxifen are menstrual changes, redness of the face, neck, arms and occasionally, upper chest, irregular or absent periods, rattling breathing, decrease in the amount of urine, swelling of the fingers, weight gain or loss feeling of warmth, skin changes, stopping of menstrual bleeding, hands, feet, or lower legs, white or brownish vaginal discharge, troubled breathing at rest. Localized surgery such as those of conserving the breast or mastectomy (surgical removal of the breast). Lymph nodes from the Underarm area are usually checked in the process to analyze the degree of spread of cancer. Breast cancer therapies may include chemotherapy (before or after surgery), radiation, aromatase inhibitors, hormone therapy or targeted therapy.

Metastasis

The process of metastasis holds great value in understanding cancer as majority of cancer related deaths account due to systemic spread of cancer rather than the localized tumor. Cancer patients with non – spread cancers show significantly higher rate of survival than patients with metastatic cancers. Latest research reports bring highlight on the idea that cancer metastasis can be an early event and the chances of metastasized cancer are almost 60% during diagnosis. Thereby, advanced treatment strategy that can target both cancer cells in the primary tumor as well as the disseminated cancer cells would be really helpful. [7]

Moreover, every patient, with or without metastasized cancer holds an equal

amount of risk for the disease to spread out. In an average, almost 1/3 of women whose lymph nodes were negative are likely to develop secondary site tumors. In the process of metastasis, to successively develop a secondary location, cancerous cell undergoes multiple steps until it forms a medically detectable tumor. A cancer cell must invade and damage surrounding tissues, reach and survive in the body circulation to reach to distant sites and form micro-colonies.[8,9](Figure 1-3)

Due to many steps to overcome in this process, therefore, metastasis at an overall rate is a process with multiple loop-holes. [7] As, the failure to successfully complete any of the multi-step process will lead to failure in the development of secondary colonies. Thus, bringing in some hope to beat the process of metastasis.

Significance of cellular Hsp.s in cancer

The generation of resistance for therapies, such as those of chemotherapy and hormonal therapy, makes the number of deaths by cancer even more higher. Exposing the cancer tissues to anti cancer therapies leads to development of cellular stress, which is biochemically regulated by a family of proteins, called Heat Shock Family Proteins (Hsp.s), In normal situations, Heat Shock Proteins deal together with multiple small proteins to help in protein folding, and recover the cell from stress, therefore, enhancing survival of cell.[10] These proteins also inhibit functioning of apoptosis related proteins by interacting with them, and thus preventing apoptosis.

Heat shock family proteins (HSPs) were initially found in the year 1962. These proteins are superiorly conserved in all kind of living organisms and are functional when cell encounters any kind of stress. These proteins are ubiquitous in nature and are identified as cyto-protective molecular chaperones.[11] 4 major heat shock families of proteins are HSP70, HSP60, , HSP90 and HSP27. These proteins play a key role in protein transport even during normal cellular

conditions. However, a requirement for these Heat Shock Proteins skyrockets when cell encounters some kind of stress to protect the cells.

HSP70 & HSP27 makeup the most significantly regulated molecular chaperones when cell encounters stress. They are excessively generated in cancerous cells and help in maintaining chemo-resistance. Increased amounts of HSP70 family proteins exhibited to be essential for protecting the cancer cells, while a loss of HSP70 in tumor cells population led to extensive cellular death or apoptosis. A major regulatory protein involved in maintaining cellular survival is Mortalin or mthsp70 or PBP74 or Grp75.[10]

Mortalin: function and significance in cancer progression & cancer metastasis

Mortalin is a key regulator of the Heat Shock Family 70 (Hsp70) proteins. This was for the first time studied in fractions of cytoplasm from fibroblastic cells of normal mouse. [10-12]

Moreover, this protein was seen to exhibit different cellular localization in case of normal cells and in case of immortal cells. cDNA study explained presence of 2 types of mortalin in a cell, Mortalin 1 (mot-1) that was localized throughout the cytoplasm in the cell, while mortalin-2 (mot-2) that was present around the nucleus in cells. It was seen that mortalin-1 and mortalin-2 were different by two amino acids, and by that difference they have completely contrasting biological activity in the cells. When the mortlin-1 cDNA was transfected to NIH 3T3 cancer cell line, the presence of this protein led to senescence of cells.

Whereas, when the cDNA of mortalin-2 was incorporated in the same cancer cells, a perinuclear protein that resulted, which enhanced the malignancy of cell, increased their survival and lifespan.[13,14]

Every cell of mammalian origin is known to possess the protein mortalin, depicting some kind of essential survival function of the protein.[15] Making it explainable that over- expression of this chaperone results in gain of extensive survival efficiency in cells, making them immortal. Upon studying the

expression levels of mortalin in both normal and tumor cells exhibited its significant upregulation in case of tumor cells. Other than this, Mortalin is also seen to interact with its binding partner p53, leading to suppression of its tumor inhibiting function, and thus inhibiting tumor growth. [16-18]

Over the past several years of research, mortalin has been shown to be a binding partner to different proteins involved in apoptosis or cell death and halt their specific function to enhance cell survival. [19,20]

Besides p53, Mortalin has been to shown to interact with apoptosis related proteins, hTERT,[21] Bcl-2[22] and Bcl-xL and inhibit cellular apoptosis and therefore, promote cell survival.[23,24] Mortalin has also been identified to be localized in the nucleus, and this nuclear mortalin was shown to promote enhanced carcinogenesis and increased metastatic ability in cancer cells. Very recently, Mortalin role in the Parkinson's disease has also been highlighted.

Bringing highlight to mortalin functioning in a cancer cell brought light on it as an important target for cancer therapy. [25]

Targeting Mortalin by anti-mortalin molecules

The above knowledge makes mortalin a good target for studying potential anticancer drugs.[26] Based on this idea, several anti-mortalin drugs are being studied.

MKT-077 is a chemical mortalin inhibitor. It's a type of rhodacyanine dye that selectively kills to cancer cells. Moreover, this drug was seen to cause arrest of growth in cancer cells, which also resulted in a shift in the staining pattern of mortalin, which is one of the major features of normal cells. Cytoplasmic localization of p53 could possibly be a method of p53 inactivation in some tumors. Mortalin is known to bind to p53 and hold it in the cytoplasm, Thereby, targeting mortalin by some kind of anti – mortalin molecules cold help release p53 to the nucleus, thereby, initiating tumor suppressor functions of p53. Use of specific mortalin small interfering - RNA has lead to yield similar results.[29,30]

Therefore, a need to investigate more anti-mortalin molecules, majorly the ones that will be less toxic, could help to prove better anti-cancer potents.

Complications in anticancer drugs and the need for natural anti-cancer therapy

Since ancient times, plant derived natural products have been of immense importance in healing various kinds of diseases. The need for a better anti-cancer drug, brings focus on the development of useful medicine from mother nature. The vast diversity of nature derived compounds and their promising potential have brought plant – derived components to a new height. The usage of these compounds promises relatively very few side – effects. Moreover, these compounds are more efficient in treating a variety of diseases and can prove as wide – spectrum drugs.

The main hurdles in the current available treatments against the cancer lie in its non- specificity, increased costs, re-occurrence of cancer and many more. The current available drugs mainly emphasize on killing the cancer cells, which in a way targets every rapidly dividing cell in the body. This leads to weak immune system and low immune response, making a patient more vulnerable to other side infections. Moreover, killing of most rapidly dividing cell in the body renders the patient different side-effects such as nausea, hair-loss, fatigue, etc. Despite these reasons, the most important being the recurrence of cancer in the patients by the low-efficiency of the drugs to target metastasized cancer or the metastatic cancer cells. These reasons leave us to seek for some safer and economic treatment option for cancer, which could beneficially also target highly invasive cancerous cell.

Research Objectives

Cancer marks as a deadly disease, which is one of the major causes of mortality across the world. Cancer is caused when the cells in the patient's body begin to divide controllably. The statistics for cancer begin to grow, despite the therapeutic advances.

Cancer is caused by a variety of different causes, ranging from age, family history to exposure to carcinogens. Biochemically, cancer is caused when there is imbalance in the functioning to 2 types of genes, the oncogenes and the tumor-suppressor genes. A loss – of –function in tumor-suppressor genes and a gain-of-function in oncogenes leads to imbalance of growth stimulatory signal leading to uncontrolled cell division.

Today, a variety of treatments based on the stage of cancer are available. These treatments are either used alone or in conjunction.

Most cancer patients that have primary cancers show better response rate than the ones whose cancers have already metastasized. Thereby, advanced treatment strategy that can target both cancerous cells in the primary tumor as well as the disseminated cancer cells would be really helpful.

Another reason for the failure of maximum anti-cancer drugs accounts for the development of chemo-resistance in the cancer cells. This is a common phenomenon as treatment of cancer induces a state of stress in the cancer cells from which to survive them, they begin to over-express a variety of stress proteins. These stress proteins help the cell to fight against the stress induced by chemical drugs, promote cell survival. One of the most common family of stress proteins is heat shock family 70 proteins (hsp70) that are the first ones to be expressed. These proteins act as a guardian to the cell by promoting cell survival. Mortalin is a hsp70 family protein, it is highly abundant in cancer cells and has been widely demonstrated for its cell survival abilities by enhancing growth of cancer cells, promoting metastasis, causing drug resistance, inactivating p53 and in halting the process of apoptosis. Based on these observations, mortalin is considered to be a therapeutic target against

cancer. Anti-mortalin molecules could therefore prove of great importance in developing anti – cancer drug.[31,32]

Moreover, the current available drugs mainly emphasize on killing the cancer cells, which in a way targets every rapidly dividing cell in the body. This leads to enhanced side –effects of the cancer treatment to the patient's health and causing various health problems such as weight loss, weak immune system, fatigue, diarrhea, pain, blood disorders, sores in the mouth and throat, nausea, vomiting, hair loss, nervous system effects such as weakness or numbness in the hands and/or feet, Loss of balance, tingling, sore, burning, tired, or achy muscles, trembling or Shaking or Stiff neck, headache, walking problem, visual problems, memory loss to name a few.

All these above factors, illuminate a need for the search and development of more safe, non-harmful, more-specific anti-cancer drugs.

Since, herbal medicines have always been used for the past hundreds of years and as of now, the incidence of cancer was very low back at the ancient times, giving us a hint that these herbs may also have an answer for developing a safe anti-cancer drug.

Several plants have been well known for their medicinal properties in the traditional medicinal system. Some of these plants are also known to possess anti-cancer properties. However, much study on the mechanism of the effectiveness of these drugs has not been done so far. As the side effects and loopholes of the conventional drugs are being illumination, more and more focus is shifting on the nature based drugs for the development of a safer and healthier society.

Based on the above information and the needs in the development for new better and safer drugs for breast cancer, we developed a research plan, which would target the following aspects for formulation of a potential candidate for breast cancer therapy,

- New candidate that would be better than the current conventional drugs.
- It would be safe to the patient and would not be an inducer of several toxic side effects.
- It could target both cancerous cells in the primary tumor as well as the metastatic cancer cells that have been disseminated or are in the process of dissemination.
- It could over come the barrier of chemo resistance by targeting the heat shock family proteins that are specifically known to enhance cell survival ability by inducing chemo resistance.
- And, finally, to study the mechanism of action of such an anti-cancer potential drug.

Based on these criteria, we thought, it would be worthwhile to study the expression levels of mortalin when the cells are treated with the potential candidates for the study. Mortalin was used as a selection marker protein as its role in imparting cell survival properties; induction of highly metastatic properties, and in the enhancement of cancer has been well documented, mainly by its binding activity with the tumor – suppressor protein, p53 and thus inducing its inactivation. [33,34]

Embelin

Embelin is the main active component, which is a type of benzo-quinone, derived from the fruits of an Indian herbal plant Embelia ribes. (Figure 1 – 4)

Embelia ribes, generally called as False Black Pepper or White-flowered Embelia, is a species in the Primulaceae. Nicolas Laurens Burman originally described it in his 1768 publication, Flora Indica. Susruta described the fruits of this plant as Anthelmintic, Alterative and Tonic and recommended its use along with liquorice root for the purpose of strengthening the body and avoiding aging effects.

Embelia ribes is a scandent shrub with thin branches and elliptic-lanceolate and gland-dotted leaves. The plant possess Embelin, quercitol, and fatty ingredients; an alkaloid, christembine, a resinoid, tannins and minute quantities of a volatile oil. Embelin occurs as golden yellow needles insoluble in water, while soluble in alcohol, chloroform and benzene. It is known to be effective against tapeworm. Embelin and its salts possess anti-inflammatory potential. Fruits in the dried form possess medicinal properties such as Anthelmintic, Carminative, Astringent and Stimulant. Its usage in India as Anthelmintic and Ascariasis is since ancient times. Fruits in the dried form are used against fevers and for chest and skin diseases. The fruits also possess Anti – bacterial property. Plant is known to heal, bronchitis, migraine, asthma, fever, skin diseases, flatulence, weakness, constipation, cough, intestinal worms.

Embelia root bark is acrid, astringent, anthelmintic, antifertility, anti-estrogenic, carminative, digestive, laxative, soothing, stimulant, stomachic, and thermogenic.

4. CHAPTER II

Initial Screening

Abstract

Cancer is a division of diseases characterized by uncontrolled cell growth. This happens mainly due to loss-of-activity of tumor-suppressor genes and enhanced activity of oncogenes. Due to increased occurrence of permanent and impermanent side effects caused by the current cancer therapy, there is a constant search for better anti-cancer drugs in order to provide significant relief to the cancer patients in a non – harmful manner.

Heat shock proteins, are known to be frequently over-expressed in a majority of cancers, owing to the enhanced survival and induced chemo-resistance in cancer cells. Mortalin is a key heat shock protein and is considered to be a key therapeutic target against cancer.

In this chapter, we select a potential anti-cancer therapy candidate, Embelin, for our further study, based on its ability to effectively decrease cellular levels of Mortalin in human breast cancer cell lines.

Introduction

Current available cancer therapies mainly focus on killing cancer cells. Since, these cancer drugs do not specifically target cancer cells, they at the time of action, also target the rapidly multiplying normal cells. This mechanism of action leads to accountability of various side effects by a cancer patient. Some of these side effects are impermanent and disappear over a period of time. Whereas, many of other side effects caused by these drugs damage a person's health in an irreparable manner, by causing a permanent damage.

These highlights over the current cancer treatment, have lead to the initiation of a search for finding better anti-cancer drugs. The search of these drugs today targets on focusing on other mechanism of targeting the cancer progression, besides using the traditional mechanism of targeting cancer cells by killing. Upon finding a suitable anti-cancer drug, encountering chemo-resistance is another critical aspect that needs to be dealt with.

Several biomolecules are over-expressed in cancer cells and these biomolecules further lead to enhanced cell survival ability of cancer cells. One such class of biomolecules which are significantly expressed in cancer cells are heat shock proteins or HSP.s. One of the key player in this family is Mortalin. The past decade of research has highlighted the importance of mortalin in enhancing cell survival ability and in enhancing the progression of cancer. Therefore, mortalin is considered to be a key therapeutic target against cancer.

In this chapter, we perform screening of a variety of commercially available drugs and two nature derived compounds, for their anti-mortalin ability. Based on the effect of following drugs on Mortalin levels, we selected Embelin as a potential anti-cancer candidate for our further study.

Materials and Methods

Cells, Drugs and Cell Culture

The breast cancer cells studied were: MCF-7 and MDA-MB-231. Human Breast Carcinoma – MCF-7 (Adenocarcinoma), MDA-MB-231 (Adenocarcinoma) were purchased from Japanese Collection of Research Bioresources (JCRB, Japan) Cell Bank. MCF-7 and MDA-MB-231 were cultured in the DMEM (Dulbecco's Modified Eagle Medium) (Life Technologies, CA) that was supplemented with 10% FBS (fetal bovine serum) at 37 °C, in a humidified incubator (5% CO2 and 95% air).

Embelin was obtained purified from Sigma Aldrich Co., Japan. Embelin stocks were then, made by dissolving the adequate amount of it in dimethyl sulfoxide (DMSO) to obtain 1 mM stocks. These 1 mM stocks were aliquoted into tubes and were preserved at -20°C for further use. Concentrations were made by diluting the 1mM Embelin stock to the complete cell culture medium (DMEM) that was used for each experiment. All biochemical and imaging assays were performed at sub-confluent (60–70%) cultures.

Immunoblotting

To identify the effect on expression level of proteins in effect to that of Embelin – treatment, Immunoblotting technique was used. Cells that were priorly treated and not-treated with Embelin were prepared as protein lysates samples for loading in SDS – PAGE gels. Equal amounts of whole cell protein samples from control and treatment groups were seperated on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane. The blots were incubated with the desired primary antibodies followed by their respective secondary antibodies. Anti β-actin antibody was used as internal loading control. Quantitation of western blots was done by ImageJ software (NIH, MA).

Immuno – cytochemical staining

For immuno - fluorescence or immuno – cytochemical staining, cells were cultured in 12-well dish containing coverslips. Control and cells from treatment groups were fixed, and stained with primary and secondary antibodies of interest for the study. In this study, antibodies for Mortalin and p53 were used. Coverslips containing stained cells, were mounted and observed using Carl Zeiss microscope (Axiovert 200 M).

Results

Embelin decreases the cellular expression levels of Mortalin

To select a potential non – harmful anti- cancer drug candidate, a variety of different anti – cancer compounds, including commercially available compounds and two nature derived compounds are tested for their anti-mortalin ability.

As seen by the immunofluorescence results, the nature derived compounds, Embelin and CAPE was seen to be very effective in reducing the cellular mortalin expression levels. A similar anti – mortalin effect was observed in both the human breast cancer cell lines, MCF7 (Figure 2 – 1) and in MDA-MB-231 (Figure 2 – 2). Their effect in reducing the cellular mortalin levels was seen to be better than Adriamycin, a drug known for it's anti – cancer property.

A similar reduction in the mortalin levels was observed by a different cellular experiment (Immunoblotting) (Figure 2 – 3) and also by the pixel calculation of these results (Figure 2 – 4).

Discussion

Current available anti - cancer treatments are inefficient in specifically targeting cancer cells, thereby accounting for multiple side effects. Moreover, anti – cancer drugs can barely target metastatic or disseminated cancer cells, leading to re-occurrence of cancer. Based on these insights, there is a constant search for a better anti-cancer drug.

This study started with the idea to screen a potential anti- cancer drug candidate, which would be less harmful for the cancer patients.

In this chapter, an initial screening of several potential candidates was performed by investigating their anti-mortalin ability. Mortalin is a heat – shock family protein and is known to be over expressed in cancers. Mortalin significantly imparts cell survival property to cancer cells, as the down – regulation of mortalin leads to enhanced cell death response.

In the study conducted, Embelin, which is a plant compound, was observed to be very promising by its ability to reduce cellular mortalin levels. Moreover, its anti-mortalin efficiency was seen to be better than Adriamycin, a drug commonly used during cancer chemotherapy. Although, Adriamycin is a very effective anti-cancer drug, its main drawback lies in its ability to cause life – threatening heart damage.

Based on its ability to significantly reduce cellular mortalin levels, Embelin was selected for further investigation, as a potential anti – cancer candidate.

5. CHAPTER III

Cytotoxic effect of Embelin on Breast Cancer cells and their metastatic derivatives

Abstract

Human Breast Cancer, despite improved treatment and surgical resection stakes millions of lives. Chemotherapy drugs provide an option for cancer treatment, but the complication arises when these drugs target all frequently multiplying cells in cancer pateint's body. These, in turn also targets the non- carcinoma quickly multiplying cells of the patient, leading to various side-effects such as bleeding, low-immune response, hair loss, fatigue, nausea, hot flushes, mood swings, infertility, hormone- imbalance, to name a few. Moreover, very few of the chemotherapy drugs target metastatic cancer cells, making the relapse of cancer more accountable, and also difficult to treat metastatic cancer through chemotherapy drugs. Natural chemicals are gaining increased limelight in the field of cancer research, due to their increased bioavailability and non-harmful nature. Embelin is a kind of benzo-quinone compound and is derived from fruits of an Indian herbal plant known as Embelia ribes Burm. f. (Primulaceae). Embelin possesses various therapeutic properties such as Anthelmintic, Anti-Tumor, Anti-Diabetic, Anti-Bacterial and Anti-Inflammatory. Based on the known effectiveness of Embelin as a therapeutic agent, we decided to investigate Anti-cancer potential of Embelin. Since, Breast cancer accounts for millions of deaths worldwide, we selected an in-vitro experimental model of Human Breast Cancer cell lines. In this chapter, we explain results showing Embelin exhibits Anti-Cancer potential by inhibiting the proliferation activity of cancer cells.

Introduction

Breast cancer accounts for millions of deaths worldwide, making it one of the deadly health problems to be dealt with. Despite the availability of a wide variety of treatments options for the disease, such as improved chemotherapy, hormonal therapy, and surgical resection, there is still a long way to conquer the deadly disease. One of the reasons for the failure of the effectiveness of the above treatments is the high ability of the breast cancer to metastasize.

Metastasis is process in the stage of cancer in which cells in the primary tumor begin to detach themselves and move out of the tumor. After moving out from the original tumor site, many of cancer cells die and the remaining cancer cells travels to different locations of the body with the help of the blood circulation or the lymph nodes in the body. And, upon reaching to different favorable locations in the body, these cells began to divide in order to form micro-metastasis. Many of the these micro-metastasis are ruled out by the immune system of the body, however the ones that survive further divide uncontrollably leading to formation of secondary tumors. Metastasis or the Breast Cancer metastasis was initially thought to be a concluding stage in the progress of cancer. However, recent reports on metastatic breast cancer depict it as an early event during cancer growth, showing escape of cells to other body parts, in an early cancer stage such as ductal carcinoma in-situ (DCIS), making breast cancer an even more important issue to be dealt with. [35,36] Chemotherapy drugs provide an option for cancer treatment, but the complication arises when these drugs target quickly multiplying cells in the body. These, in turn also targets the non- carcinoma quickly multiplying cells in the body of the patient, leading to various side-effects such as bleeding, low-immune response, hair loss, fatigue, nausea, hot flushes, mood swings, infertility, hormone- imbalance, to name a few.

Moreover, very few of the chemotherapy drugs target metastatic cancer cells, therefore leaving the metastatic cancer cells still healthy. This results into making the relapse of cancer more accountable, and also difficult to treat metastatic cancer through chemotherapy drugs. [37]

Herbal plants that are known to have therapeutic properties are used since ancient times as a solution for different ailments and to help the mankind. Ayurveda or the Indian traditional medicine system consists of several such natural products that have been used in ancient times and are also very popular today to treat a variety of dreadful diseases.

Natural chemicals are gaining increased limelight in the field of cancer research, due to their increased bioavailability and non-harmful nature. Biochemical level to understand their exact mechanism of action, of several such natural chemicals is under investigations, which are proving to be promising. One such naturally-derived compound is Embelin.

Embelin is a type of benzo-quinone that is derived from the fruits of an Indian herbal plant known as Embelia ribes burm. The fruits of this plant are known to variety of therapeutic activity. Therefore, as Embelin is the active component from the fruits of this plant, it has so far seen to have anti-helmenthic, anti-diabetic, anti-inflammatory, analgesic, immuno-modulatory activities. Based on its immense health benefits, the effect of Embelin on cancer cells was studied.

The anti-cancer effect of Embelin was mainly studied upon breast cancer cells in-vitro, since breast cancer is one of most fatal cancer accounting for the maximum number for cancer related deaths among women.

In this chapter, we show that Embelin has potential anti-cancer properties, which can prove very beneficial. Embelin was seen to reduce the cancer cell proliferation, making it a useful and promising compound in halting the cancer progression.

Materials and Methods

Cells, Drugs and Cell Culture

The breast cancer cells studied were: MCF-7 and MDA-MB-231. Human Breast Carcinoma – MCF-7 (Adenocarcinoma), MDA-MB-231 (Adenocarcinoma) were purchased from Japanese Collection of Research Bioresources (JCRB, Japan) Cell Bank. To understand the effect of Embelin on the process of metastasis, we used highly metastatic breast cancer cell lines. For this work, Mortalin over-expressing highly metastatic cell lines were used. These stable Mortalin overexpressing metastatic derivatives were obtained by retro-viral transfections as described earlier (Wadhwa et al) [27,28]. MCF-7 and MDA-MB-231 were cultured in the DMEM (Dulbecco's Modified Eagle Medium) (Life Technologies, CA) that was supplemented with 10% FBS (fetal bovine serum) at 37 °C, in a humidified incubator (5% CO2 and 95% air).

Embelin was obtained purified from Sigma Aldrich Co., Japan. Embelin stocks were then, made by dissolving the adequate amount of it in dimethyl sulfoxide (DMSO) to obtain 1 mM stocks. These 1 mM stocks were aliquoted into tubes and were preserved at -20°C for further use. Concentrations were made by diluting the 1mM Embelin stock to the complete cell culture medium (DMEM) that was used for each experiment. All biochemical and imaging assays were performed at sub-confluent (60–70%) cultures.

Cytotoxicity and Growth Inhibition Assay

To determine the cytotoxic effect of Embelin on Human Breast cancer cell lines, an in-vitro cell viability experiment was performed. The effect of Embelin on cancer cell survival ability was determined using the MTT assay (Sigma Aldrich, Japan). Cells initially cultured in 10-cm dish were trypsinized and harvested. These cells were then counted using a cell counter and the cells were

then diluted accordingly to reach a desired cell number for plating. An estimated equal number of cells were plated in each well. A Cell number of 5×10^3 cells/well was plated in 96-well cell culture dishes. Following 24h, cells were supplemented with increasing concentrations of Embelin for an incubation period of about 48h or an incubation period as indicated. After the completion of the determined incubation period the cell viability was assessed. Cell viability was studied by the degree of conversion of yellow MTT by mitochondrial dehydrogenases of living cells to purple formazan. A working concentration of MTT (0.5 mg/mL) was supplemented to the cell culture medium for an incubation period of 4h following the treatment of cells as indicated. MTT-containing medium was aspirated and 100µL DMSO was added to each well to completely dissolve formazan crystals. The results were recorded at an absorbance of 550 nanometer using a multiwall plate reader by Tecan, Switzerland. Absorbance values was recorded from 3 individual experiments and analyzed. The P-value was estimated using by t-test calculator (GraphPad Software, Inc., CA).

Morphological observations

For understanding the affect of Embelin on cancer cells, a morphology examination experiment was performed. In this experiment, the cells were plated to 60-70% confluency in 6-well dishes and following the completion of 24h; the cells were treated with Embelin. Morphological study cells in treatment and non-treatment groups was performed at intervals of every 24h for 4 days using a phase contrast microscope.

Colony forming assays

To understand the efficiency of Embelin to reduce cell transformation ability and the capability of a single cancer cell to multiply and generate its clones, a colony formation study was performed. For this experiment, cells initially being cultured in 10-cm dish were trypsinized and harvested. The cells were then, counted and an estimated number of 500 cells were plated per well of a 6-well dish. Following 24h, the cells were supplemented with medium containing Embelin. The culture plate was then incubated to multiply and form colonies for the next 10 – 15 days, with a regular change in media every alternate day. When colonies were formed, they were washed 2-3 times with 1X PBS, followed by fixing with cold methanol for 10-15 minutes. Followed by fixing with methanol, the cell colonies were stained using 0.1% crystal violet for overnight. The stain was rinsed with water to remove all excess stain, dried and photographed. The number of colonies in each well was counted and analyzed.

Data recorded from 3 independent experiments were analyzed, and the P-value were calculated by t-test calculator (GraphPad Software, Inc., CA).

Results

Embelin is efficient in decreasing the cell viability of breast cancer cells and their highly metastatic derivatives.

To understand the anti-cancer potential of Embelin, this is a quinone derivative from the fruit of Embelia ribes Burm. f., its effect on the cell viability of Human Breast cancer cells was analyzed. Embelin was seen to reduce the percentage cell viability of both MCF7 and MDA-MB-231 cells with increase in concentration.

A clear decline in the percentage cell survival efficiency was observed in the cells with response to increasing concentration of Embelin. (Figure 3-1 A, B)

Moreover, as failure to effect metastatic cancer cells leads to be the main reason for the failure of maximum anti-cancer drugs, therefore, the effect of Embelin on highly metastatic derivatives of these breast cancer cells were developed and tested for the anti-cancer effectiveness of Embelin. These highly metastatic derivatives of cancer cells was developed by inducing stable over-expression of Mortalin, an Heat shock family protein in the cells. Over the past several years of research in our lab, it has been established that mortalin over-expression in cells leads to increase in the hallmarks of cancer, and a reverse of down-regulation can lead to p53-mediated apoptosis causing the death of cancer cells. Therefore, targeting mortalin could prove as a promising anti-cancer therapy. [25]

Based on these understandings, highly metastatic derivatives of MCF7 and MDA-MB-231 were tested for the effectiveness of Embelin.

Embelin induced similar anti-proliferative response in these cells, depicting that Embelin may proof beneficial in effecting both cancer cells and the metastatic cancer cells. (Figure 3-2 A, B)

Morphologically non- Cytotoxic low doses of Embelin reduce the division efficiency in breast cancer cells

To understand the effectiveness of non-cytotoxic low doses of Embelin on cancer cells, a morphology analysis was performed. When the cells were treated with non – cytotoxic concentrations of Embelin, the division ability of cancer cells was reduced. However, there was no cell death seen. On analyzing the cells morphologically, the cells seemed healthy, but their growth or the rapid cell division was greatly reduced in comparison to untreated cells with Embelin. This similar response was seen in both the cancer cell lines, that is, MCF7 and MDA-MB-231, showing that the low concentration treatment of Embelin to cancer cells lead them to a growth arrest response. (Figure 3-3 A, B; 3-4 A, B) Also, a similar growth arrest response was observed in highly metastatic derivatives of cancer cells. (Figure 3-5, 3-6)

Embelin halts the ability of a single cell to divide and generate its clones

We next determined the efficiency of Embelin to inhibit or reduce the ability of a single cancer cell to divide and generate its clones. Upon completion of experimentation, Embelin showed significant reduction in the colony forming ability. The ability in cells that were supplemented with Embelin was reduced upto almost 80% when compared to the control. Moreover, similar decrease was seen in both the breast cancer cell lines, MCF7 and MDA-MB-231. (Figure 3-7 A, B; 3-8 A, B)

We next checked Embelin against highly metastatic mortalin over-expressing derivatives of cancer cells, where we observed similar inhibitory effect of Embelin on the colony formation ability of cells. (Figure 3-9 A, B; 3-10 A, B)

The difference between the control and Embelin treated cancer cells was very significant, showing that Embelin can be a promising potential candidate as an anti-cancer drug.

Discussions

Cancer is a disease that accounts for millions of deaths worldwide every year. Despite various treatment options, the recurrence of cancer in patients has been the main cause of cancer related deaths.

Moreover, the popular treatment options that are available in the market, target on the killing of cancer cells, but by doing that they account for multiple side-effects as these drugs target every rapidly dividing cell in the patient's body.

Due to the harmful side-effects caused, therapies involving use of natural products is gaining insight. The use of natural products in ancient medicinal system has been well quoted, mainly due to its non-harmful nature and the high abundance of resources.

Based on these prospects, we selected a compound called Embelin that is derived from the fruits of a plant called Embelia ribes burm. Embelin is the key component from the fruits of this plant, and has been known for its various therapeutic properties.

In this chapter, we tested Embelin on breast cancer cells to check its anti-cancer potential.

It was seen that Embelin possessed anti-cancer potential as it greatly reduced the cell viability of breast cancer cells. Since, the failure of the several current cancer treatment options reasons to their inability to target metastatic cancer cells, which in turn leads to relapse of cancer in the patients. Therefore, the effectiveness of Embelin was checked against highly metastatic derivatives of breast cancer cells where, similar reduction in the cell viability was observed. Upon understanding the cytotoxic effect of Embelin on breast cancer cells and also similar effect on highly metastatic derivatives of breast cancer cells, we next considered to analyze the non-cytotoxic dose of Embelin.

As there are several key proteins that are involved in the functioning of both cancer and normal cells. Biochemically, the overexpression or the abundance of these proteins leads to uncontrollable division of cancer cells. Therefore, cytotoxic dose of

a product can also be responsible for targeting similar biomolecules also in the normal cells. So, based on this assumption and with the idea of developing a drug for cancer that is not lethal to the normal cells, we therefore, thought to study the effects of the non-cytotoxic dose of Embelin, for our further study.

The understanding of the effect of non-cytotoxic dose of Embelin on breast cancer cell by their morphology study was then taken up next for analyses. Morphologically, It was observed that Embelin halts the multiplication of cells, showing a growth arrest response, leading to a clear decline in the population doubling efficiency of breast cancer cells.

Moreover, the non-cytotoxic dose of Embelin was found to also be capable of reducing the efficiency of single cells to generate its clones.

6. CHAPTER IV

Embelin exhibits Anti - metastatic potential

Abstract

Cancer is a dangerous disease, for which research everyday is extending hands, to find a suitable cure. Today, understanding the disease and its functioning has become a prime area of research which can help solve the mystery and give the patients suffering with this deadly disease a new life. Side effects from the conventional drugs add to another harmful dimension in the cancer treatment. Based on these observations and statistics, more and more focus is moving to natural compounds that can be used as an anti-cancer drug.

In this study, I have selected one such plant derived compound, Embelin that is the active component from the fruits of a plant, Embelia ribes burm. In the earlier chapter, Embelin was investigated for its anti-cancer potential. Embelin exhibited very promising anti-proliferative activity, where it showed a remarkable significant decline in the cell viability with increasing concentration. To avoid any cytotoxic effect to normal cells, we selected a non-cytotoxic low dose, for our further study. At low concentrations also, Embelin significantly reduced the proliferation ability of cancer cells and also caused significant reduction in the colony forming efficiency of cancer cells.

The availability of treatment options is constantly increasing, but sadly to give only a time-bound relief to most of the patients. The key reason for the failure: the spread of cancer. This is because the conventional medicines or treatment therapies practiced against cancer fail to target any metastatic or migrating cancer cells. This leads to the relapse of cancer. Therefore, targeting metastatic cells along with cancer cells is the most crucial part.

With the understanding of this critical aspect of targeting the metastatic cells, in this chapter we investigated the Anti-metastatic potential of Embelin.

Introduction

Cancer is a disease for which solutions are being searched every day. Besides this, cancer still targets a huge number of people worldwide, every year and the statistics seem to keep increasing.

One possible reason for the increasing statistics is, the time of detection of cancer. By the time cancer is detected, it is no more in its preliminary stage and has already advanced far more than can be controlled. This means that the cancer has already metastasized or started spreading to other parts of the patient's body.

Metastasis is a highly complicated procedure in the progression and spread of cancer. In order to efficiently invade another location, a cancer cell needs to go through multi-step procedure, which include separating from the primary tumor by invading through the surrounding tissues and basement membranes, survival in the circulation to reach to distant organs and develop secondary tumors.[38]

Therefore, by the time a patient is diagnosed with cancer and the starts receiving anti-cancer medications, the disease is far more invasive to be controlled. This leaves the patient with very less positive hope, and accounts for the increasing statistics. The prime question to address lies in the effectiveness of the anti-cancer drugs. Meanwhile, the most effective anti-cancer drugs do their best to stop cancer, but their main mode of action lies onto killing the cancer cells. On one hand, these drugs target every rapidly diving cancer cells, leaving the patient with a multiple varieties of side effects to be tolerated. Whereas, on the other hand, these drugs are incapable of targeting metastatic cells, leading to relapse of cancer.

Understanding the importance of targeting metastatic cancer cells, in this chapter, we investigated the anti-metastatic potential of Embelin.

Materials and Methods

Cells, Drugs and Cell Culture

The breast cancer cells studied were: MCF-7 and MDA-MB-231. Human Breast Carcinoma – MCF-7 (Adenocarcinoma), MDA-MB-231 (Adenocarcinoma) were purchased from Japanese Collection of Research Bioresources (JCRB, Japan) Cell Bank. MCF-7 and MDA-MB-231 were cultured in the DMEM (Dulbecco's Modified Eagle Medium) (Life Technologies, CA) that was supplemented with 10% FBS (fetal bovine serum) at 37 °C, in a humidified incubator (5% CO2 and 95% air).

Embelin was obtained purified from Sigma Aldrich Co., Japan. Embelin stocks were then, made by dissolving the adequate amount of it in dimethyl sulfoxide (DMSO) to obtain 1 mM stocks. These 1 mM stocks were aliquoted into tubes and were preserved at -20°C for further use. Concentrations were made by diluting the 1mM Embelin stock to the complete cell culture medium (DMEM) that was used for each experiment. All biochemical and imaging assays were performed at sub-confluent (60–70%) cultures.

Wound scratch assay

To analyze the effect of Embelin on metastasis, the *in vitro* cancer cell migration ability in control and Embelin treatment, was studied utilizing Wound-scratch assay. Cell growing in 10-cm dish were trypsinized and seeded in 6-well culture dish. The cells were cultured until, in a monolayer. This monolayer was then wounded by uniformly damaging cells in a straight line using a pipette tip of 200µl. Wound was rinsed using 1X PBS, 2-3 times, to wash away any partially attached cells, and then added with only medium and medium supplemented with Embelin in control and treated wells, respectively. The time of wounding was taken 0 h. The experimental dishes are then kept heal by covering the scratched surface. The efficiency of cancer cell migration was observed in the form of

pictures (after 24h) using a microscope at a magnification of 10X. The results were analyzed using Wimsratch software (Wimasis Image Analysis, Germany).

In vitro cancer cell chemotaxis assay

To understand the effect of Embelin on in – vitro cancer cell invasion ability, an in – vitro chemotaxis assay was done. For this experiment, the cells that were initially being cultured in 10-cm dish were washed with PBS, and replated in matrigel invasion chambers from BD Biosciences at a cell number of 50,000 cell/ml. These chambers were then in control and treatment groups, incubated in a humidified CO_2 incubator for 20h. After the completion of the incubation period, invasion chambers were removed and stained. The stained membranes were washed for the removal of any excess stain, and allowed to dry at room temperature. The dried membranes were visualized under the microscope and pictured at a magnification of 10X. Migrated cells was dissolved using acetic acid dilution (10%) and results were quantitated using spectrophotometer at absorbance of 590 nanometer wavelength.

Results

Embelin reduces the cancer cell migration ability

To understand the anti-metastatic potential of Embelin, its inhibitory effect on the migration ability of breast cancer cells was investigated. For this purpose a wound scratch assay was performed. After wounding the cells in a straight line, cells in both control and Embelin treated well were allowed to migrate and heal the wound. After 24h duration, it was observed that the wound in control well, where the cells were supplemented with only the culture media was almost coved with the migrated cells. However, in the wells where the cells were supplemented with Embelin containing, the wound was very slightly healed. Upon visual examination, similar results in both the cancer cells lines that is, MCF7 and MDA-MB-231 were observed. (Figure 4-1 A; 4-2 A) Further on, a comparative analysis of results in control and Embelin – treated wells was performed using Wimasis software and based on the quantitative values given by the software, a quantitative plot explaining the results was prepared. This resulting plot also gave similar results as seen by the wound scratch assay pictures. (Figure 4-1 B; 4-2 B)

Embelin exhibits anti-invasive potential against cancer cells

To understand the Anti-invasive potential of Embelin against breast cancer cells, an in-vitro chemotaxis assay was performed. The cells were seeded in a chemotaxis assay chambers and allowed to degrade the matrigel, that was coated onto a membrane and migrate to the other side of the membrane. The number of total cells that migrate through the membrane by degrading the matrigel tells the invasive potential present in the cells. More invasive cells degrade the matrigel - coated membrane faster and travel to the other side of the chamber that consists of

a chemo attractant (in this experiment FBS). Upon performing an invasion assay on control (non-treated) cells and the Embelin – treated cells, we observed the number of cells that were Embelin – treated showed significant reduction in their invasion ability, as compared to the control. (Figure 4-3 A; 4-4 A) More over, to confirm the results of visual examination, migrated cells were stained and extracted and their absorbance was measured. The results of plotted percentage absorbance explained a similar result on reduction in the cell invasion ability of cancer cells when they were treated with Embelin with respect to control. (Figure 4-3 B; 4-4 B)

Discussion

Metastasis is the key step in the progression of cancer, and also the key reason for the failure of convention anti-cancer drugs. Because metastasis is such a complex process, it still remains to be completely understood. Therefore, drugs targeting the metastatic cells or reducing metastatic nature of cancer cells is greatly in demand.

Since Embelin showed promising reduction in the cell viability of highly metastatic derivatives of cancer cells, we assumed that Embelin could also possibly reduce the metastatic properties of cancer cells. Based on this idea, we checked Embelin against the metastatic properties of breast cancer cells by investigating its effect on cancer cell migration and on cancer cell invasion ability.

Migration of a cancer cell help it to travel through circulation, from the source of primary tumor to distant body parts, thus making it an essential process to be targeted by any anti-metastatic potential drug. Embelin exhibited strong response against cancer cell migration potential. Cancer cells that were treated with Embelin showed reduced migration ability with respect to control.

Invasive property of a cancer cell helps the cancer cell to degrade and invade through the surrounding tissues and the basement membrane to come in contact with the circulation, lymphatics or the peritoneal space. This is a very important step in the process of metastasis, through which a cancer cell can render itself free and move to different other tissues in the body. We found that Embelin treated cancer cells exhibited reduced invasive potential as depicted by a reduced degradation of the matrigel coating on the membrane. This further leads to reduced migration of cells through the matrigel coated membrane. Whereas, the control cells exhibited enhanced invasive property and were seen to migrate effectively through the matrigel coated membrane. These results depict that Embelin shows promising anti-metastatic potential against breast cancer cells.

7. CHAPTER V

Embelin targets Mortalin-p53 interactions

Abstract

This study was initiated with an idea of finding a better drug to target breast cancer, which would be less toxic to the patients and account for fewer side – effects, could also target disseminated cancer cells, besides targeting the primary tumor cells, and also, could overcome the barrier of chemo – resistance in cancer therapy.

Based on all these ideas, we selected a plant derived compound, Embelin through our initial results of screening different anti – cancer drugs.

Embelin is a type of benzo-quinone compound which is found in fruits of Embelia ribes Burm. f.(Primulaceae). Embelin has been reported to exhibit multiple therapeutic properties such as including Anthelmintic, Anti-Tumor, Anti-Diabetic, Anti-Bacterial and Anti-Inflammatory.

Embelin was observed to reduce cancer cell proliferation, cancer cell migration and invasion. Embelin was also seen to reduce mortalin expression levels significantly during the initial screening of various anti-cancer drugs. Mortalin has been well documented to bind to tumor – suppressor protein, p53 and inactivate its tumor – suppressor functions.

In this chapter, we investigate Embelin’s ability to disrupt mortalin – p53 binding in cancer cells.

Introduction

Cancer stakes millions of lives worldwide, every year. An important criterion in the cancer treatment is the resistance developed by the cancer cells against the anti-cancer therapy.

Heat shock proteins are generally expressed in a cell when they are in a state of stress. In the case of cancer, a cancer cell is abundant in the expression of heat shock proteins. These proteins protect the cell from any external stress, accounting for cell survival functions. The two heat shock family proteins that are first to be released in a cell are hsp70 and hsp27 family proteins. [10]

Mortalin is a heat – shock family 70 protein. Mortalin functions as a cell survival protein, by protecting the cancer cell from the stress generated by anti-cancer drugs. Mortalin is known to bind to p53, a tumor suppressor proteins and thus inactivate its tumor suppressor functions. In common case, a functional p53 goes to the nucleus and induces tumor suppressive functions in a cancer cell, by halting its division and executing apoptosis. However, when mortalin binds to p53, it holds it in the cytoplasm preventing its nuclear localization and thus, inactivating its tumor-suppressor function. [16,32]

In this chapter, since, we have earlier observed that Embelin is capable of reducing the cellular levels of mortalin. We assumed that it might be possible that Embelin could be potential in disrupting the mortalin-p53 interaction leading to a release of p53, which could thereby localize itself to the nucleus and perform its tumor-suppressor functions.

Materials and Methods

Cells, Drugs and Cell Culture

The breast cancer cells studied were: MCF-7 and MDA-MB-231. Human Breast Carcinoma – MCF-7 (Adenocarcinoma), MDA-MB-231 (Adenocarcinoma) were purchased from Japanese Collection of Research Bioresources (JCRB, Japan) Cell Bank. MCF-7 and MDA-MB-231 were cultured in the DMEM (Dulbecco's Modified Eagle Medium) (Life Technologies, CA) that was supplemented with 10% FBS (fetal bovine serum) at 37 °C, in a humidified incubator (5% CO2 and 95% air).

Embelin was obtained purified from Sigma Aldrich Co., Japan. Embelin stocks were then, made by dissolving the adequate amount of it in dimethyl sulfoxide (DMSO) to obtain 1 mM stocks. These 1 mM stocks were aliquoted into tubes and were preserved at -20°C for further use. Concentrations were made by diluting the 1mM Embelin stock to the complete cell culture medium (DMEM) that was used for each experiment. All biochemical and imaging assays were performed at sub-confluent (60–70%) cultures.

Immunoblotting

To identify the effect on expression level of proteins in effect to that of Embelin – treatment, Immunoblotting technique was used. Cells that were priorly treated and not-treated with Embelin were prepared as protein lysates samples for loading in SDS – PAGE gels. Equal amounts of whole cell protein samples from control and treatment groups were seperated on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane. The blots were incubated with the desired primary antibodies (Mortalin) followed by their respective secondary antibodies. Anti β-actin antibody was used as internal loading control. Quantitation of western blots was done by ImageJ software (NIH, MA).

Immuno – cytochemical staining

For immuno - fluorescence or immuno – cytochemical staining, cells were cultured in 12-well dish containing coverslips. Control and cells from treatment groups were fixed, and stained with primary and secondary antibodies of interest for the study. In this study, antibodies for Mortalin and p53 were used. Coverslips containing stained cells, were mounted and observed using Carl Zeiss microscope (Axiovert 200 M).

Luciferase reporter assay

MCF-7 cells were plated in 6-well cell culture dish. Following overnight incubation, cells were then transfected with p53 - luciferase plasmid reporter, using X-tremeGENE 9 DNA Transfection Reagent. Control and treatment group cells, were kept for an incubation period of 48h. The cells were then used for studying the luciferase activity.

Quantitative Real – Time Polymerase Chain Reaction (qRT-PCR)

To estimate the modulation at the mRNA level, quantitative Reverse Transcriptase PCR (qRT-PCR) was done. Total RNA extracted was reverse transcribed to cDNA, The resultant cDNA was subjected to amplification using gene specific primers. The analysis of the results was done using the relative analysis method of gene expression by the $2^{-\Delta\Delta C}T$ method, as described by Kenneth J. Livak and Thomas D. Schmittgen [41] The gene specific primer sequences were as follows:

Mortalin	Sense	5′- AGCTGGAATGGCCTTAGTCAT-3′
Mortalin	Antisense	5′- CAGGAGTTGGTAGTACCCAAATC -3′
MMP-9	Sense	5′-TGTACCGCTATGGTTACACTCG -3′
MMP-9	Antisense	5′- GGCAGGGACAGTTGCTTCT -3′

MMP-3	Sense	5′- CTGGACTCCCGACACTCTGGA -3′
MMP-3	Antisense	5′- CAGGAAAGGTTCTGAAGTGACC-3′
18s (internal control)	Sense	5′-CAGGGTTCGATTCCGTAGAG -3′
18s (internal control)	Antisense	5′-CCTCCAGTGGATCCTCGTTA - 3′

Results

Embelin reduces cellular Mortalin expression

Mortalin is known to be over-expressed in cancer cells, which results in imparting the cancer cells with cell survival properties. We checked the effect on mortalin expression levels on cells upon treatment with Embelin. There was a reduction in the mortalin expression levels in the cells that were treated with Embelin, when compared to the cells that were not treated with Embelin. A similar response was observed in both the breast cancer cell lines, MCF7 and MDA-MB-231, as seen by the results of Immuno blotting and its quantitation and also by Immunofluorescence. (Figure 5-1 A, B; 5-2 A, B; 5-3, 4)

Embelin causes down-regulation of Mortalin at the transcriptional level

To understand whether this reduction of mortalin by Embelin is due to the down-regulation of mortalin gene or only at the translational level, we performed quantitative Real time – polymerase chain reaction (qRT-PCR). Upon analysis of results, it was understood that this reduction of mortalin expression levels is mediated by its transcriptional down-regulation, showing that Embelin was responsible for down-regulation of mortalin at the transcriptional level.

Embelin caused significant down-regulation of mortalin in both the breast cancer cell lines that is, in MCF7 and in MDA-MB-231 as seen in Figure 5-5, 5-6.

Reduction of mortalin by Embelin causes translocation of p53 to the nucleus

To understand whether the down-regulation of mortalin by Embelin, is capable of rendering free p53 to the nucleus, the presence of nuclear p53 was investigated by Immunofluorescence. Embelin was seen to enhance the

translocation of p53 to nucleus, predicatively by disrupting the binding of mortalin to p53 and thereby, activating its tumor-suppressor functions. (Figure 5-7) A very similar result was also seen in p53-luciferese reporter assay. (Figure 5-8)

Embelin causes down-regulation of MMP.s at the transcriptional level

As mortalin is known to enhance the invasive character of cancer cells, we next determined the status of Matrix Metalloproteinase in the cancer cells in response to presence and absence of Embelin treatment. Upon Embelin treatment, MMP-3 and 9 were seen to be down regulated in cancer cells, as depicted by the quantitative real time polymerase chain reaction. (qRT-PCR) (Figure 5-9), which explains the inhibitory effect of Embelin against cancer cell invasion and cancer cell migration.

Discussion

In the present study, we investigated the molecular mechanism of the effect of Embelin on Mortalin. As mortalin is a heat shock protein, it has been well documented for its cell survival properties, to protect the cancer cell, and therefore, making it a potential therapeutic target.

Embelin showed reduction in the expression levels of mortalin in cancer cells. Moreover, similar effect was seen at the transcriptional level, where a significant down-regulation of mortalin was observed.

Mortalin-p53 binding is response for the inactivation of the tumor-suppressor functions of the p53 protein. Disruption of this binding could render p53 free, which then translocate to the nucleus to execute its tumor suppressor functions. Embelin was seen to disrupt the mortalin-p53 interaction, as it's down – regulation of mortalin led to nuclear localization of p53.

Role of mortalin in enhancing cancer metastasis and invasion is well known, so an inhibitor would mortalin would also reduce the above properties. MMP are widely known for their ability to enhance invasive characteristics to a cancer cell, so the effect of MMP was checked in effect to Embelin treatment. Embelin down regulated the transcription of MMP.s, thus explaining the inhibitory effect of Embelin on cancer cell migration and cancer cell invasion.

8. CHAPTER VI

Embelin down regulates growth factors involved in cancer progression

Abstract

Cancer accounts for millions of deaths worldwide. A search for a safe anti-cancer drugs is still in process. A wide degree of families and classes of proteins involved in cancer have been disclosed so far, depicting a blurred image of the complexity of this disease.

In this study, we have tried to target some of the main hallmarks of cancer and some of the main hurdles that account for the failure of maximum anti-cancer drugs. In order to do this, we have used a plant – derived compound, Embelin for the entire study, based on the initial screening as an anti-mortalin molecule.

In this chapter, we target the large variety of growth factors that are involved in the process of cancer and understand the effect on their expression levels with effect of Embelin.

Growth factors are very critical in cancer progression, as a stimulated cancer cell begins to release a variety of growth inducing factors and also respond to the similar factors leading to the formation of an autocrine signaling mechanism, which leads to enhanced dis-regulation in proliferation signal, indicating the cell as cell for proliferation; in response to which the cell undergoes repetitive cycles of cell divisions. [39]

In the earlier chapters, we have witnessed that Embelin is capable of reducing percentage cell viability. And at lower dose, induce growth arrest (morphologically) and also reduce cell migration and cell invasion ability.

So, in order to understand the molecular mechanism underlying into these effects, we next checked the changes in the expression levels of variety of growth factors involved in cancer.

Introduction

It has been well quoted that the exposure of cancer cells to high levels of well-known growth factors significantly increase the risks of colorectal, breast, and prostate cancer to name a few.

Growth factors are proteins that are released from a cell. These proteins bind to their receptors present on the cell membrane, and this binding of the ligand and receptor leads to the initiation of a cascade of molecular events inside the cells, which leads to the expression of proteins that regulate cell proliferation. Therefore, the basic function of these growth factors is to enhance cell survival, to initiate proliferation and to enhance growth, as their name calls them, ‘growth factors’.

However, a misbalance in these growth factors can lead to enhanced or uncontrollable growth in cells that is also regarded as uncontrollable cell division or multiplication in cells. [39]

This is a common phenomenon occurring in the case of cancer cells, where the loss of depency for specific growth factors and the cells begin to manufacture their own Growth Factor synthesis ("autocrine" activation), this is a case where a cell starts generation of its own growth factors and starts responding to the similar growth factors leading to an enhanced autocrine signaling. [40]

Based on these understandings, in this chapter, we investigated the mechanism of action of Embelin’s anti-proliferative and anti-metastatic effect by analyzing the expression levels of growth factors involved in cancer.

Materials and Methods

Cells, Drugs and Cell Culture

The breast cancer cells studied were: MCF-7 and MDA-MB-231. Human Breast Carcinoma – MCF-7 (Adenocarcinoma), MDA-MB-231 (Adenocarcinoma) were purchased from Japanese Collection of Research Bioresources (JCRB, Japan) Cell Bank. MCF-7 and MDA-MB-231 were cultured in the DMEM (Dulbecco's Modified Eagle Medium) (Life Technologies, CA) that was supplemented with 10% FBS (fetal bovine serum) at 37 °C, in a humidified incubator (5% CO2 and 95% air).

Embelin was obtained purified from Sigma Aldrich Co., Japan. Embelin stocks were then, made by dissolving the adequate amount of it in dimethyl sulfoxide (DMSO) to obtain 1 mM stocks. These 1 mM stocks were aliquoted into tubes and were preserved at -20°C for further use. Concentrations were made by diluting the 1mM Embelin stock to the complete cell culture medium (DMEM) that was used for each experiment. All biochemical and imaging assays were performed at sub-confluent (60–70%) cultures.

Growth factors membrane array

For understanding the regulation of growth factors involved in cancer in response to absence and presence of Embelin, a human growth factor membrane array experiment was performed. Initially, the cells were cultured in 10-cm dish. Following overnight incubation, the cells were either supplemented with only medium or treated with Embelin for the next 48h. After completion of the incubation period, the culture media was harvested, to be used for the experiment. This culture media was centrifuged at full speed for 3 min.s to remove off any debris or cells. After centrifugation, the supernatant was harvested and used for the experiment.

For the membrane array experiment, the Human Growth Factor Antibody

Array kit – membrane by Abcam, Cambridge, UK was used, and the experiment was performed using manufacturer's guidelines. Briefly, each membrane was priorly blocked for 30 min.s at Room Temperature, following this; the membranes were incubated with the samples (cell culture supernatant) for 2h at the RT. The samples were then aspirated and the membranes were washed using the supplied washing buffers. Following this, the membranes were incubated with 1X Biotin-conjugated Anti-cytokines for 2h at RT. After the completion of the incubation, 1X Biotin-conjugated Anti-cytokines were aspirated and the membranes were washed as earlier. Then, 1X HRP-Conjugated Streptavidin was supplemented to each membrane – containing well containing a membrane and incubated for 2h at RT. Following the incubation time, the membranes were again washed using the washing buffer and then, visualized using chemiluminescence detection. The exposure images were recorded and the pixel calculation was done using ImageJ software (NIH). The interpretation of the results was performed acc. to the manufacturer's instructions.

Quantitative Real – Time Polymerase Chain Reaction (qRT-PCR)

To estimate the modulation at the mRNA level, quantitative Reverse Transcriptase PCR (qRT-PCR) was done. Total RNA extracted was reverse transcribed to cDNA, The resultant cDNA was subjected to amplification using gene specific primers. The analysis of the results was done using the relative analysis method of gene expression by the $2^{-\Delta\Delta C}$T method, as described by Kenneth J. Livak and Thomas D. Schmittgen [41] The gene specific primer sequences were as follows:

TGF-β	Sense	5′- CAATTCCTGGCGATACCTCAG-3′
TGF-β	Antisense	5′- GCACAACTCCGGTGACATCAA-3′
Wnt3a	Sense	5′- CAAGATTGGATCCAGGAGT -3′
Wnt3a	Antisense	5′- TCCCTGGTAGCTTTGTCCAG-3′
β-catenin	Sense	5′- AAAGCGGCTGTTAGTCACTGG -3′
β-catenin	Antisense	5′- GACTTGGGAGGTATCCACATCC -3′
Vimentin	Sense	5′- CCTTGAACGCAAAGTGGAATC-3′
Vimentin	Antisense	5′- GACATGCTGTTCCTGAATCTGAG -3′
18s (internal control)	Sense	5′-CAGGGTTCGATTCCGTAGAG -3′
18s (internal control)	Antisense	5′-CCTCCAGTGGATCCTCGTTA - 3′

Results

Embelin reduces the secretion levels of growth factors involved in cancer progression

To understand the mechanism of action of Embelin on cancer cells, we checked the expression status of various growth factors involved in cancer. Since, a majority of growth factors are secreted from the cells, therefore, their expression levels was analyzed by investigating the cell culture media. The cell culture media of both control and cells treated with Embelin was harvested and used for the assay. Upon analysis, it was indicated that out of 41 targets analyzed in the study, more than 15 growth factors were seen to be down regulated upon action with Embelin. (Figure 6 – 1)

Reduction of TGF-β by Embelin is through transcriptional down - regulation

Understanding the above results, we next checked the transcriptional status of these two genes. Upon quantitative Real time PCR analysis, it was observed that transcription of TGF-β was significantly down regulated in cells that were treated with Embelin when compared to those that were not treated with Embelin. Thus, explaining the enhanced decline in the secretion levels of TGF-β in the Embelin treated cells. (Figure 6 – 2, 6 – 3)

Embelin reduces the process of Epithelial to Mesenchymal transition (EMT) by down-regulating Wnt, β-catenin and Vimentin

Epithelial to Mesenchymal Transition (EMT) is basically a developmental process. However, now EMT has been known to enhance the process of metastasis, which involves invasion. [60,61] Cancer cells present in primary tumor lose their cell-cell binding causing an increase in invasive properties, which help them to break through the basement membrane. Moreover, Embelin induced down – regulation of TGF- β, which plays a prime role in epithelial to

mesenchymal Transition. Embelin was so far also seen to reduce the levels of MMP.s. Based on these observations, we thought it would be worthwhile to study the effects of Embelin of the process of Epithelial to Mesenchymal transition, a process that is so closely linked in the regulation of Metastasis. For this, we studied the effect of Embelin on the regulation of three key regulators of the EMT process, Wnt, β-catenin and Vimentin.

The regulation of Wnt, β-catenin and Vimentin was investigated by quantitative real time PCR analysis. Embelin was seen to down – regulate, Wnt in the treated cells, when compared to the control. (Figure 6-4) To confirm the suppression in the process of EMT, we investigated the regulation of its down-stream regulators, β-catenin and Vimentin, which were also down regulated at the transcriptional level. (Figure 6-5, 6)

Discussion

The role of Growth factors in cancer progression has been well stated now. One of the first lines of evidence associating cancer with soluble growth factors (GFs) emerged from studies performed in the 1950s, in the laboratory of Victor Hamburger, by two fellows, Rita Levi-Montalcini and Stanley Cohen.

Growth factors are known to enhance cell proliferation and is said to be essential for the survival of any type of cells. However, the over-reception of ligands such as growth factors leads to initiation of increased or uncontrollable proliferation signaling in a cell. This leads to cells to divide in an uncontrollable manner, which is also a common case in cancer.[39]

However, in most cases, the cells rely on other cells for receiving growth factors like signals. But, in the exceptional case of tumor cells, is it observed that the cells derived from human tumors, often secrete Growth Factors, which are responsible for self-stimulation (autocrine) of growth.

Based on this speculations and understandings, the expression level of different growth factors involved in cancer was checked. Embelin was seen to significantly reduce levels of several growth factors at the secretion level. The regulation of the modulated factors was then checked at both translational and transcriptional level, where TGF-β was down regulated. Moreover, as TGF- β is significantly involved in EMT, we checked the regulation of other important regulators of EMT, Wnt, β-catenin and Vimentin, which were also down regulated.

These findings bring some light on the mechanism of anti-proliferatory and anti-metastatic action of Embelin on cancer cells.

9. CHAPTER VII

TACE is a target of Embelin

Abstract and Introduction

Embelin is type of benzo-quinone compound, commonly found in the fruits of Embelia ribes Burm. f. (Primulaceae). Embelin exhibits a variety of therapeutic properties such as Anti-Diabetic, Anti-Bacterial, Anti-Tumor, Anti-Inflammatory and Anthelmintic.

The anti-inflammatory response of Embelin is known to be mainly due to its inhibitory effect against tumor – necrosis factor – alpha. TACE is a key proteolytic enzyme that leads to generation of tumor-necrosis factor-alpha. Therefore, the study in this chapter was performed to check if there is an existing link between tumor necrosis factor alpha and TACE.

Materials and Methods

Cells, Drugs and Cell Culture

The breast cancer cells studied were: MCF-7 and MDA-MB-231. Human Breast Carcinoma – MCF-7 (Adenocarcinoma), MDA-MB-231 (Adenocarcinoma) were purchased from Japanese Collection of Research Bioresources (JCRB, Japan) Cell Bank. MCF-7 and MDA-MB-231 were cultured in the DMEM (Dulbecco's Modified Eagle Medium) (Life Technologies, CA) that was supplemented with 10% FBS (fetal bovine serum) at 37 °C, in a humidified incubator (5% CO2 and 95% air).

Embelin was obtained purified from Sigma Aldrich Co., Japan. Embelin stocks were then, made by dissolving the adequate amount of it in dimethyl sulfoxide (DMSO) to obtain 1 mM stocks. These 1 mM stocks were aliquoted into tubes and were preserved at -20°C for further use. Concentrations were made by diluting the 1mM Embelin stock to the complete cell culture medium (DMEM) that was used for each experiment. All biochemical and imaging assays were performed at sub-confluent (60–70%) cultures.

Immunoblotting

To identify the effect on expression level of proteins in effect to that of Embelin – treatment, Immunoblotting technique was used. Cells that were priorly treated and not-treated with Embelin were prepared as protein lysates samples for loading in SDS – PAGE gels. Equal amounts of whole cell protein samples from control and treatment groups were seperated on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane. The blots were incubated with the desired primary antibodies (TACE, MMP- 9, MMP- 2, VEGF) followed by their respective secondary antibodies. Anti β-actin antibody was used as internal loading control. Quantitation of western blots was done by ImageJ software (NIH, MA).

TACE activity assay

In order to understand the effect of Embelin on cellular TACE activity, a TACE activity assay was performed. Cells in 6-well dish were plated at a concentration of 5×10^4/well. Following overnight incubation, cells were incubated with Embelin for 24 h, and then, rinsed with 1X PBS. The cells were then used for studying the cellular TACE activity .

TNF-α ELISA

An Enzyme-Linked Immunosorbent Assay technique was used to estimate functional TNF-alpha levels, in control and Embelin – treated cells. Briefly, Cell suspension from cells with or without treated were tested for secreted tumor-necrosis factor – alpha levels.

*RT-PCR (*Reverse Transcriptase Polymerase Chain Reaction)

To estimate the modulation at the mRNA level, Reverse Transcriptase PCR (RT-PCR) was done. Total RNA extracted was reverse transcribed to cDNA, The resultant cDNA was subjected to amplification using gene specific primers.
PCR products were separated on 1% agarose gel, which was visualized by Gel green staining. Pixel calculation of PCR results was done using with ImageJ and statistical analysis was done out using Student's t test, where p values scores ≤0.05 was considered significant. The gene specific primer sequences were as follows:

TNF-α	Sense	5′-GGAGAAGGGTGA CCGACTCA-3′
TNF-α	Antisense	5′- CTGCCCAGACTCGGC AA-3′
TGF-α	Sense	5′-CACACTCAGTTCTGCTTCCA-3′
TGF-α	Antisense	5′-TCAGACCACTGTTTCTGAGTGGC- 3′

AREG	Sense	5′ GACCTCAATGACACCTACTCTGG- 3'
AREG	Antisense	5′-AAATATTCTTGCTGACATTTGC-3’
GAPDH *(internal control)*	Sense	5′-ACCTGACCTGCCGTCTAGAA-3′
GAPDH *(internal control)*	Antisense	5′-TCCACCACCCTGTTGCTGTA-3′

Immuno – cytochemical staining

For immuno - fluorescence or immuno – cytochemical staining, cells were cultured in 12-well dish containing coverslips. Control and cells from treatment groups were fixed, and stained with primary and secondary antibodies of interest for the study. In this study, antibodies for Mortalin and p53 were used. Coverslips containing stained cells, were mounted and observed using Carl Zeiss microscope (Axiovert 200 M).

Results

Embelin reduces cellular TACE expression levels

Embelin treatment to cancer cells was seen to significantly reduce cellular TACE expression levels as observed by experimental techiniques of western blotting and immuno-cytochemistry. (Figure 7-2)

Embelin reduces TACE Activity

For the next stage, effect of Embelin on TACE activity. Upon analysis, following Embelin treatment, a significant decline in TACE activity was observed. Moreover, Embelin at 20μM concentration showed even more significant effect than the commercially available TACE inhibitor. (Figure 7-3).

Embelin treatment leads to reduction in TNF-α expression levels

Furtheron, the resultant decline in TACE activity and cellular TACE levels by Embelin further led to significant decrease in TNF-α levels, as observed by TNF-α ELISA. (Figure 7-4)

Embelin causes down-regulation of TACE at a translational level

The decrease of TACE expression levels by Embelin was seen at only at translational level, as Embelin seemed to effect only yhe protein levels of TACE and not the mRNA levels of TACE. Morevoer, mRNA levels of TACE downstream targets were also not affected. (Figure 7-5)

Down-regulation of TACE does not directly effect the transcriptional activation of its down-stream targets

We had learned so far that Embelin is capable of reducing the cellular TACE levels, which leads to decline in its functional activity and thereby, causing a reduction in the TNF-α levels. So, we wanted to investigate whether down-regulation of TACE affected the transcriptional levels of its down-stream targets. Therefore, the mRNA level of TACE down-stream targets, TNF-α, TGF-α and AREG was checked using the technique of RT-PCR. However, no significant decrease in RNA levels was observed in Embelin - treated cells with compared to control, showing that there may be other factors responsible for maintaining the RNA levels of these genes or TACE levels do not affect their transcriptional levels directly. (Figure 7-6)

Embelin down – regulates the expression levels of Matrix Metalloproteinases

In light of this data, and as we have earlier witnessed the reduction of cell migration and cell invasion ability in the cancer cells, when they were treated with Embelin, as compared to control. We next examined the expression of metastatic markers that include Matrix Metalloproteinases. As Matrix Metalloproteinases are superiorly involved in enhancing the cancer cell invasion and cancer cell migration and Embelin treatment earlier significantly reduced cell invasion and cell migration ability. We next checked the expression levels of MMP proteins. Amongst the family of MMP proteins, MMP-2, MMP-9 in

control and treated cells was seen to show reduction (60% and 25% respectively) in their level of expression (Figure 7-7 A, B).

Embelin reduces the expression levels of Vascular Endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a signal protein. When it is produced, it stimulates vasculogenesis and angiogenesis. When VEGF is overexpressed in cancer cells, it can help the tumors by contributing in creating new blood vessels, leading to enhanced nutrient supply to tumors. This will help them the tumors to metastasize. Understanding the contribution of VEGF in cancer development and metastasis, we examined the expression of VEGF in control and treated cells and found reduction of about 48% in their level of expression (Figure 7-8 A, B).

Embelin decreases the expression levels of hnRNP-K

Heterogeneous nuclear ribonucleoprotein – K (hnRNP-K) is a protein that has widely been studied in our lab, and is known to contribute to metastasis in cancer. We, next, examined the expression status of an upstream regulator, hnRNP-K that has also been shown to play critical role in cell migration. We found that in response to Embelin treatment there was a significant reduction in hnRNP-K (Figure 7-9 A, B, Figure 7-10), supporting its potency for treatment of metastatic cancer.

Discussion

In this chapter, I have found that Embelin exhibits inhibitory effect on TACE and multiple related proteins in the TACE involved pathway. An initial molecular dynamic study was performed, which showed that Embelin docks strongly into the active site of TACE, thereby, avoiding any other ligand to bind to its active site. The active site of TACE contains a zinc atom, coordinated by a zinc – binding motif. This complex of Embelin and TACE seemed very stable soon after 6ns of interaction. Molecular interactions with histidine and glutamate residues was observed which are known to play a significant role in managing the proteolytic functioning of TACE.

Further with this biochemical effect of Embelin was studied on human breast cancer cell lines. Following the biochemical analysis, Embelin was seen to reduce cellular TACE expression levels. This reduction in cellular TACE expression levels and in its activity by Embelin led to an enhanced decline in the cellular TNF-alpha expression levels. However, upon observing the mRNA levels of TACE down – stream targets, TNF-alpha, TGF-alpha and Amphiregulin, Embelin was not seen to affect their mRNA levels.

Moreover, expression levels of MMP-2 and MMP-9 proteins were seen to be down-regulated in cancer cells that were treated with Embelin as compared to that of their control counter –parts. Further on, hnRNP-K, which is known to be primly involved in enhancing the process of metastasis, and VEGF [57-59], an angiogenesis factor, was also observed to decrease in Embelin – treated cells when compared to control.

10. Conclusions & Future works

Conclusions & Future works

Cancer is a deadly disease. It accounts for millions of deaths worldwide. Finding a suitable drug for cancer is one of the major missions of cancer research.

Breast cancer is the second leading cause of deaths from cancer amongst women. The therapies available provide a temporary relief to the patients, which mainly target on the removal of a part of the tissue or the complete removal. However, the main problem arises when despite the removal of the primary tumor, the patient begins to witness the secondary tumors in other parts of the body. Chemotherapy comes to rescue for the patient in such circumstances.

However, chemotherapy targets the cancer by killing the cancer cells in the body, but in such a condition it also begins to target many other rapidly dividing cells in the patients' body, accounting for multiple side effects, suppressed immune response and along with failure to target highly invasive cancer cell that have been disseminated from the primary tumor.

Another problem arises when the prolonged exposure to chemotherapy, makes the cancer cells in a patient chemo – resistant. Failure to kill the cancer cells by extended chemotherapy along with multiple side – effects and occurrence of multiple tumors overall worsens the situation.

Based on these understandings, this study started with the idea for developing a potential anti-cancer drug that could possibly target the following criteria during its treatment mechanism:

- New candidate that would be better than the current conventional drugs.
- It would be safe to the patient and would not be an inducer of several toxic side effects.

- It could target both cancer cells in the primary tumor as well as the metastatic cancer cells that have been disseminated or are in the process of dissemination.
- It could over come the barrier of chemo resistance by targeting the heat shock family proteins that are specifically known to enhance cell survival ability by inducing chemo resistance.
- And, finally, to study the mechanism of action of such an anti-cancer potential drug.

Based on the above criteria, we initially screened multiple convention anti-cancer drugs and two nature derived compounds for their anti-mortalin potential. Based upon the screening results, Embelin was selected for the further study.

Embelin was seen to reduce the breast cancer cell proliferation with increasing dose. On analyzing the effect of its action, it was observed that Embelin induced growth arrest response in the cancer cells, at lower concentrations. Moreover, Embelin was also seen to reduce colony-forming ability in cancer cells, explain its anti-cancer potential.

Since, failure to effect the metastatic cancer cells accounts for the ineffectiveness of maximum anti-cancer drugs, so the anti-metastatic potential of Embelin was investigated, where Embelin reduced cancer cell migration and cancer cell invasion.

To understand the mechanism of action of Embelin, its effect on growth factors was analyzed where Embelin reduced expression levels of various growth factors. Moreover, TGF-β was also down regulated at the transcriptional level.

Moreover, as TNF-α is key protein in the process of cancer and inflammation, Embelin's effect on it was studied. TACE is known to release the soluble active form of TNF-α, therefore, TACE is considered a key therapeutic target in inflammation and cancer. Embelin reduced expression levels of TACE, which further led to a decline in the shedding activity of TACE, thus reducing the release of active TNF-α. A reduction in the levels of proteins involved in

angiogenesis, VEGF, and metastasis, MMP.s and hnRNP-K was also seen.

As in the start of this project seen, that Embelin was capable of reducing the expression levels of Mortalin. We investigated if this reduction at the transcriptional level, where Embelin was observed to significantly reduce the transcription of Mortalin. Moreover, Embelin also disrupted the Mortalin-p53 interaction, as observed by translocation of p53 to the nucleus.

As Epithelial to Mesenchymal transition (EMT) is an important step in the process of metastasis. The importance of Wnt signaling is well known in the process of EMT, therefore, the effect of Embelin on this signaling was observed. Embelin was seen to reduce the Wnt 3a. Followed by this its, downstream genes, β-catenin and Vimentin were also down regulated by Embelin.

Through the above observations, we understand that Embelin is a promising anti-cancer compound. And, with further investigation in its anti – cancer potential, this compound can be a very useful Bioindustrial product anti-cancer drugs. Since, Embelin is of plant origin, it is non – harmful, a criteria very important for the selection of various anti-cancer drugs. Moreover, this study started with Embelin's selection by assessing its anti-mortalin ability. Embelin also showed inhibitory effect against metastasis by targeting some key molecules involved in cancer.

We therefore propose Embelin as a promising Bioindustrial anti cancer compound, which requires further study.

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LIST OF PUBLICATIONS, PAPER AND POSTER PRESENTATIONS

Publications:

1. Renu Wadhwa, Nupur Nigam, Yoshiyuki Ishida, Sukriti Goyal, Priyanshu Bhargava, Abhinav Grover, Keiji Terao and Sunil C Kaul 'Molecular Characterization and Enhancement of Anticancer Activity of CAPE'. Molecular Carcinogenesis (Submitted)
2. Renu Wadhwa, Didik Priyandoko, Ran Gao, Nashi Widodo, Nupur Nigam, Ling Li, Hyo Min Ahn, Chae-Ok Yun, Nobuhiro Ando, Christian Mahe 'Stress chaperone mortalin regulates human Melanogenesis' Cell Stress and Chaperones (Under Minor Revision)
3. Nupur Nigam, Abhinav Grover, Sukriti Goyal, Shashank P. Katiyar, Priyanshu Bhargava, Pi-Chao Wang, Durai Sundar, Sunil C. Kaul and Renu Wadhwa, 'Targeting of mortalin by Embelin causes activation of tumor suppressor p53 and deactivation of metastatic signaling in human breast cancer cells'. PLOS ONE, DOI: 10.1371/journal.pone.0138192, September 16, 2015
4. Jaspreet Kaur Dhanjal†, Nupur Nigam†, Sudhanshu Sharma, Anupama Chaudhary, Sunil C Kaul, Abhinav Grover and Renu Wadhwa, 'Embelin inhibits TNF-α converting enzyme and cancer cell metastasis: molecular dynamics and experimental evidence' BMC Cancer, 14:775, 1471-2407, 10, 2014. †: Equal contribution.

Presentations (Oral & Posters):

Oral:

1. Nupur Nigam, Pi Chao Wang, Sunil Kaul, Renu Wadhwa, 'Molecular mechanism of anticancer activity in Embelin, a quinone derivative of Embelia ribes', 6th ISAJ Conference, Tokyo, Japan, 2015.
2. Nupur Nigam, Li Ling, Yue Yu, Pi Chao Wang, Sunil Kaul, and Renu Wadhwa, 'Mortalin Expression level as an indicator of Anti-metastasis potential of Breast Cancer drugs', DAILAB CAFÉ PLUS - 01, DAILAB Biomedical Research Institute, AIST, Tsukuba, Japan, 2015. 'AWARDED – Best Presentation Award'
3. Nupur Nigam, Li Ling, Yue Yu, Pi Chao Wang, Sunil Kaul, and Renu Wadhwa, 'Mortalin Expression level as an indicator of Anti-metastasis potential of Breast Cancer drugs', 5th India – Japan Symposium, ISAJ, Tokyo, Japan, 2014.

4. Nupur Nigam, Li Ling, Yue Yu, Pi Chao Wang, Sunil Kaul, and Renu Wadhwa, 'Mortalin Expression Level as an Indicator of Anti-metastasis Potential of Breast Cancer Drugs', IRAGO Conference Graduate Student Session, Tsukuba, Japan, 2014.
5. Manish Biyani, Nupur Nigam, 'On- chip cell analysis system to study cancer preventive phytochemical in Indian herbal plant extracts", 99th Indian Science Congress, KIIT University, Bhubaneshwar, India, 2012.
6. Nupur Nigam, 'High throughput on-chip cell analysis system to study cancer chemoprevention', BICON – 11, Biyani Institute of Science and Management, Jaipur, India, 2011.
7. Manish Biyani, Nupur Nigam, 'High – Throughput on – chip cell analysis to study cancer chemoprevention', 34th Annual meeting - Molecular Biology Society of Japan, Yokohama, Japan, 2011.

Poster:

1. Nupur Nigam, Pi Chao Wang, Sunil Kaul, Renu Wadhwa, 'Molecular mechanism of anticancer activity in Embelin, a quinone derivative of Embelia ribes', 6th India-Japan Symposium, ISAJ, Tokyo, Japan, 2015 (Selected Posters from the Oral presentations).
2. Priyanshu Bhargava, Nupur Nigam, Yoshiyuki Ishida, Keiji Terao, Renu Wadhwa, Sunil C Kaul, 'Caffeic acid phenethyl ester (CAPE) possesses anticancer activity: Molecular Characterization and its enhancement with γCD', 6th India-Japan Symposium, ISAJ, Tokyo, Japan, 2015.
3. Nupur Nigam, Pi Chao Wang, Sunil Kaul, Renu Wadhwa, 'Molecular mechanism of anticancer activity in Embelin, a quinone derivative of Embelia ribes', DAILAB Biomedical Research Institute, AIST, Tsukuba, Japan, 2014. 'AWARDED – Best Poster Presentation Award'
4. Nobuhiro Ando, Rumani Singh, Sunil Kaul, Nupur Nigam, Christian Mahe, Renu Wadhwa, 'Molecular Characterization Of The Whitening Effect Of Tranexamic Acid Cetyl Ester (Txc) Using Human Melanoma And Primary Melanocytes', XXII International Pigment Cell Conference, Singapore, 2014.
5. Sunil Kaul, Ran Gao, Nupur Nigam, Ling Li, Renu Wadhwa, 'Ashwagandha-Derived Phytochemicals Possess Skin Whitening Potential: Evidence From Human Cell Based Assays', XXII International Pigment Cell Conference, Singapore, 2014.

6. Renu Wadhwa, Nashi Widodo, Navjot Shah, Nupur Nigam, Tomoko Yaguchi, Nobuhiro Ando, Christian Mahe, Sunil Kaul, 'Integrated Cell Based ShRNA Screening And Bioinformatics Approaches Identified Mitochondrial Stress Chaperones Hsp60 And Mortalin As New Regulators Of Melanogenesis', XXII International Pigment Cell Conference, Singapore, 2014.
7. Nupur Nigam, Pi Chao Wang, Sunil Kaul, Renu Wadhwa, 'Molecular mechanism of anticancer activity in Embelin, a quinone derivative of Embelia ribes', 87th annual meeting of JAPANESE TISSUE CULTURE ASSOCIATION (JTCA), Japan, 2014.
8. Rumani Singh, Nupur NIGAM, Caroline Cheung, Chao-Ok Yun, Sunil Kaul, Renu Wadhwa, 'p53 compromised cells escape CARF overexpression mediated growth arrest of cancer cells', 4th India-Japan Symposium, ISAJ, Tokyo, Japan, 2013.
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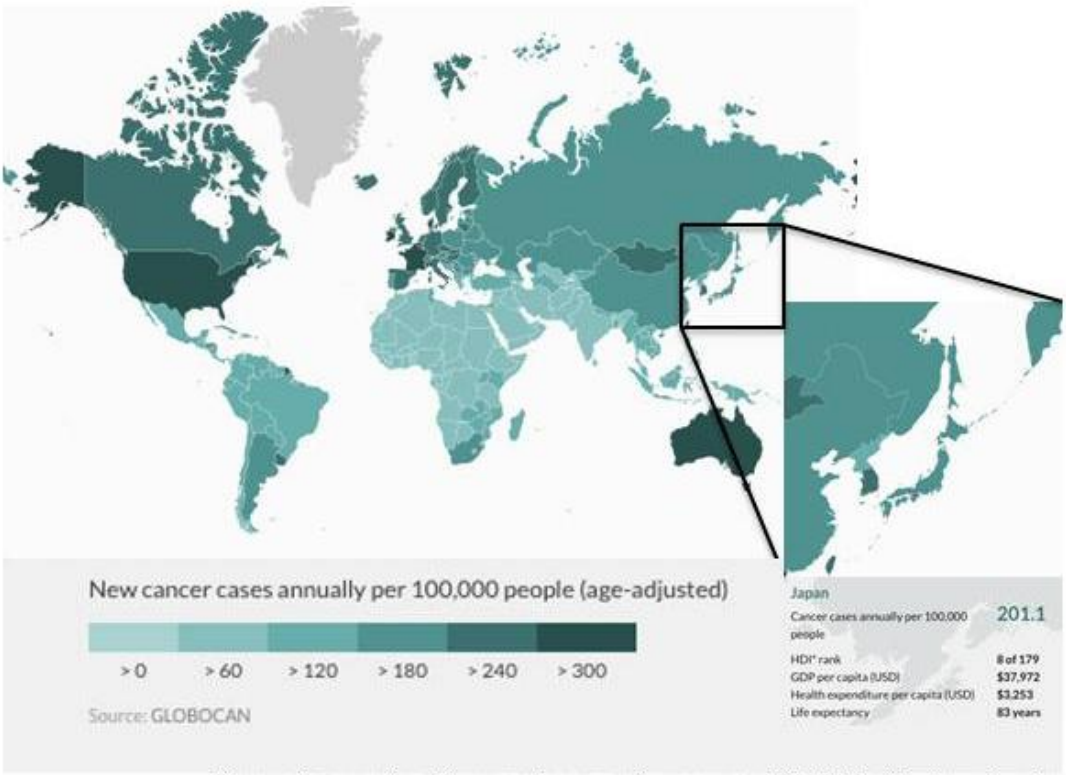
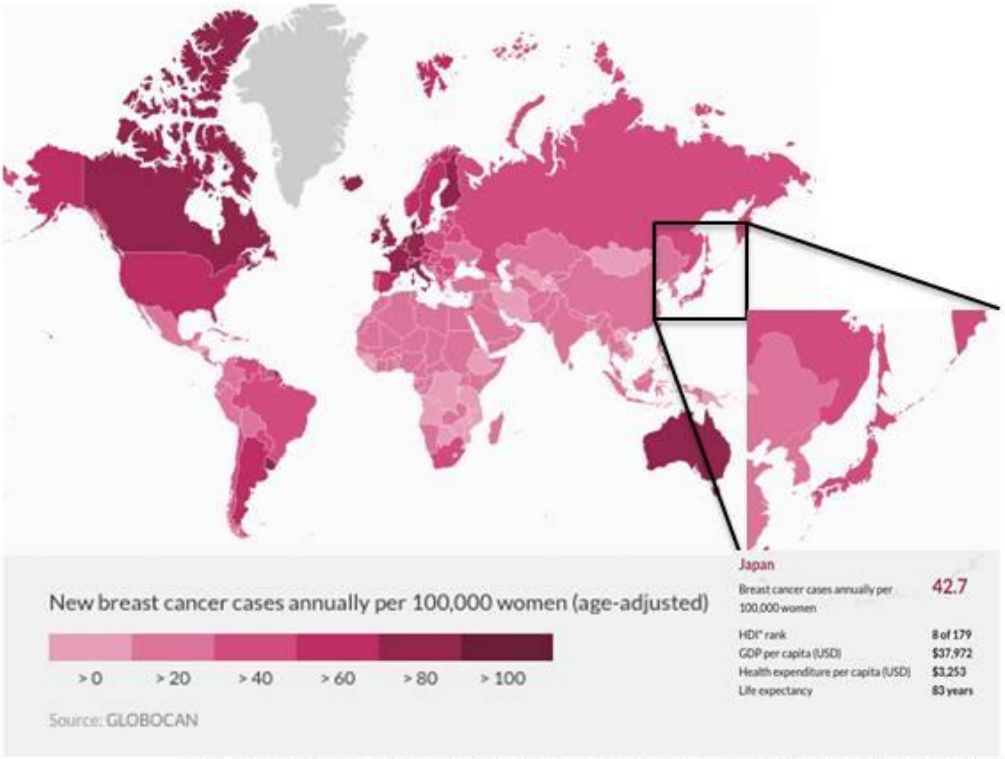
Figures: Chapter 1- Figure 1



Source: International Agency for research on cancer, World Health Organization

Figure 1-1 World Cancer statistics (regional) color indicated for showing the number of new cases annually per 100,000 people region. In the box, highlighted incidence of cancer in Japan.

Figures: Chapter 1- Figure 2



Source: International Agency for research on cancer, World Health Organization

Figure 1-2 World Breast Cancer statistics (regional) color indicated for showing the number of new cases annually per 100,000 people region. In the box, highlighted incidence of cancer in Japan.

Figures: Chapter 1- Figure 3

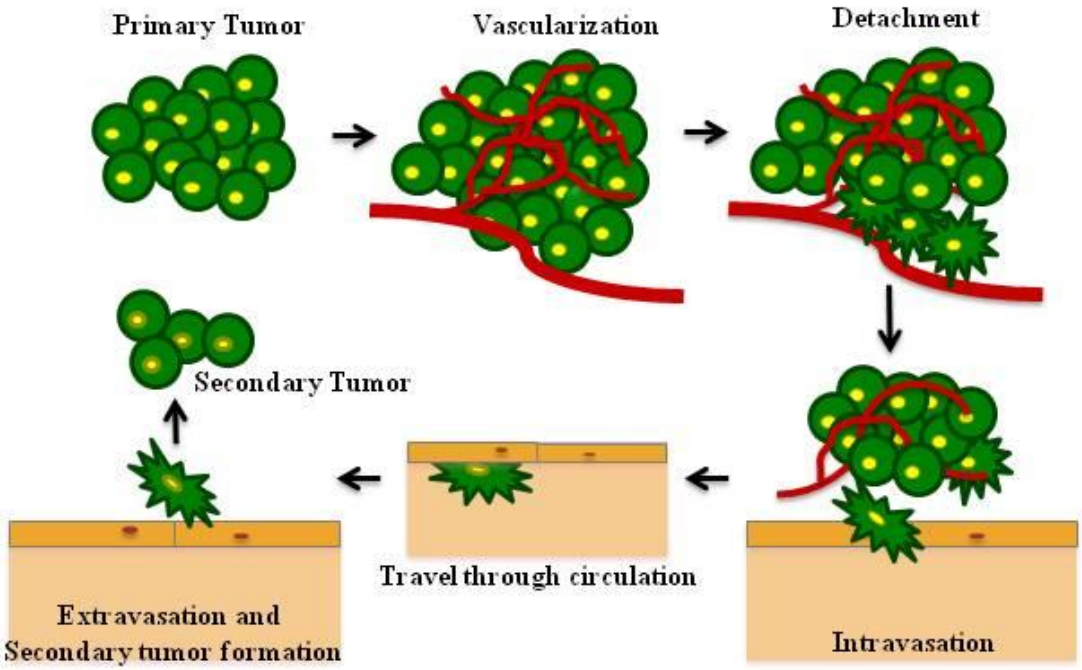


Figure 1-3 Schematic representation of the process of metastasis

Figures: Chapter 1- Figure 4

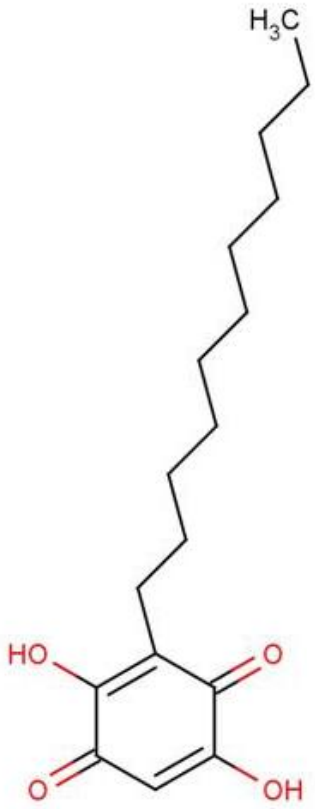


Figure 1-4 Chemical structure of Embelin

Figures: Chapter 2- Figure 1

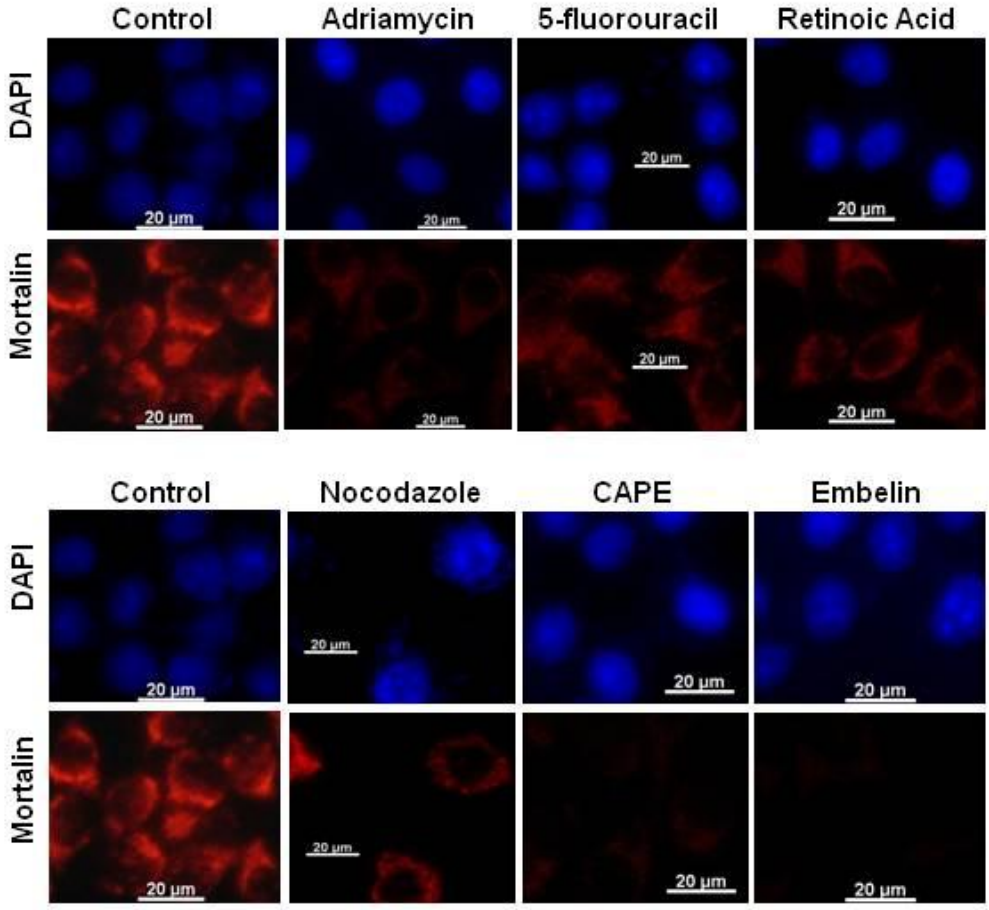


Figure 2-1 Mortalin expression levels in MCF7 cells as seen by immunocytochemistry in control and treated cells

Figures: Chapter 2- Figure 2

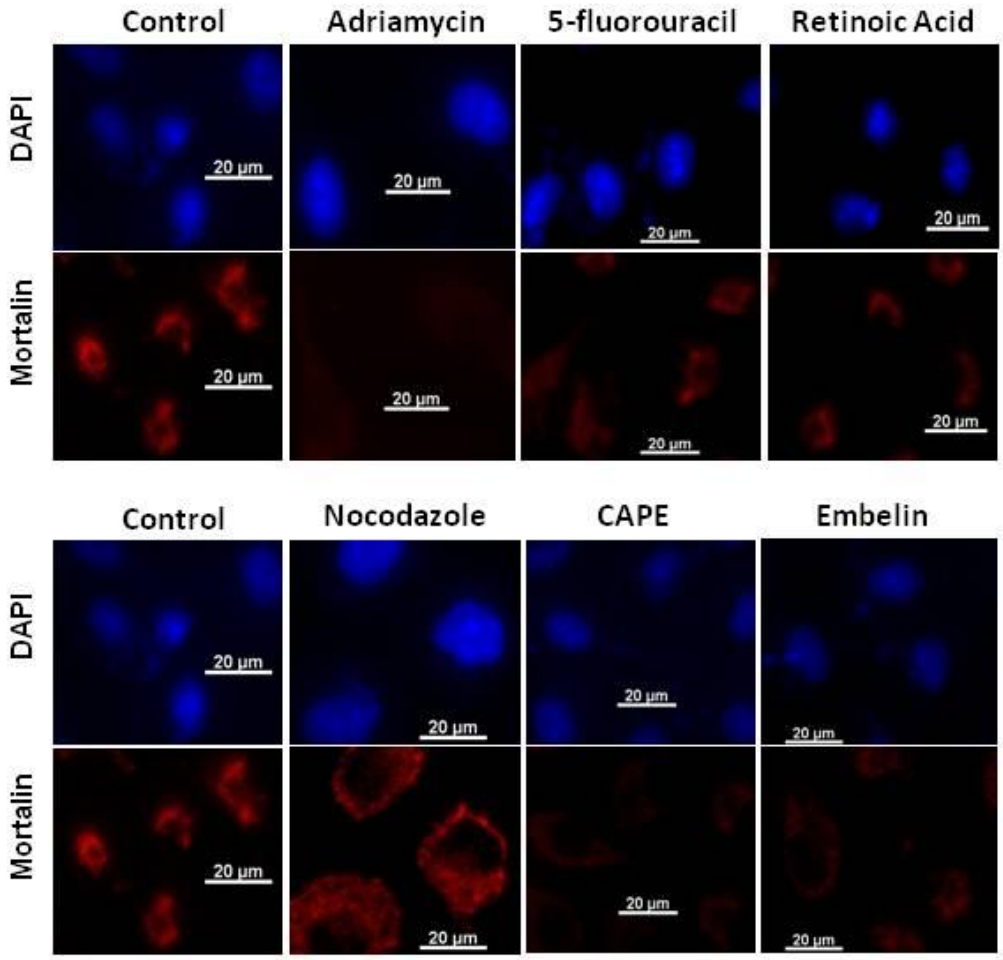


Figure 2-2 Mortalin expression levels in MDA-MB-231 cells as seen by immunocytochemistry in control and treated cells

Figures: Chapter 2- Figure 3

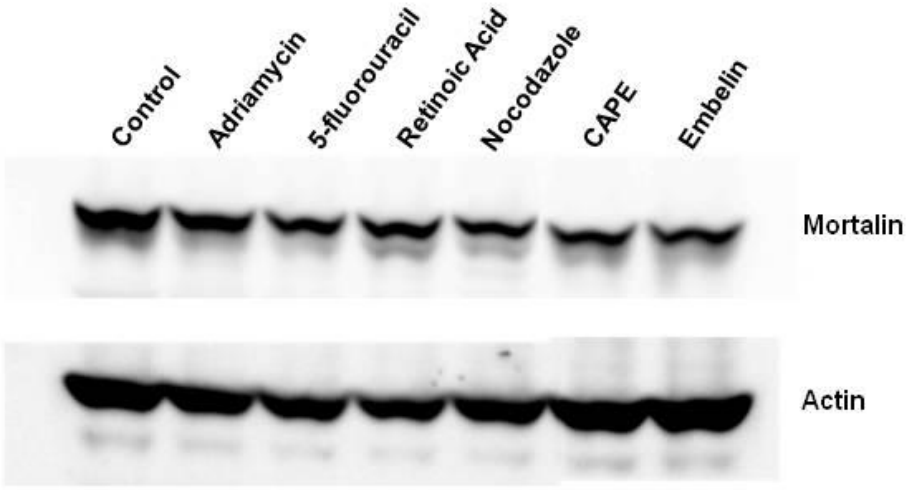


Figure 2-3 Reduction in cellular Mortalin expression levels as seen by western blotting in control and treated cells

Figures: Chapter 2- Figure 4

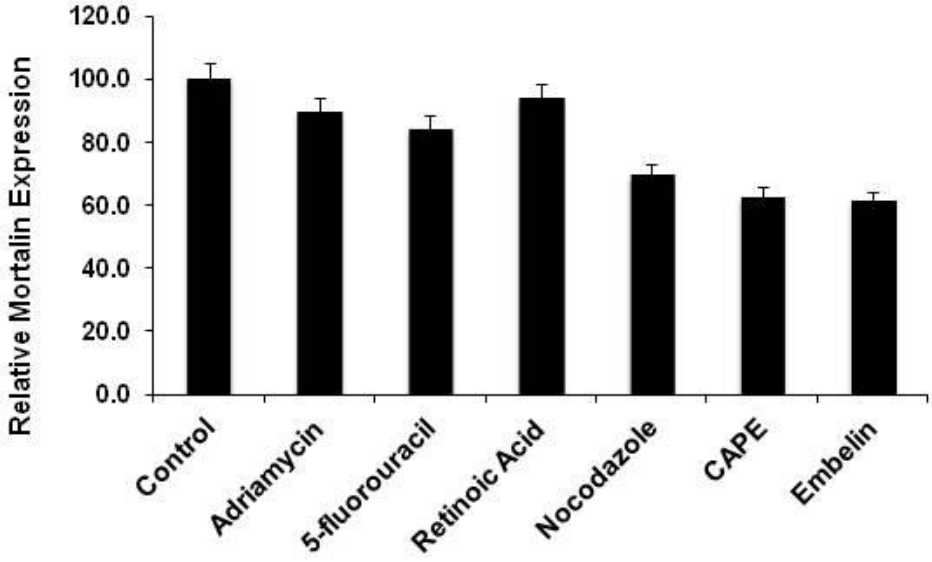


Figure 2-4 Relative Mortalin expression levels in control cells and in cells treated with indicated treatments

Figures: Chapter3- Figure 1

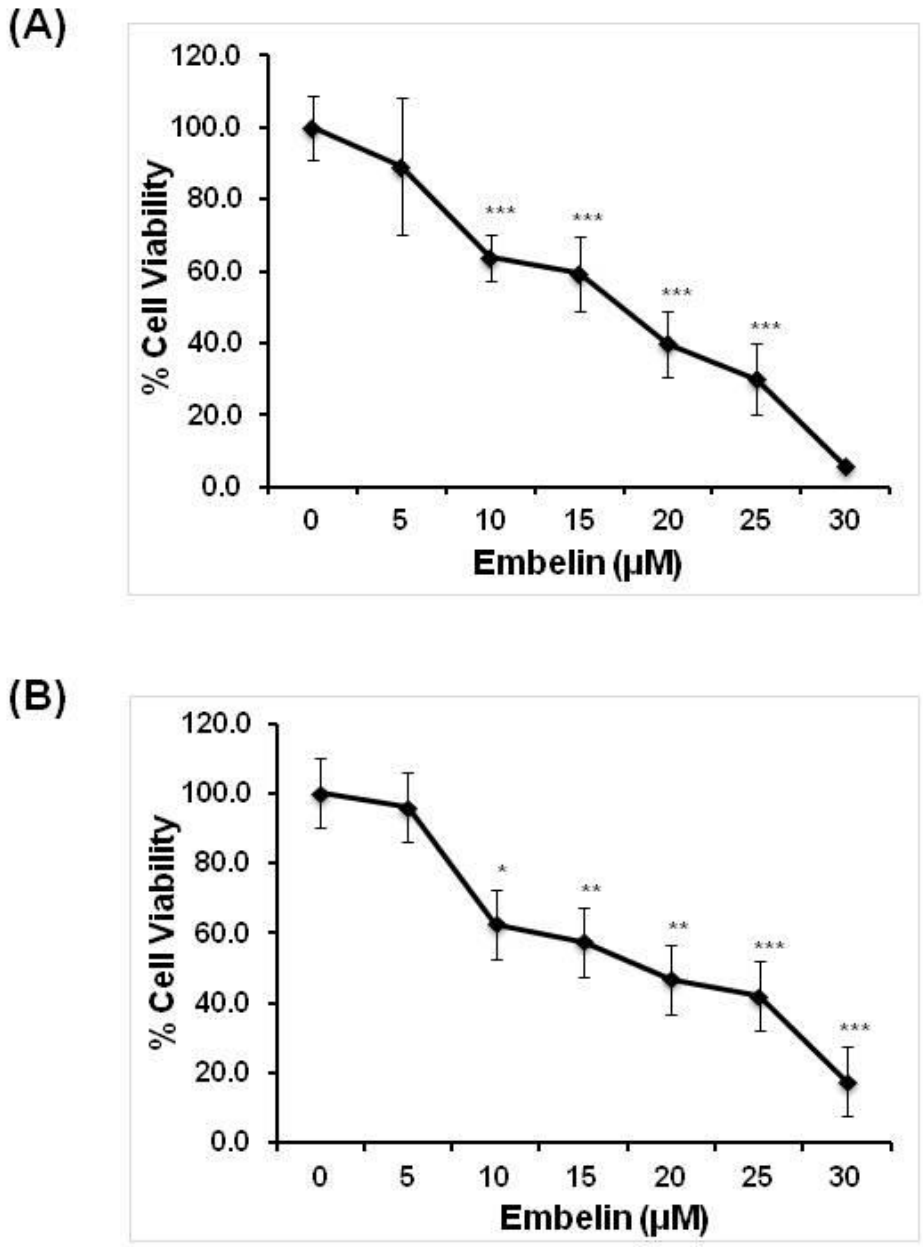


Fig. 3 – 1 Anti-proliferative cytotoxic effect of Embelin on Human Breast cancer cells, MCF7 (A) and MDA-MB-231 (B)
[Plotted average of three individual experiments]

Figures: Chapter3- Figure 2

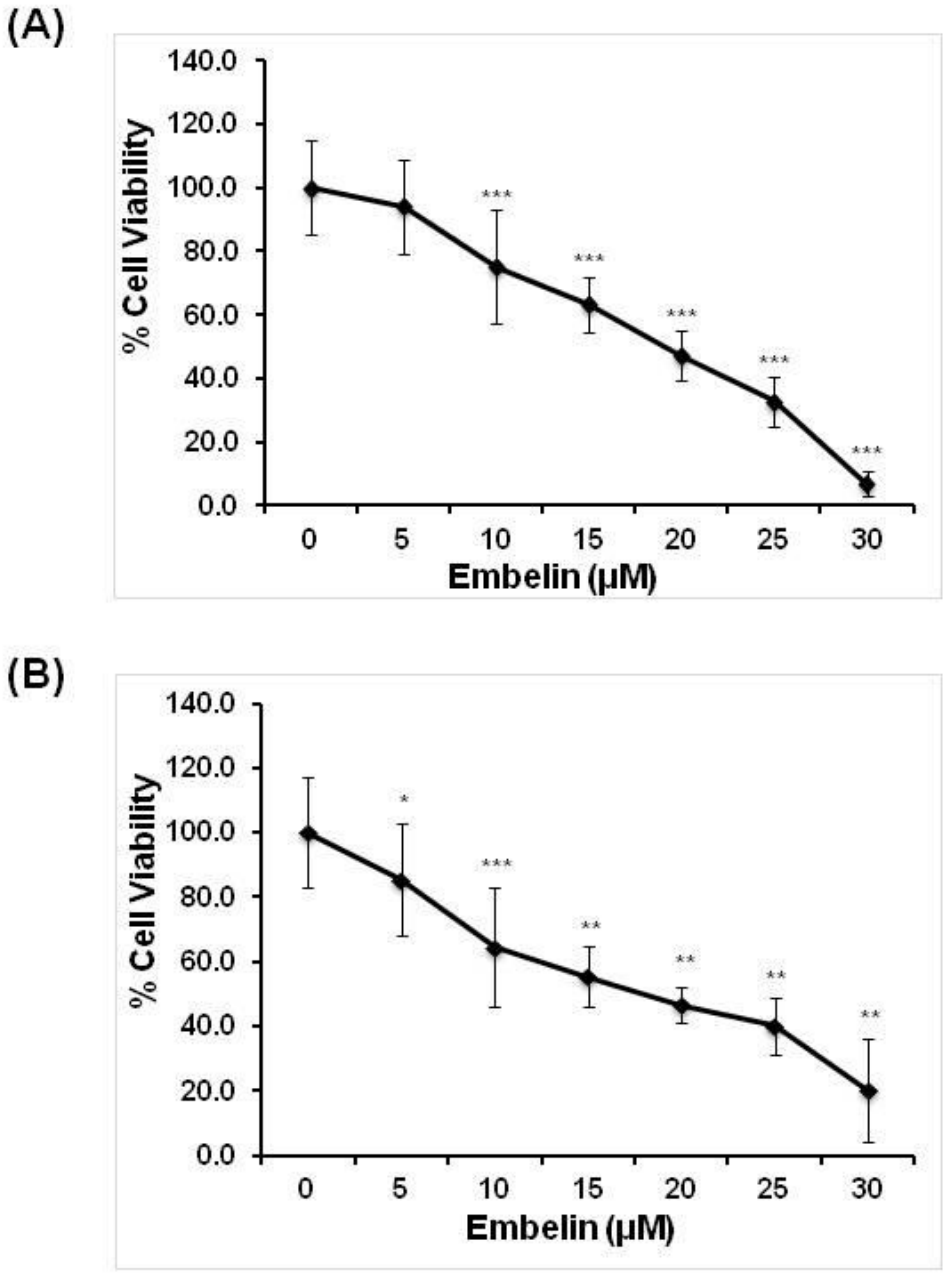


Fig. 3 – 2 Anti-proliferative cytotoxic effect of Embelin on highly metastatic derivatives of Human Breast cancer cells, MCF7 (A) and MDA-MB-231 (B)
[Plotted average of three individual experiments]

Figures: Chapter3- Figure 3

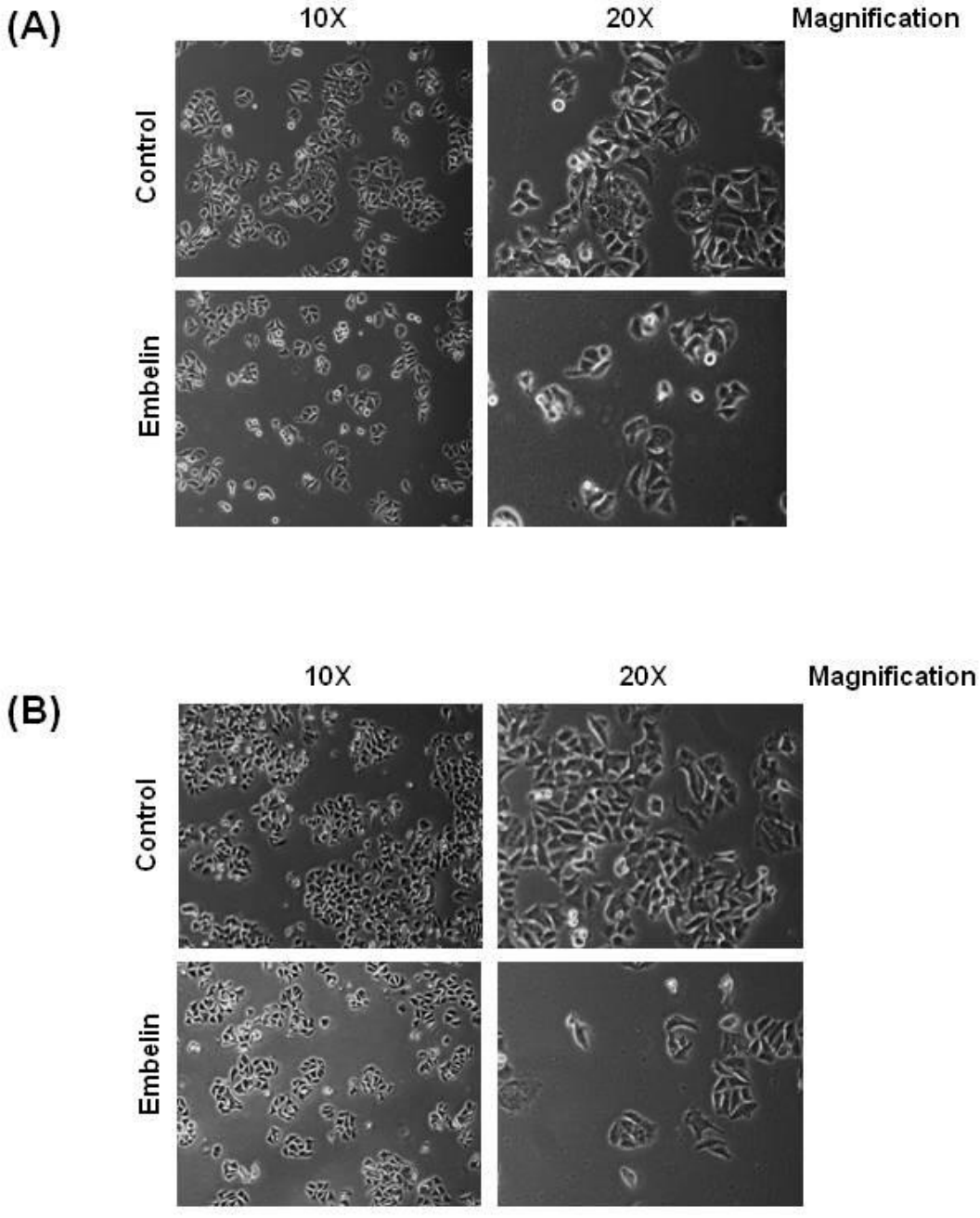


Fig. 3 – 3 Morphological observations of the Anti-proliferatory response of Embelin in Human Breast cancer cells, MCF7 after an incubation of 24h (A) and 48h (B)

Figures: Chapter3- Figure 4

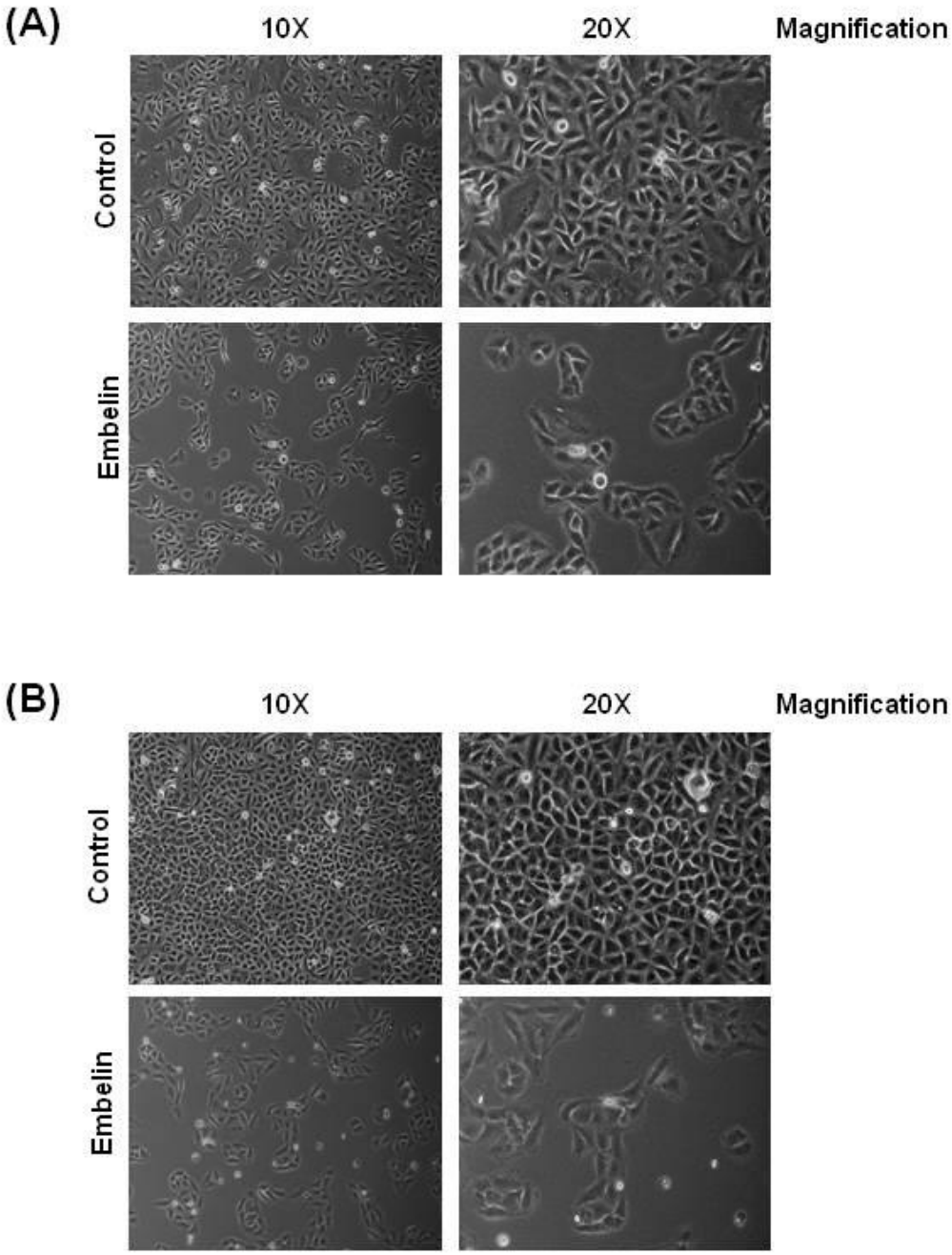


Fig. 3 – 4 Morphological observations of the Anti-proliferatory response of Embelin in Human Breast cancer cells, MDA-MB-231 after an incubation of 24h (A) and 48h (B)

Figures: Chapter3- Figure 5

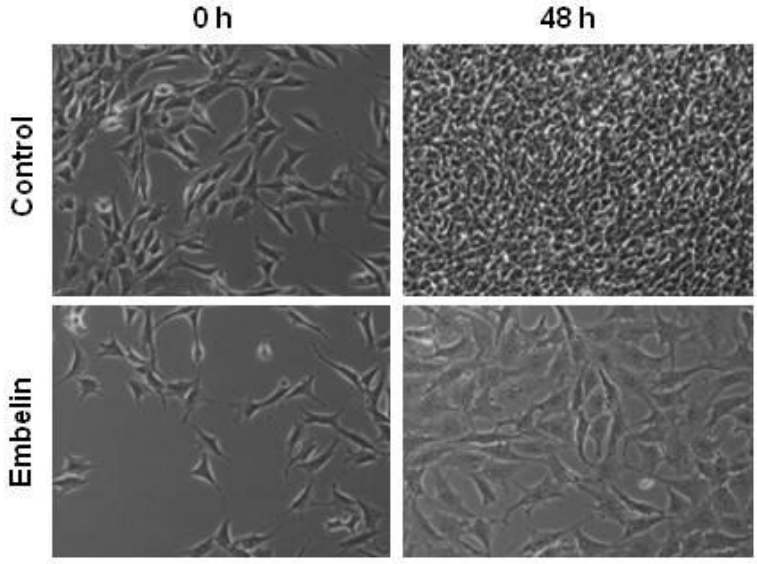


Fig. 3 – 5 Morphological observations of the Anti-proliferatory response of Embelin in highly metastatic derivatives of Human Breast cancer cells, MCF7

Figures: Chapter3- Figure 6

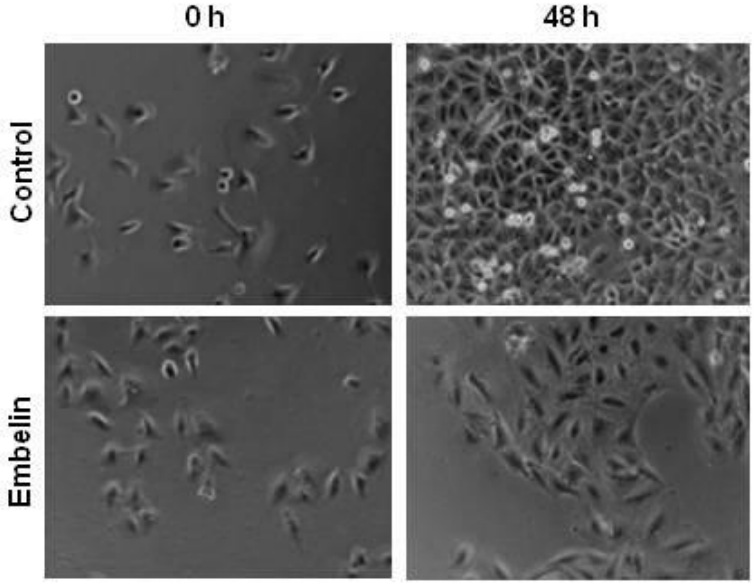


Fig. 3 – 6 Morphological observations of the Anti-proliferatory response of Embelin in highly metastatic derivatives of Human Breast cancer cells, MDA-MB-231

Figures: Chapter3- Figure 7

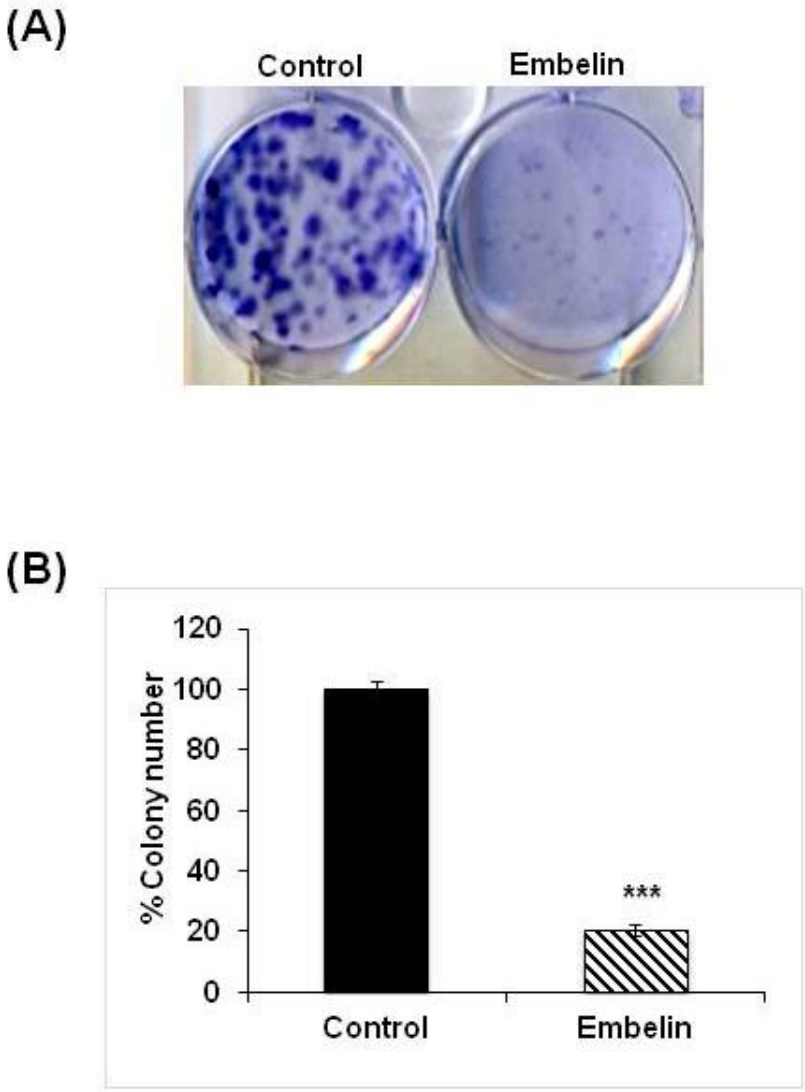


Fig. 3 – 7 Embelin inhibits the efficiency of a cancer cell to develop its clones in Human Breast cancer cells, MCF7 (A) and its quantitative analysis (B)

Figures: Chapter3- Figure 8

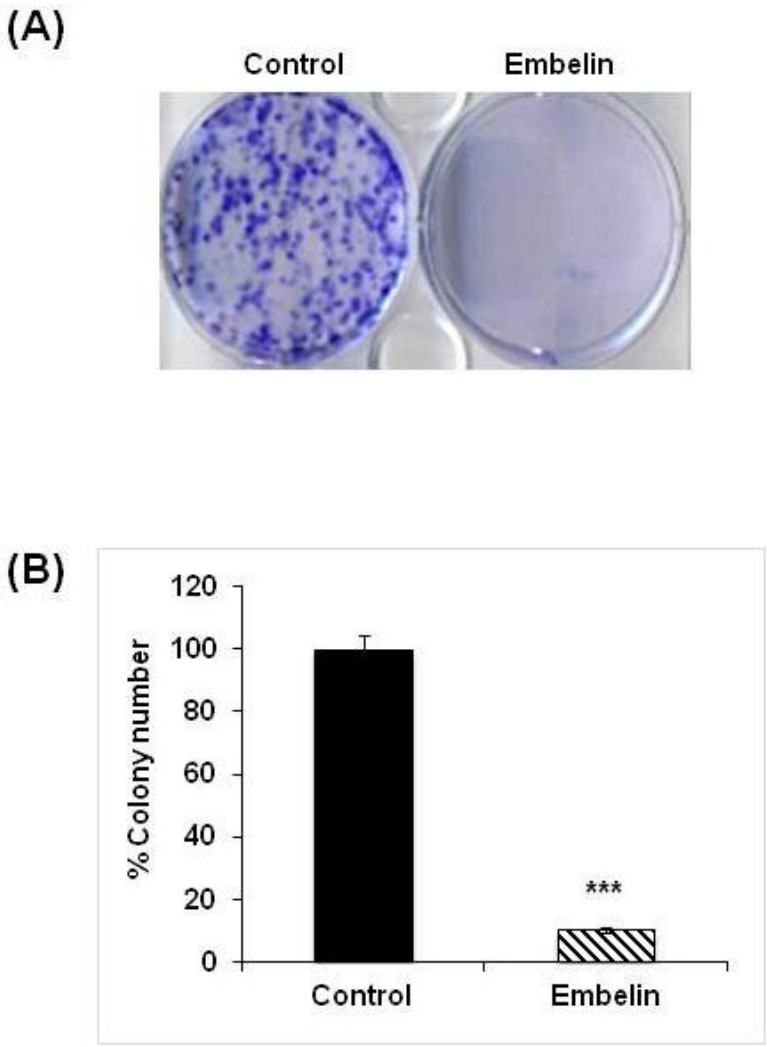


Fig. 3 – 8 Embelin inhibits the efficiency of a cancer cell to develop its clones in Human Breast cancer cells, MDA-MB-231 (A) and its quantitative analysis (B)

Figures: Chapter3- Figure 9

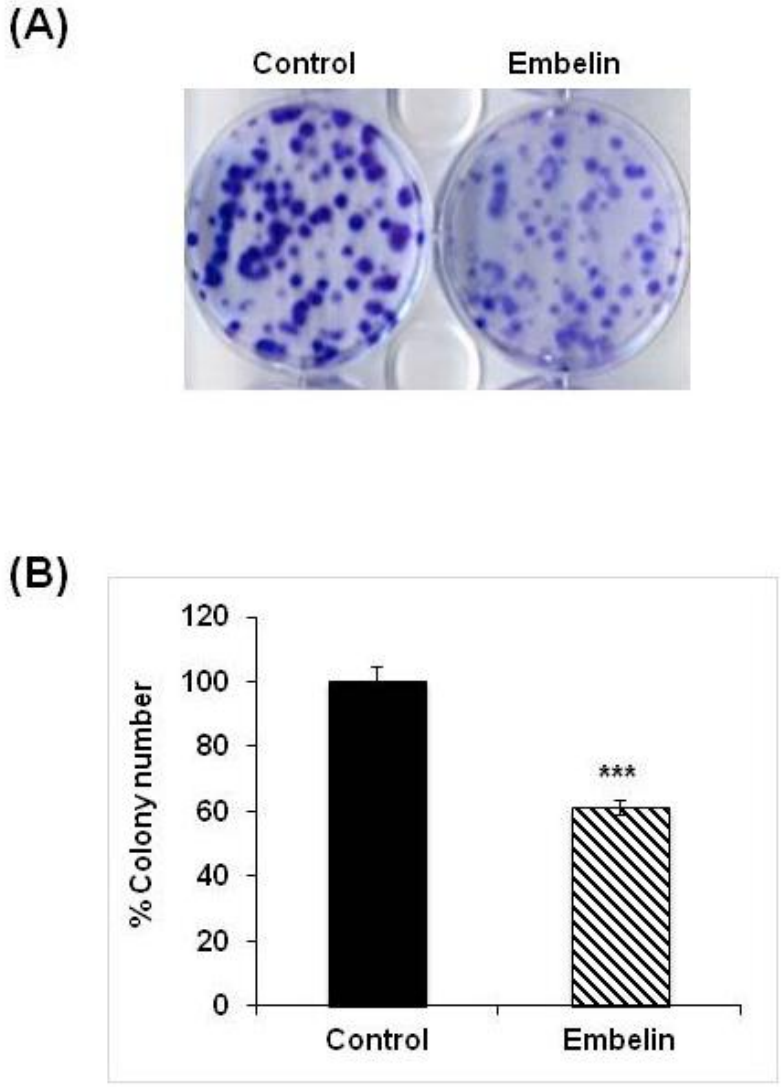


Fig. 3 – 9 Embelin inhibits the efficiency of a cancer cell to develop its clones in highly metastatic derivatives of Human Breast cancer cells, MCF7 (A) and its quantitative analysis (B)

Figures: Chapter3- Figure 10

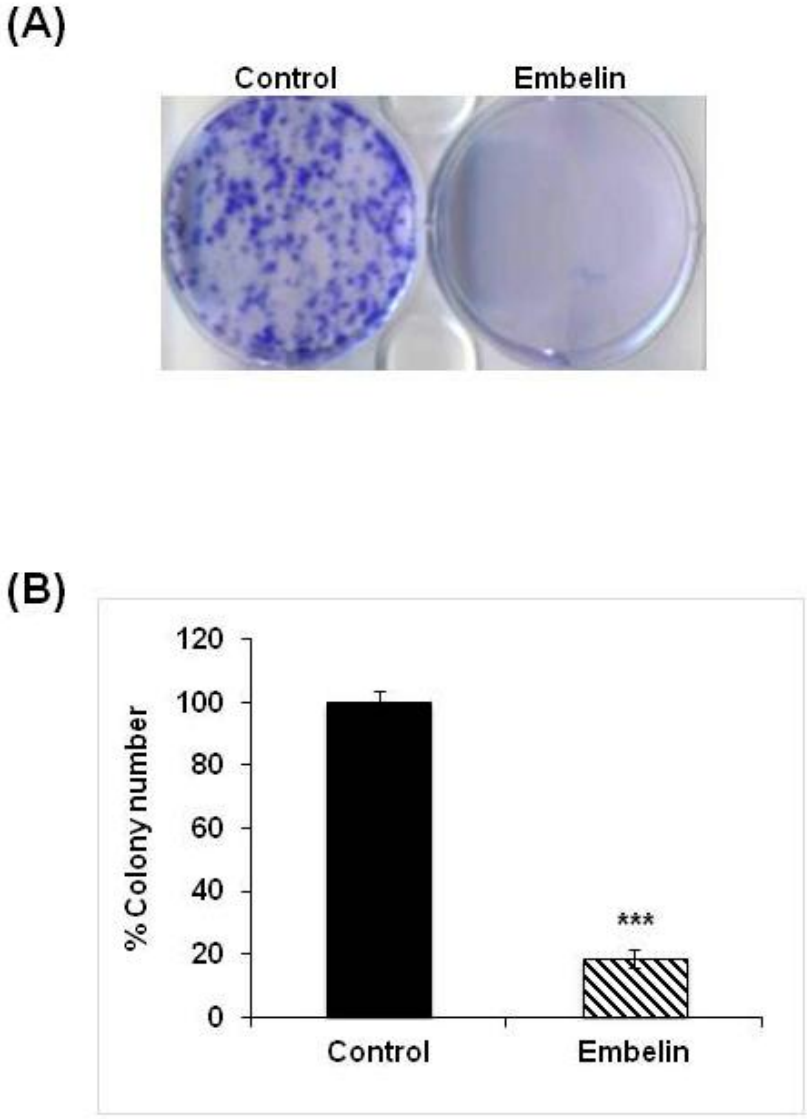
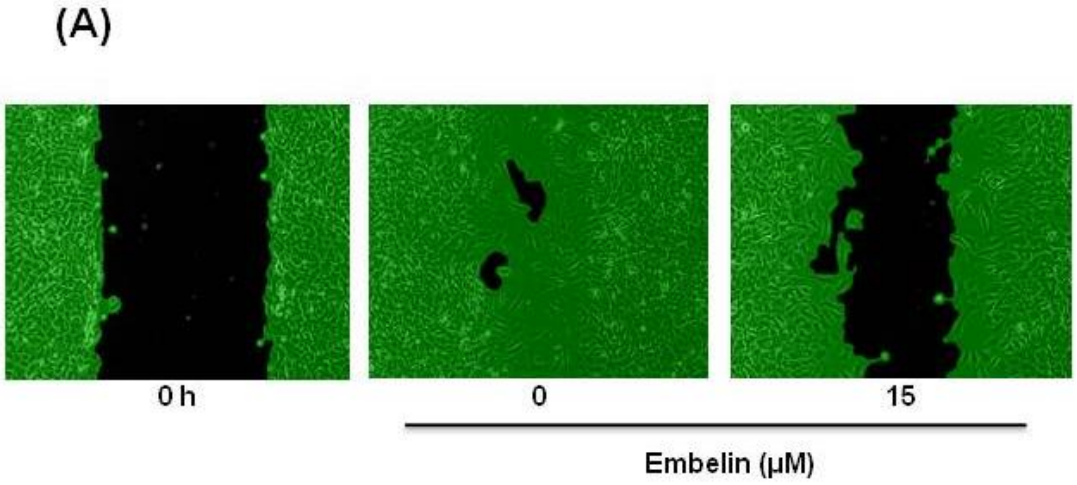


Fig. 3 – 10 Embelin inhibits the efficiency of a cancer cell to develop its clones in highly metastatic derivatives of Human Breast cancer cells, MDA-MB-231 (A) and its quantitative analysis (B)

Figures: Chapter 4- Figure 1



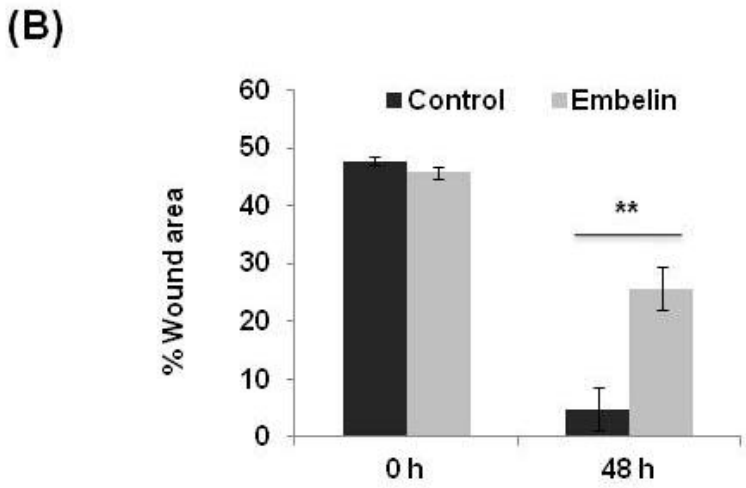


Fig. 4 – 1 Embelin reduced cell migration ability in Human Breast cancer cells, MCF7 (A) and its quantitation (B)

Figures: Chapter 4- Figure 2

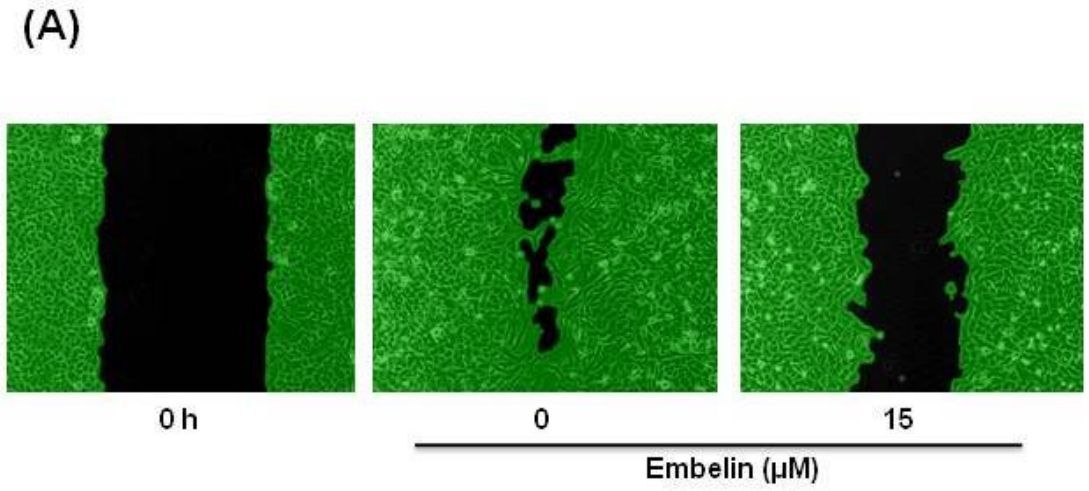


Fig. 4 – 2 Embelin reduced cell migration ability in Human Breast cancer cells, MDA-MB-231 (A) and its quantitation (B)

Figures: Chapter 4- Figure 3

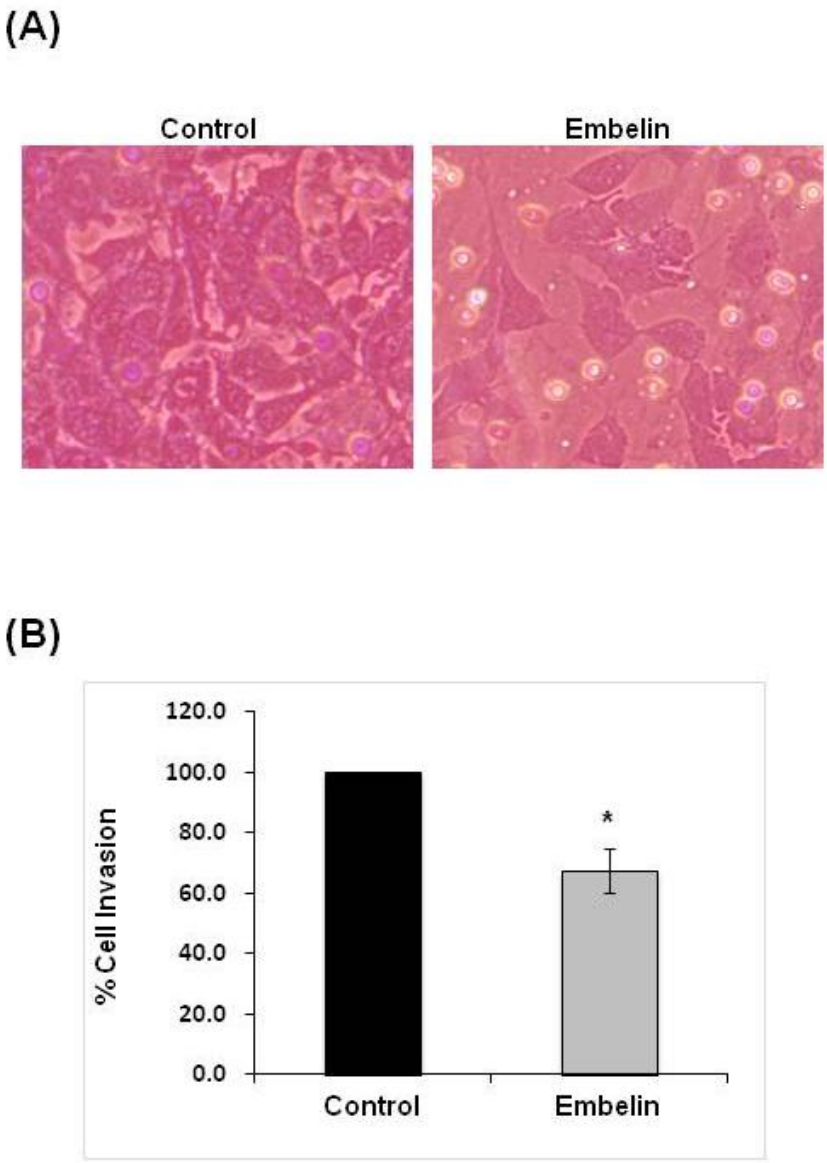


Fig. 4 – 3 Embelin reduced cell invasion ability in Human Breast cancer cells, MCF7 (A) and its quantitation (B)

Figures: Chapter 4- Figure 4

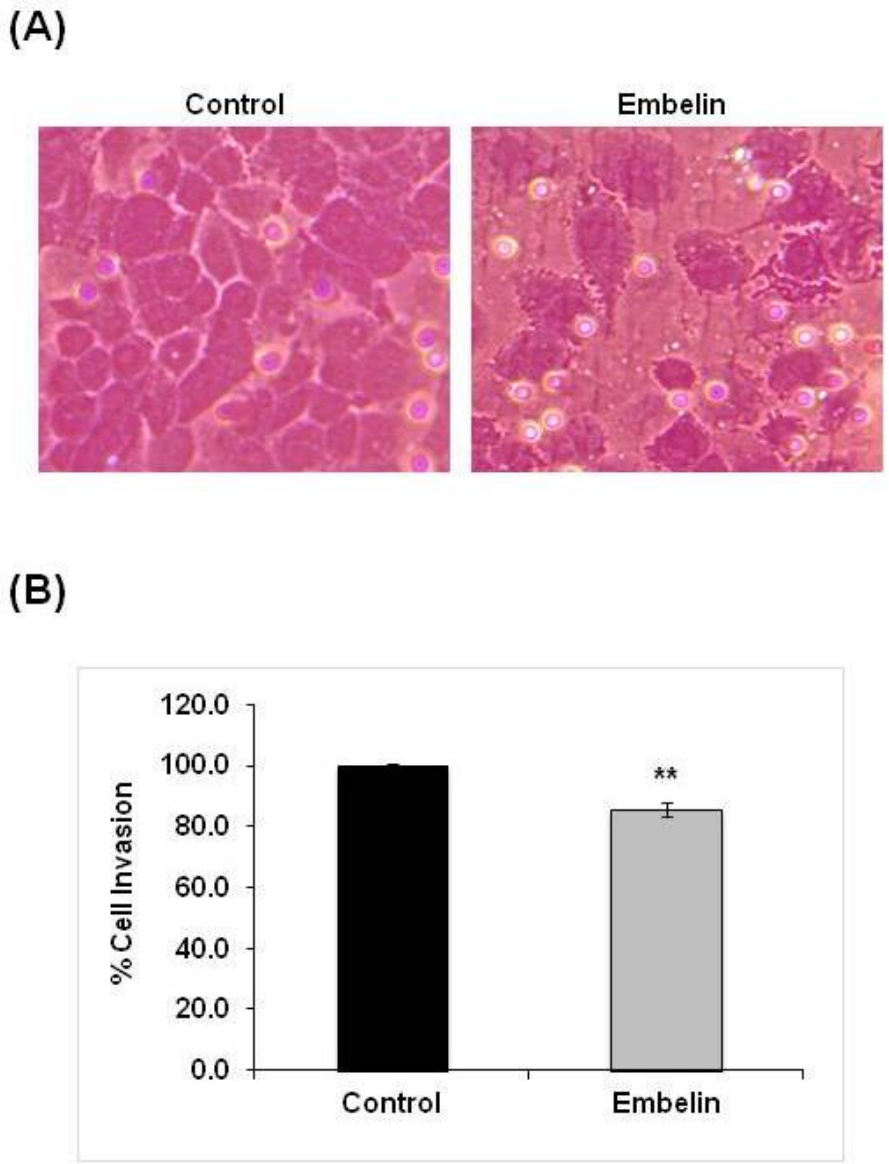


Fig. 4 – 4 Embelin reduced cell invasion ability in Human Breast cancer cells, MDA-MB-231 (A) and its quantitation (B)

Figures: Chapter 5- Figure 1

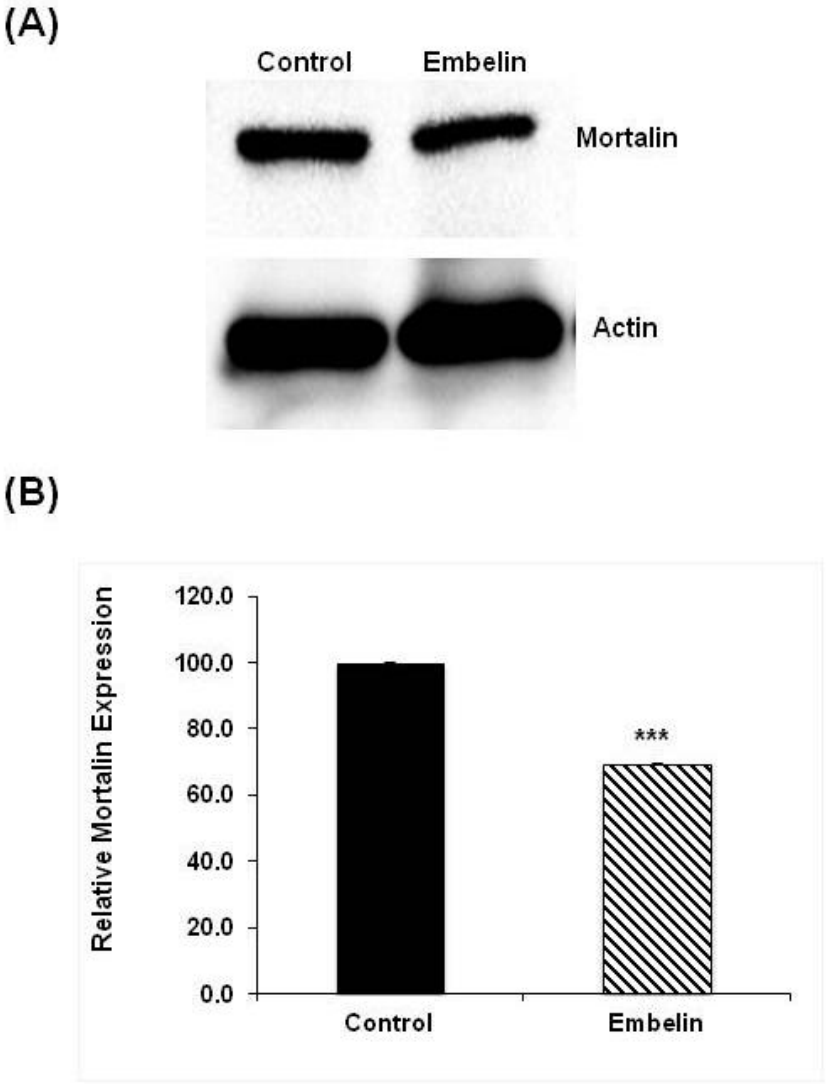


Fig. 5 – 1 Embelin reduces cellular Mortalin expression levels in Human Breast cancer cells, MCF7 (A) and its quantitation (B)

Figures: Chapter 5- Figure 2

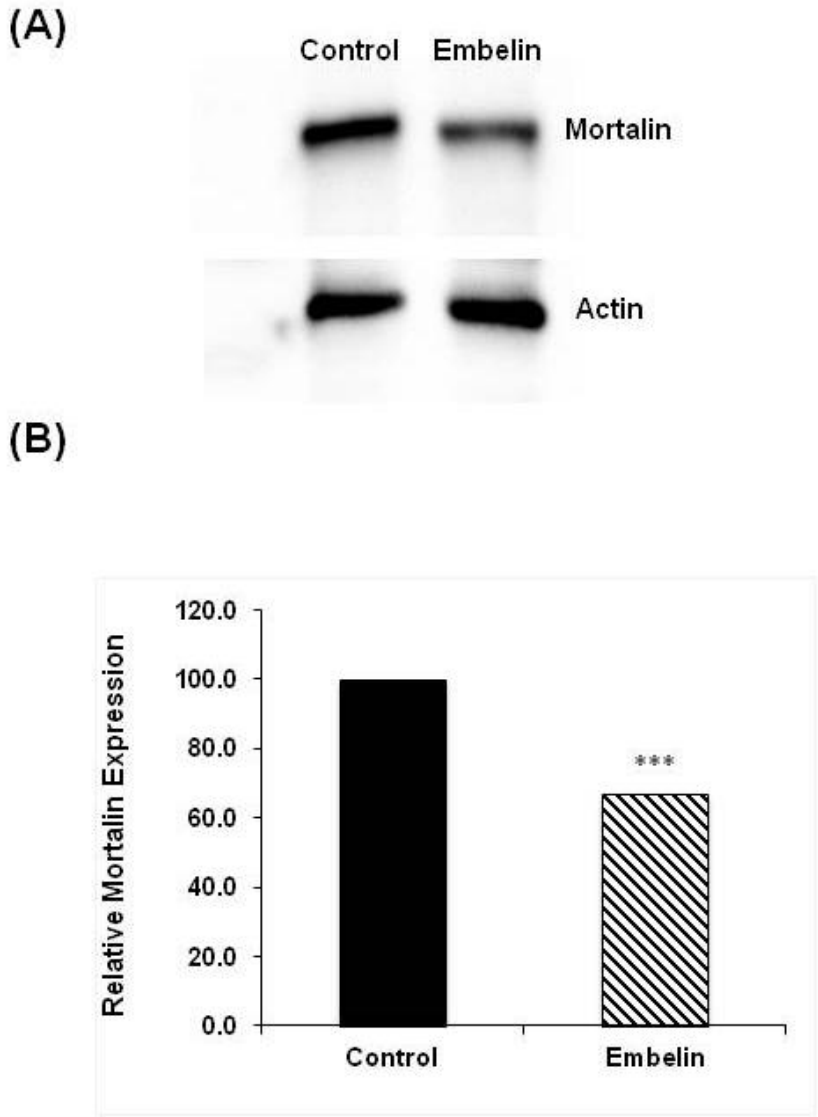


Fig. 5 – 2 Embelin reduces cellular Mortalin expression levels in Human Breast cancer cells, MDA-MB-231 (A) and its quantitation (B)

Figures: Chapter 5- Figure 3

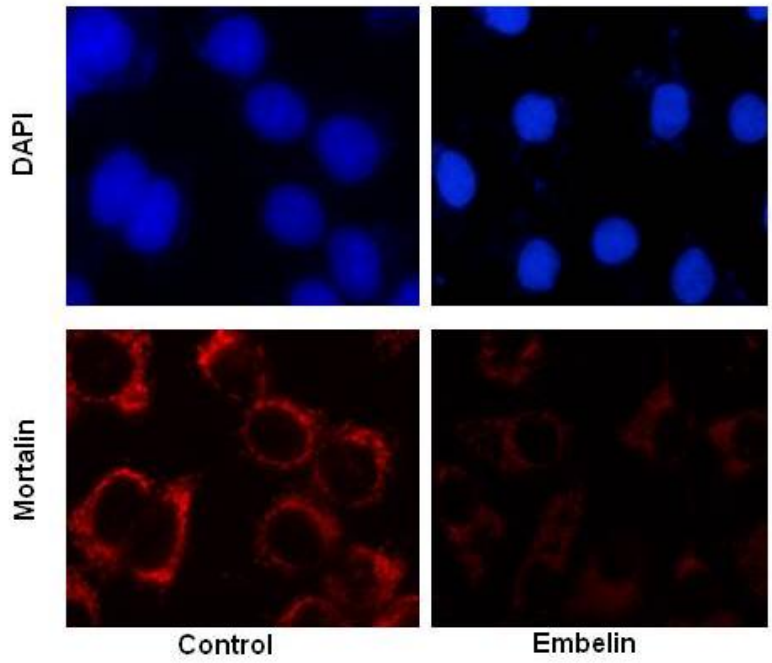


Fig. 5 – 3 Embelin reduces cellular Mortalin expression levels in Human Breast cancer cells, MCF7

Figures: Chapter 5- Figure 4

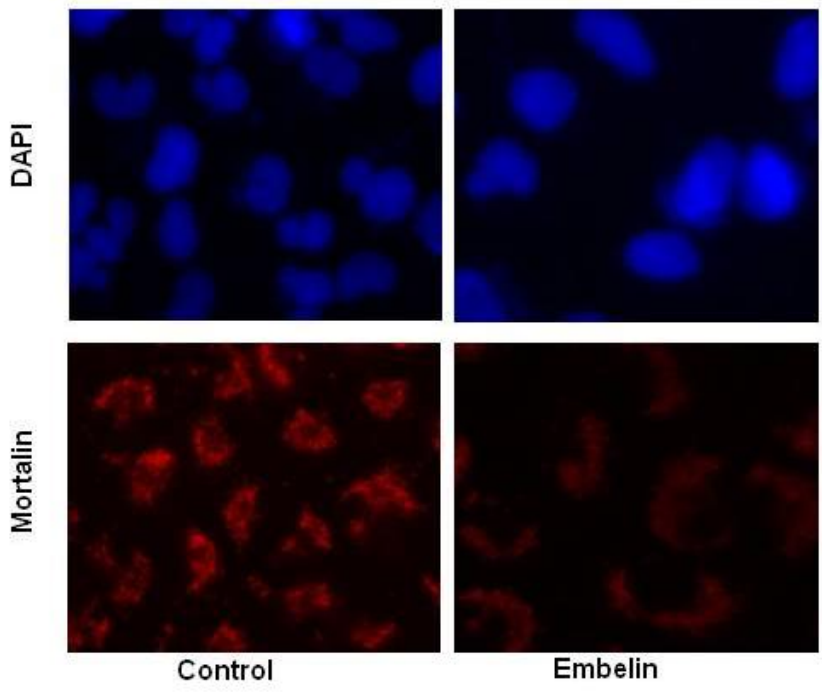


Fig. 5 – 4 Embelin reduces cellular Mortalin expression levels in Human Breast cancer cells, MDA-MB-231

Figures: Chapter 5- Figure 5

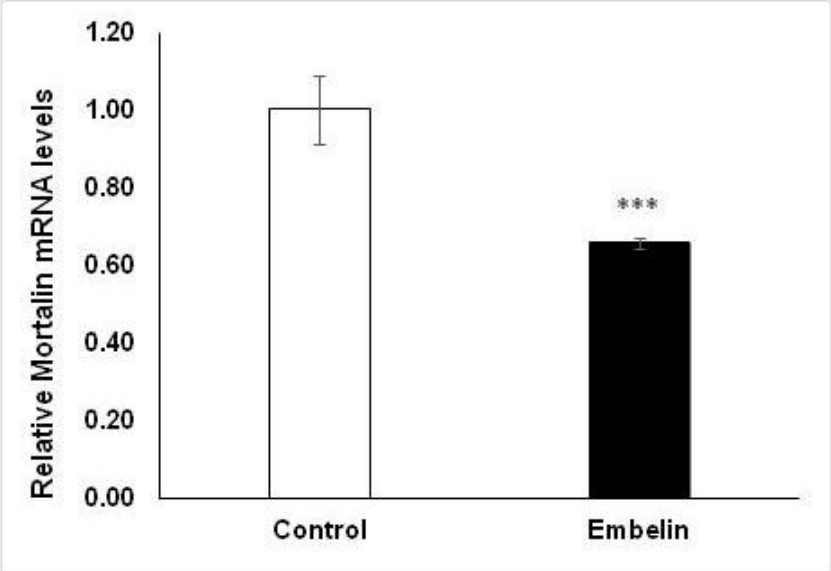


Fig. 5 – 5 Embelin down regulates Mortalin at the transcriptional level in Human Breast cancer cells, MCF7

Figures: Chapter 5- Figure 6

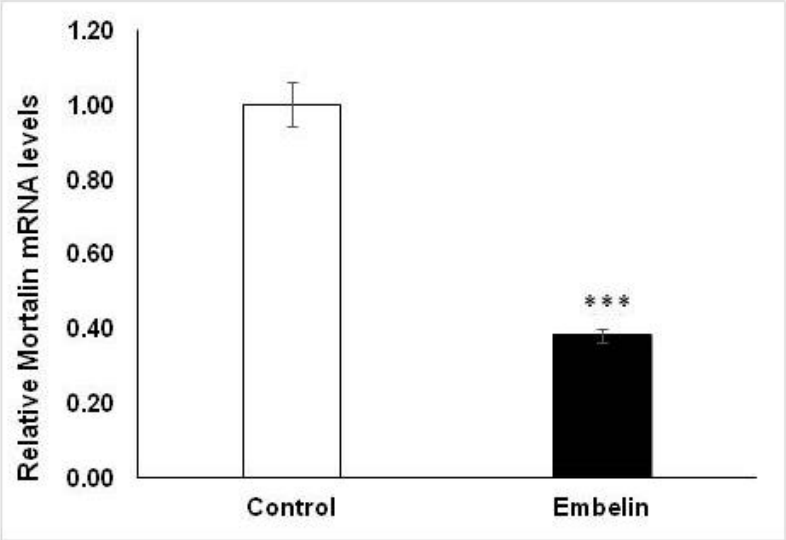


Fig. 5 – 6 Embelin down regulates Mortalin at the transcriptional level in Human Breast cancer cells, MDA-MB-231

Figures: Chapter 5- Figure 7

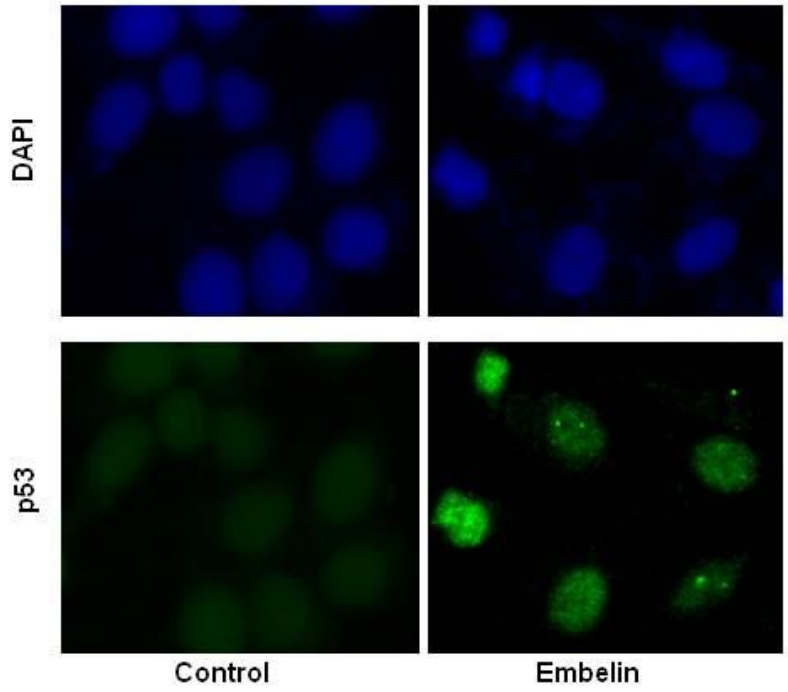


Fig. 5 – 7 Embelin induces translocation of p53 to nucleus in Human Breast cancer cells, MCF7

Figures: Chapter 5- Figure 8

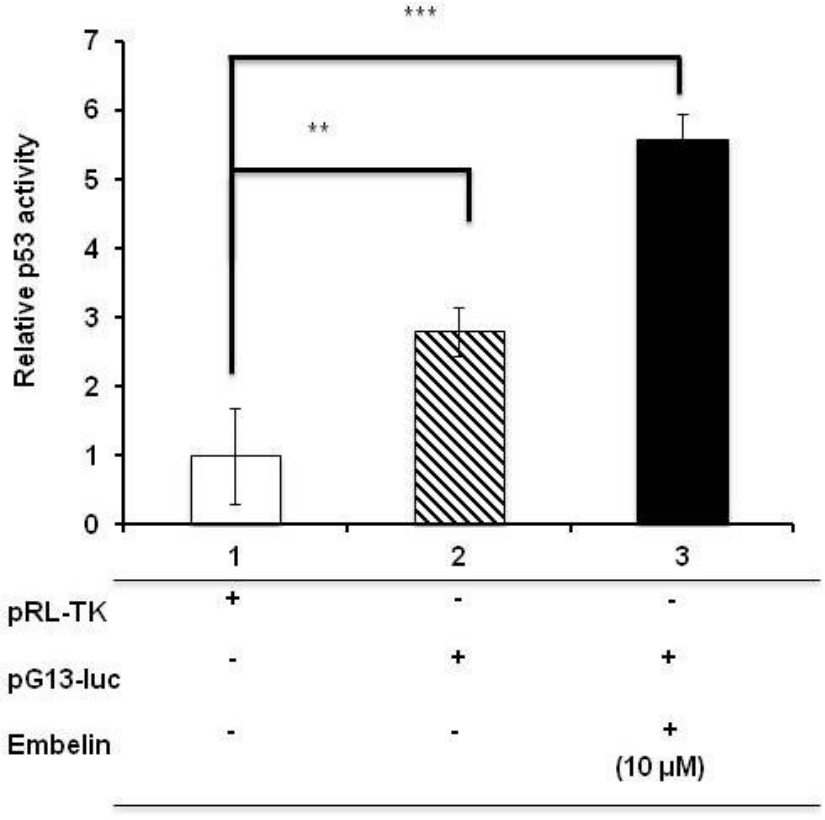


Fig. 5 – 8 Embelin causes activation of p53 in Human Breast cancer cells, MCF7, as seen by Luciferase reporter assay

Figures: Chapter 5- Figure 9

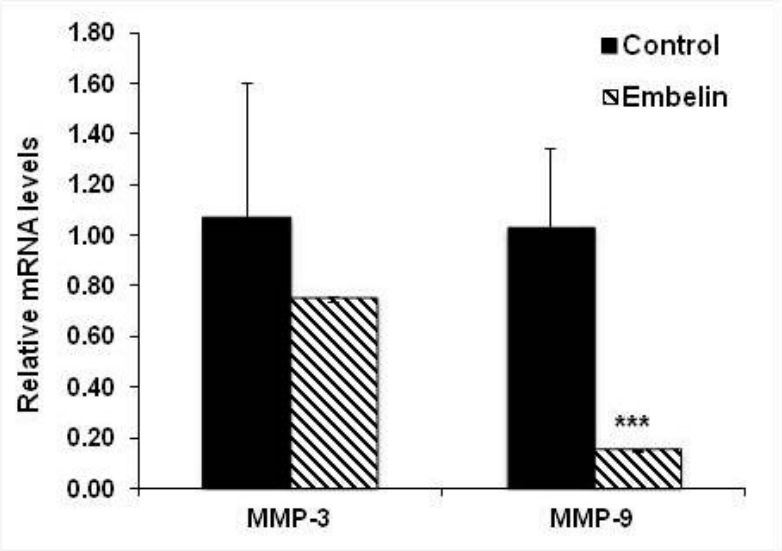


Fig. 5 – 9 Down regulation of MMP-3 and MMP-9 by Embelin in Human Breast cancer cells, MCF7

Figures: Chapter 6- Figure1

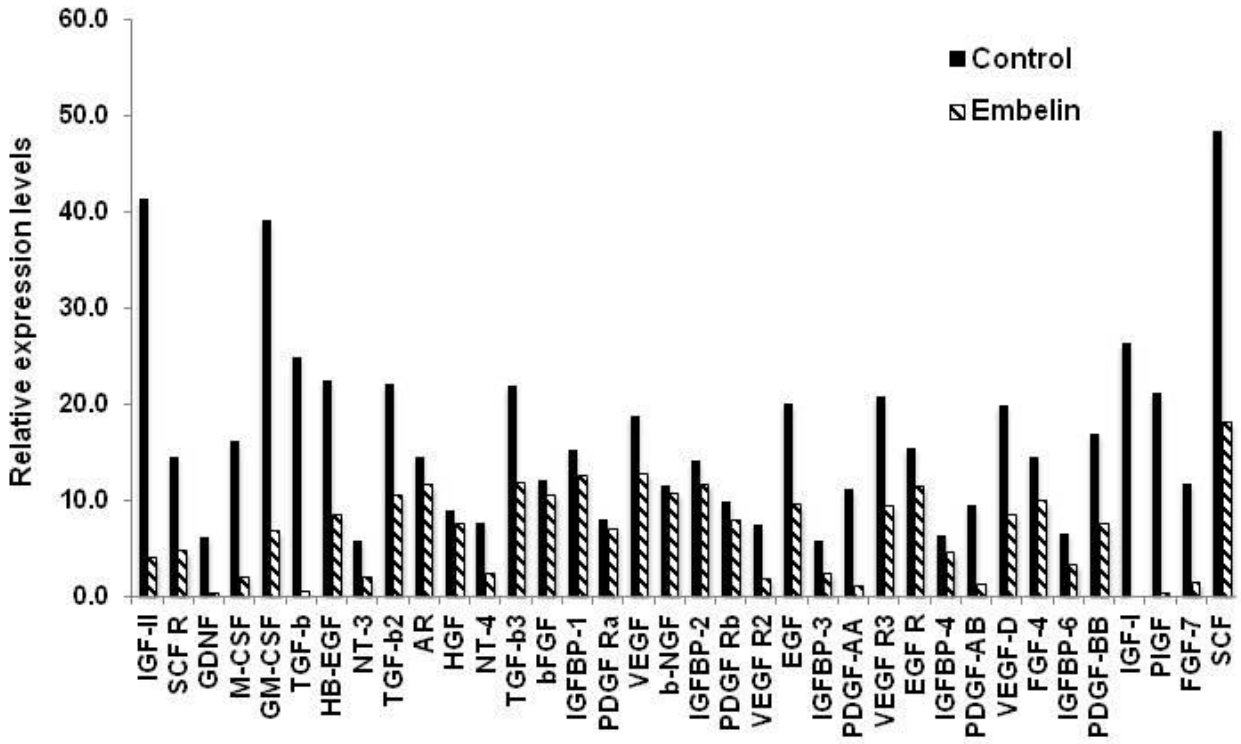


Figure 6 – 1 Embelin reduced the expression levels of growth factors involved in Human Breast cancer cells, MCF7

Figures: Chapter 6- Figure2

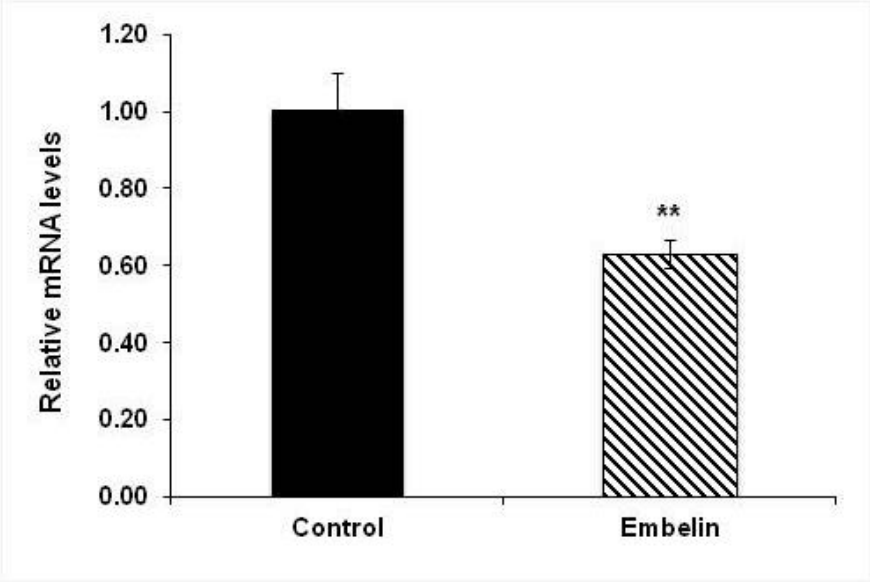


Fig. 6 – 2 Transcriptional down regulation of TGF-β by Embelin in Human Breast cancer cells, MCF7

Figures: Chapter 6- Figure3

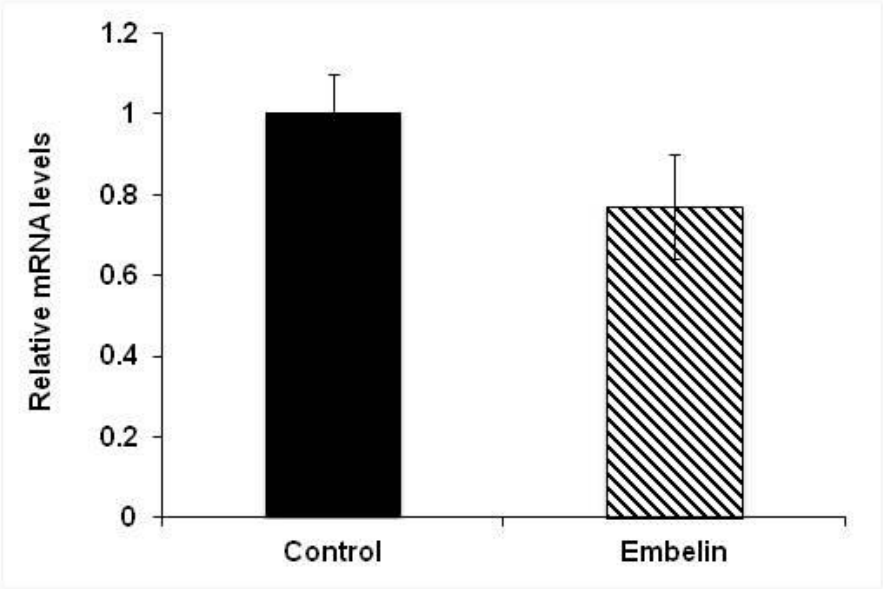
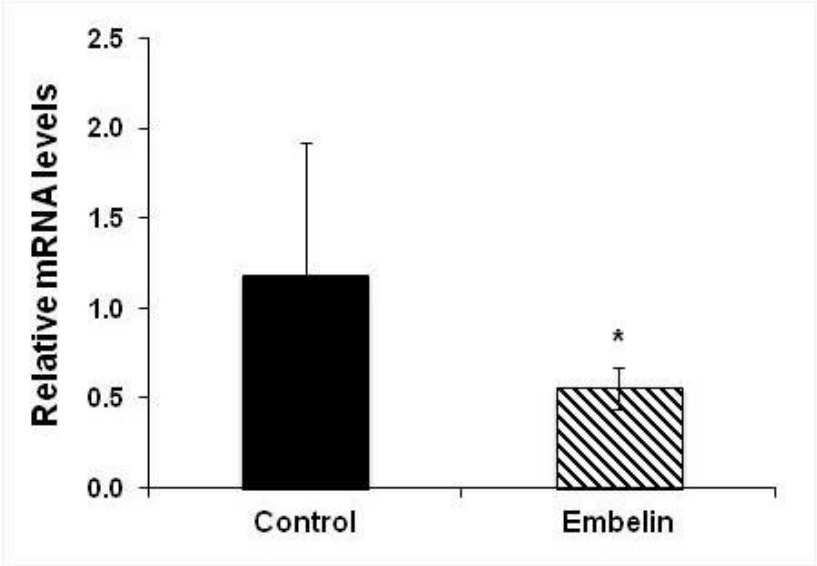


Fig. 6 – 3 Transcriptional down regulation of TGF-β by Embelin in Human Breast cancer cells, MDA-MB-231

Figures: Chapter 6- Figure 4

(A)



(B)

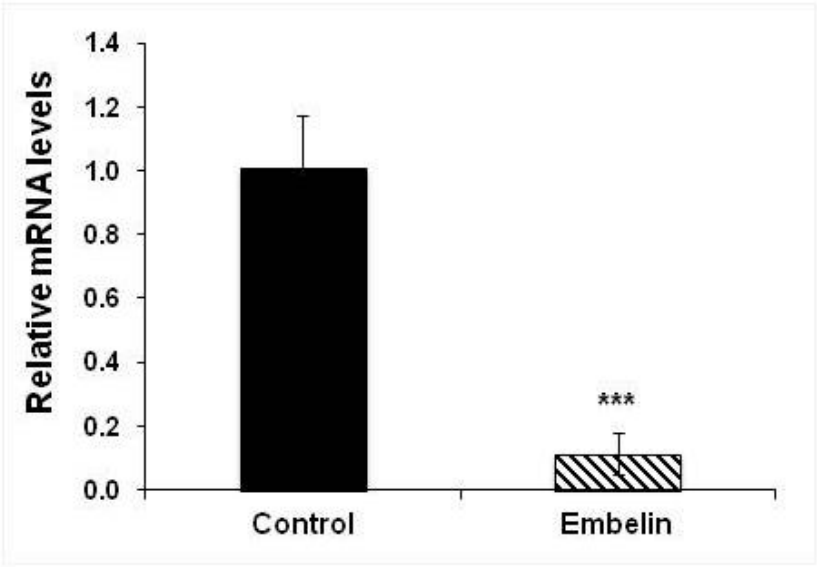


Fig. 6 – 4 Transcriptional down regulation of Wnt by Embelin in Human Breast cancer cells, MCF7 (A) and MDA-MB-231 (B)

Figures: Chapter 6- Figure 5

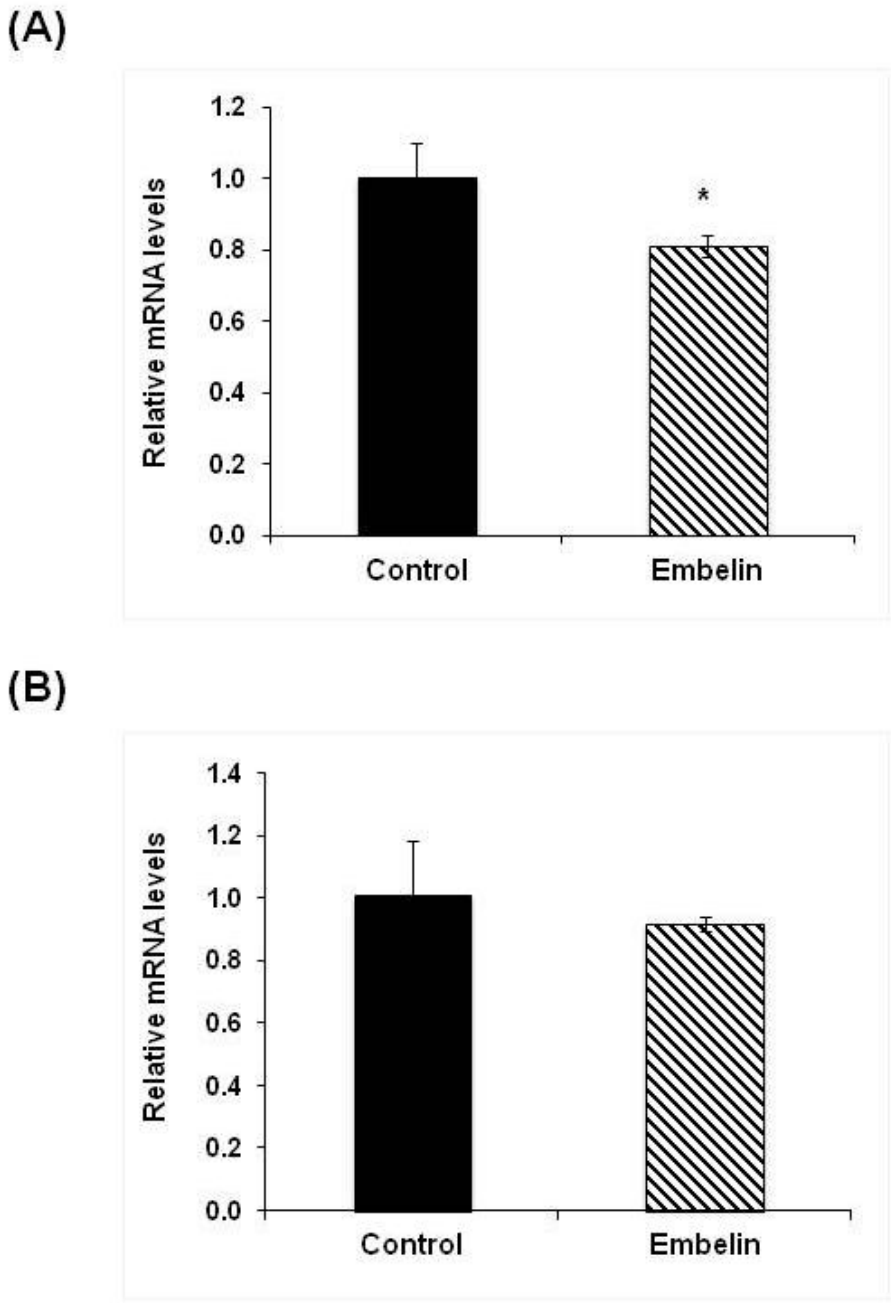
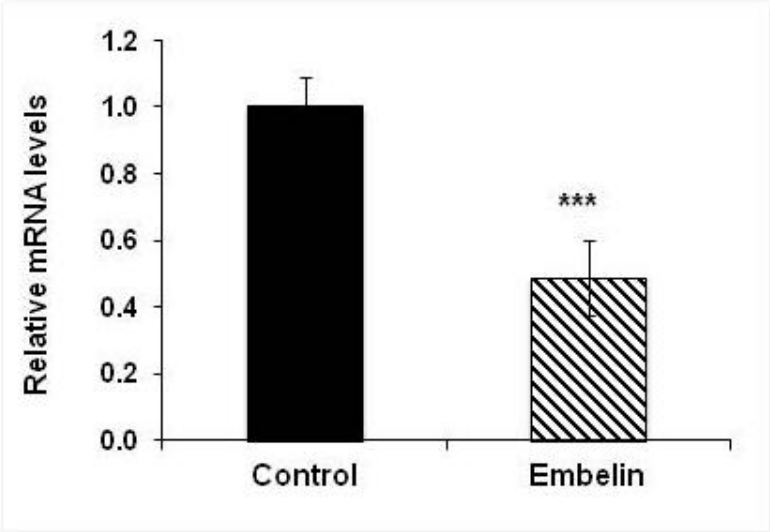


Fig. 6 – 5 Transcriptional down regulation of β-catenin by Embelin in Human Breast cancer cells, MCF7 (A) and MDA-MB-231 (B)

Figures: Chapter 6- Figure 6

(A)



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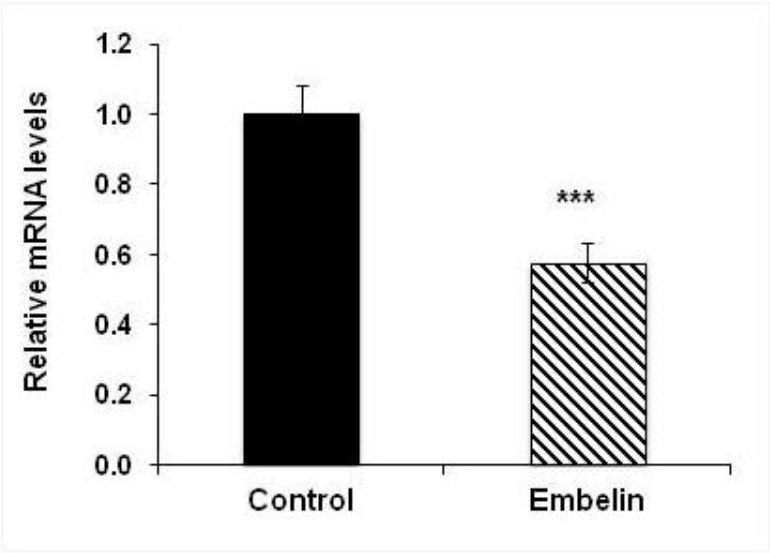

Fig. 6 – 6 Transcriptional down regulation of Vimentin by Embelin in Human Breast cancer cells, MCF7 (A) and MDA-MB-231 (B)

Figures: Chapter 7- Figure 1

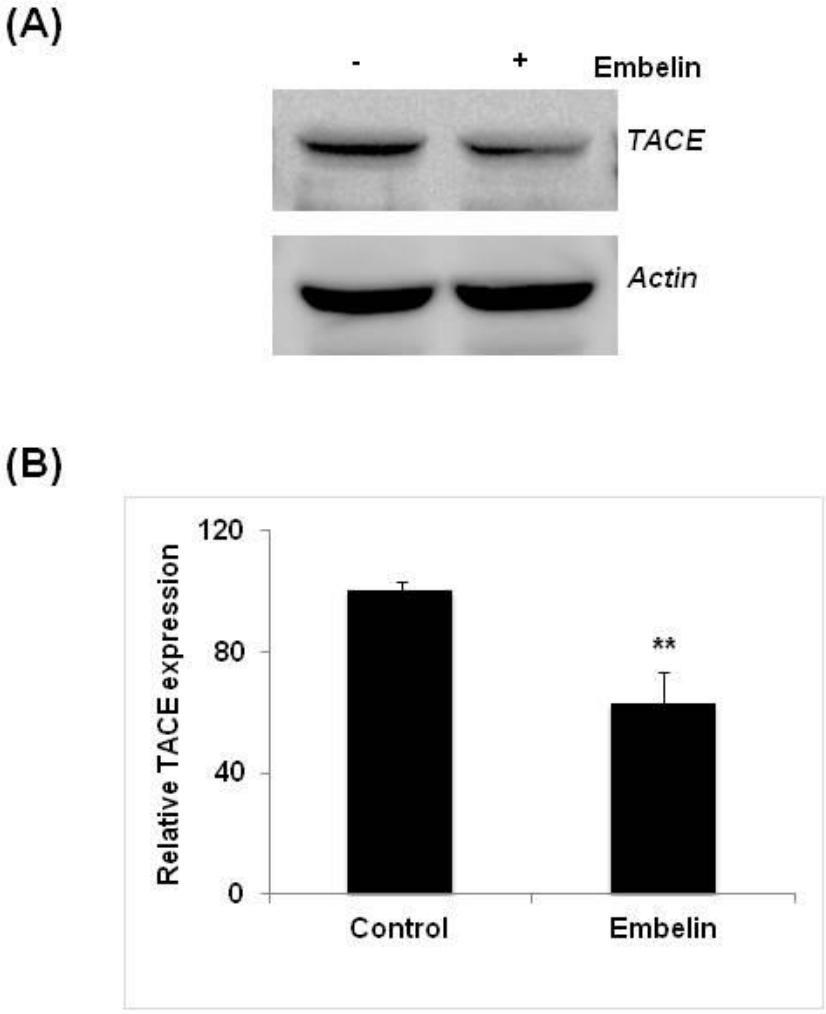

Fig. 7 – 1 Embelin reduces cellular TACE expression levels in Human Breast cancer cells, MCF7 (A) and its quantitation (B)

Figures: Chapter 7- Figure 2

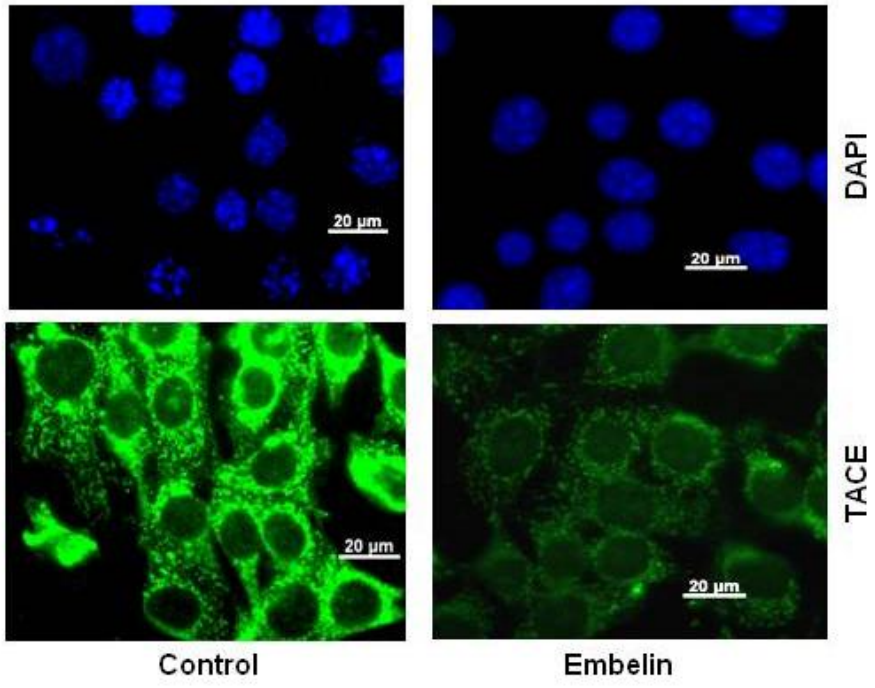

Fig. 7 – 2 Embelin reduces cellular TACE expression levels in Human Breast cancer cells, MCF7

Figures: Chapter 7- Figure 3

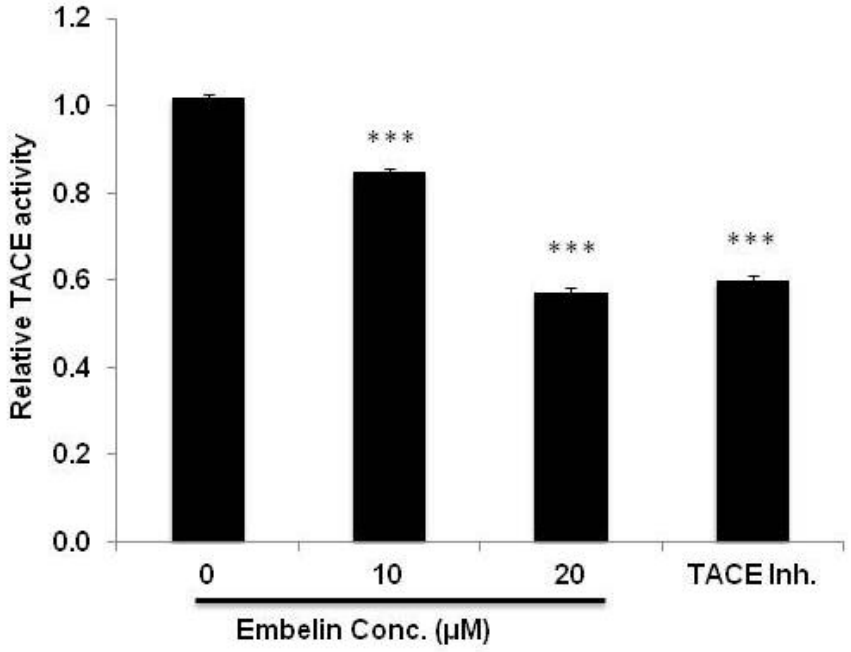

Fig. 7 – 3 Embelin reduces TACE activity in Human Breast cancer cells

Figures: Chapter 7- Figure 4

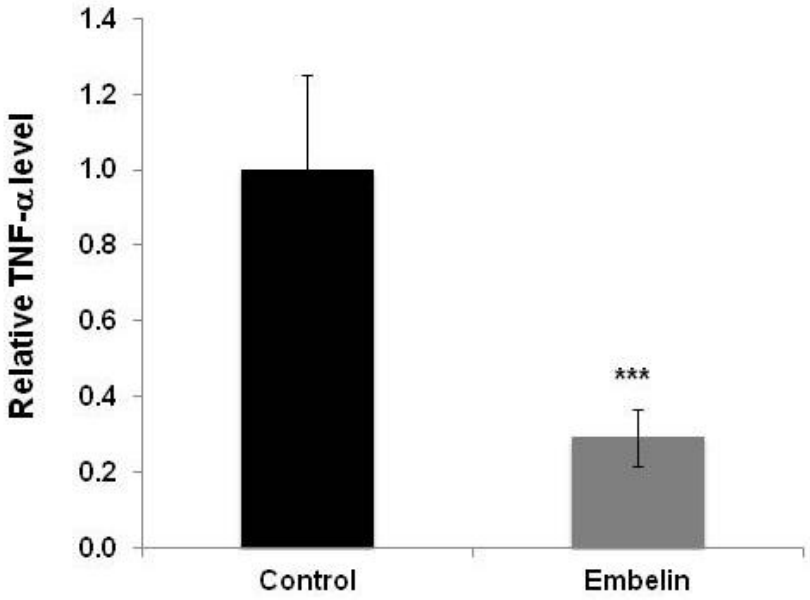

Fig. 7 – 4 Embelin reduces TNF-α expression levels in Human Breast cancer cells

Figures: Chapter 7- Figure 5

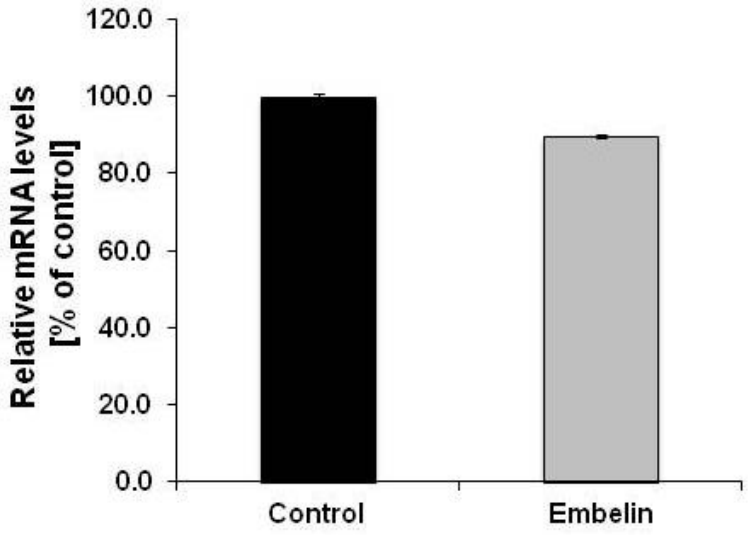

Fig. 7 – 5 RNA levels of TACE remain unaltered with Embelin treatment in Human Breast cancer cells

Figures: Chapter 7- Figure 6

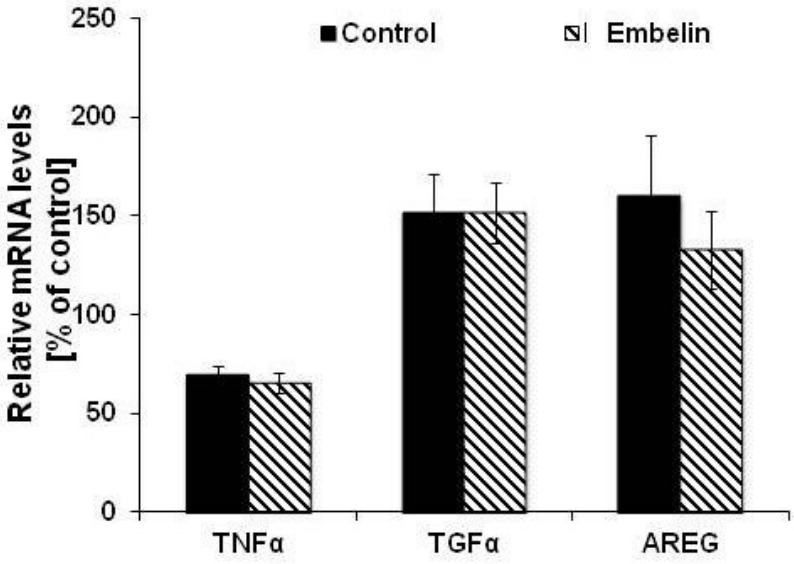

Fig. 7 – 6 Down-regulation of TACE does not directly effect the transcriptional activation of its down-stream targets

Figures: Chapter 7- Figure 7

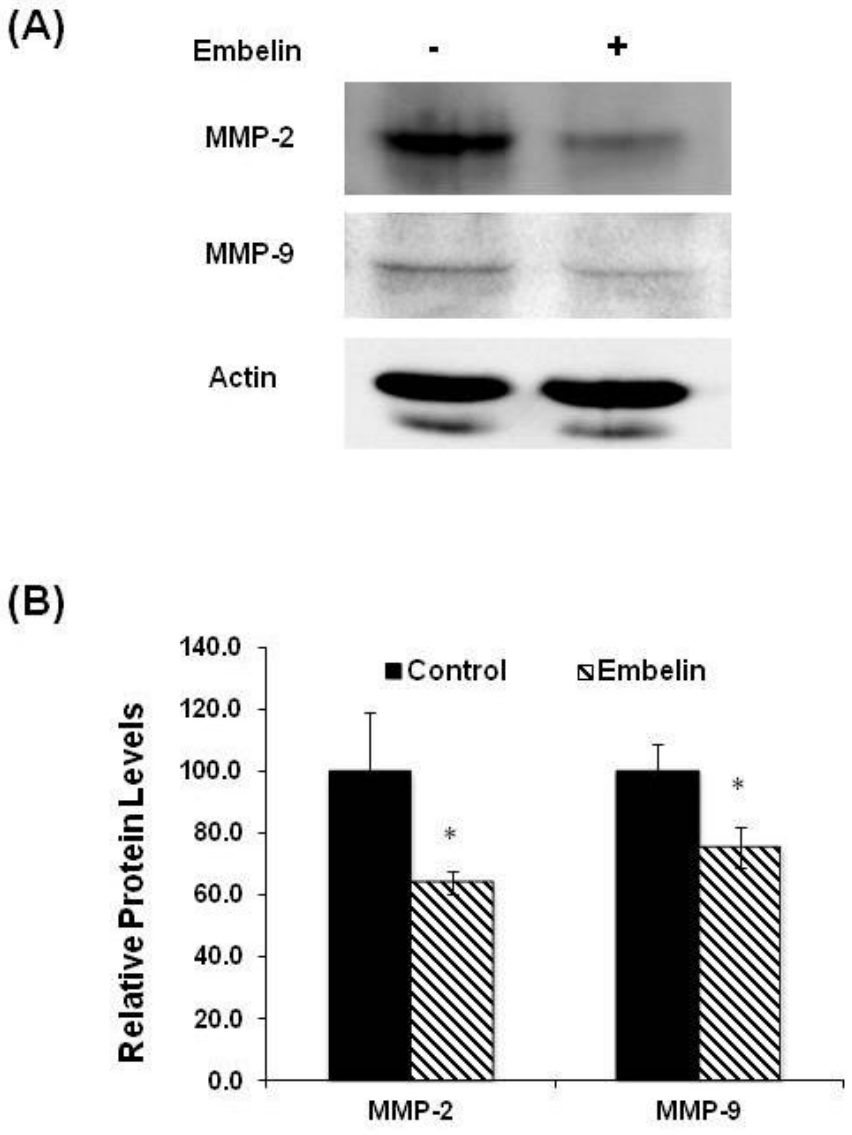

Fig. 7 – 7 Embelin down-regulates the expression level of Matrix Metalloproteinase (A) and its quantitation (B)

Figures: Chapter 7- Figure 8

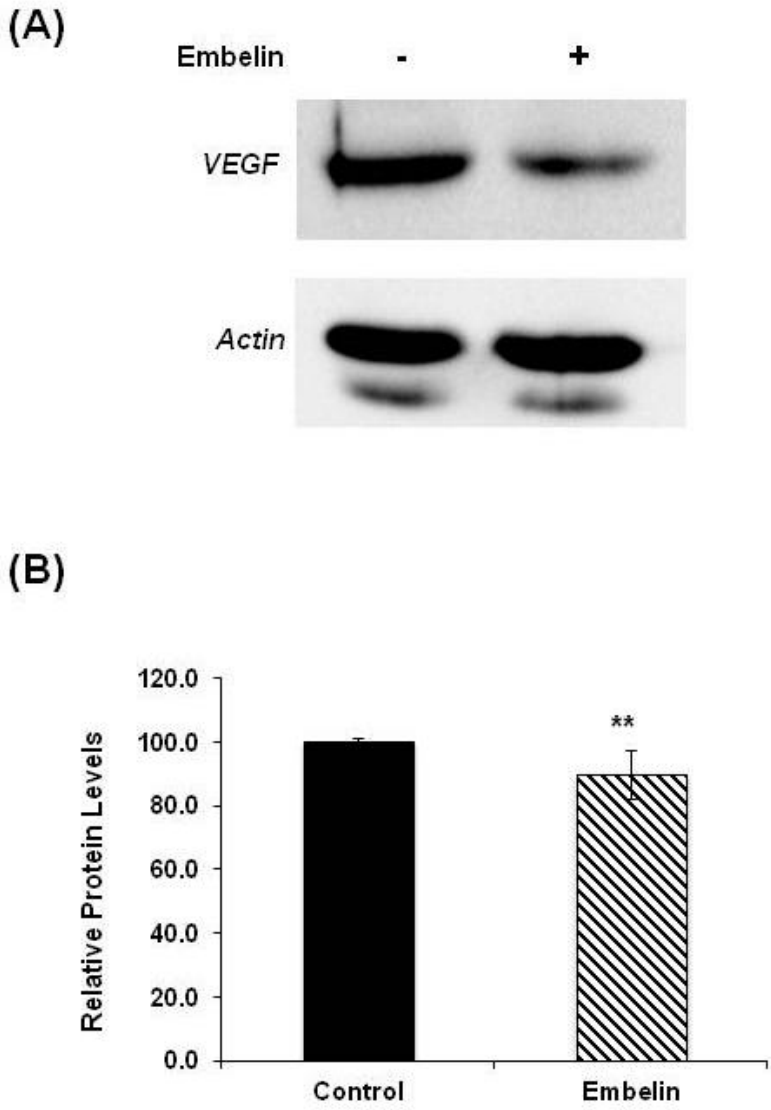

Fig. 7 – 8 Embelin down-regulates the expression level of VEGF (A) and its quantitation (B)

Figures: Chapter 7- Figure 9

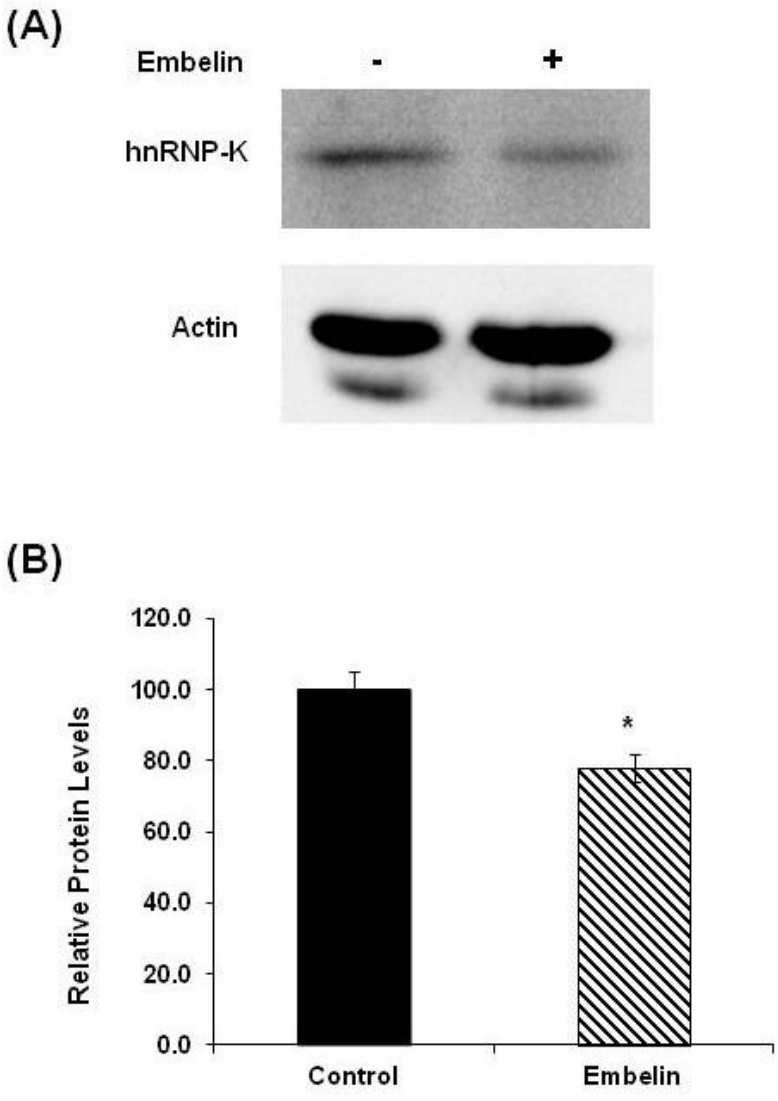

Fig. 7 – 9 Embelin Embelin decreases the expression levels of hnRNP-K (A) and its quantitation (B)

Figures: Chapter 7- Figure 10

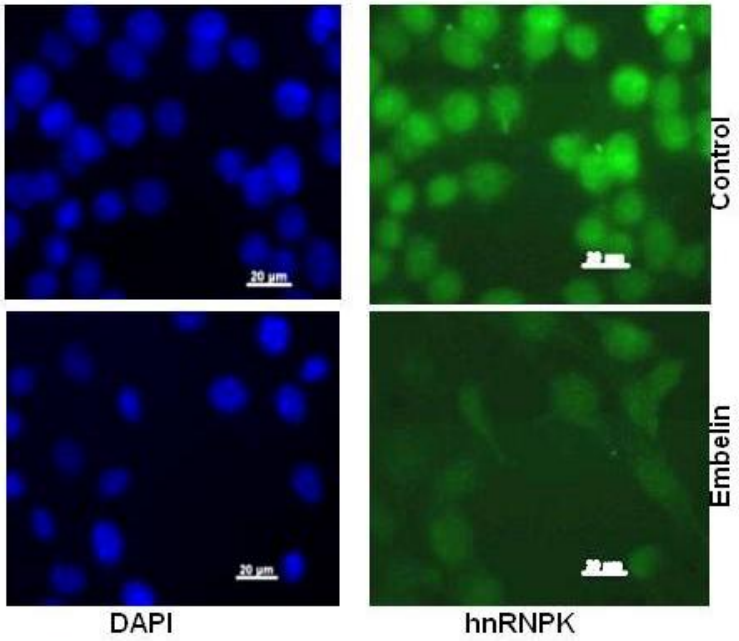

Fig. 7 – 10 Embelin decreases the expression levels of hnRNP-K in Human Breast cancer cells