IN-VITRO INVESTIGATION OF METFORMIN IN COMBINATION WITH MANNOSE AS AN ADJUVANT THERAPY FOR BREAST CANCER TREATMENT

A Dissertation Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

CHENNAI-600 032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

Submitted By

ATHIRA SHAJI

REGISTRATION NO.261725908

Under the Guidance of

Dr .C. JAIKANTH, M Pharm., Ph.D.,

Department of Pharmacology

PSG COLLEGE OF PHARMACY

PEELAMEDU

COIMBATORE -641004

OCTOBER 2019

Certificates

Dr. M. Ramanathan,
M. Pharm, Ph.D.,
Principal and Head of the Department
PSG College of Pharmacy,
Peelamedu, Coimbatore – 641004.

CERTIFICATE

This is to certify that the dissertation work entitled **"*In-Vitro* Investigation of Metformin in combination with Mannose as an Adjuvant therapy for Breast cancer treatment."** submitted by University **Reg. No. 261725908** is a bonafide work carried out by the candidate under the guidance of **Dr.C. Jaikanth, M. Pharm., Ph.D** and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2018-2019.

Dr.M. Ramanathan, M. Pharm., Ph.D.,

Principal & Head of the Department

Dr.C. Jaikanth, M. Pharm., Ph.D.,
AssociateProfessor
Department of Pharmacology
PSG College of Pharmacy,
Peelamedu, Coimbatore - 641 004

CERTIFICATE

This is to certify that the dissertation work **"*In-Vitro* Investigation of Metformin in combination with Mannose as an Adjuvant therapy for Breast cancer treatment."** submitted by University **Reg. No. 261725908** is a bonafide work carried out by the candidate under my guidance and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfilment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2018-2019.

Dr.C.Jaikanth, M. Pharm.,Ph.D.,
Associate Professor,
Department of Pharmacology

DECLARATION

I do hereby declare that the dissertation work entitled **"*In-Vitro* Investigation of Metformin in combination with Mannose as an Adjuvant therapy for Breast cancer treatment"** submitted to The Tamil Nadu DR.M.G.R. Medical University, Chennai, in partial fulfilment for the Degree of Master of Pharmacy in Pharmacology**,** was done by me under the guidance of **Dr.C.Jaikanth, M. Pharm., Ph.D.,** Associate Professor, Department of Pharmacology **,**PSG College of Pharmacy, Coimbatore, during the academic year 2018- 2019**.**

University Reg no:261725908

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"*In-Vitro* Investigation of Metformin in combination with Mannose as an Adjuvant therapy for Breast cancer treatment"** submitted by University **Reg. No. 261725908** to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfilment for the Degree of Master of Pharmacy in Pharmacology is a bonafide work carried out by the candidate at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore and was evaluated by us during the year 2018- 2019.

Examination Centre: PSG College of Pharmacy, Coimbatore.

Date:

Internal Examiner: **External Examiner:**

Acknowledgment

ACKNOWLEDGEMENT

First and foremost I express bow down before lord Almighty for his blessings in completing my project work and throughout my life till this very second.
I owe my sincere thanks to my parents who cared for my well being and had spent their times in shaping my character, conduct and my life.

I would like to render my gratitude and special thanks to my beloved guide ***Dr.C. Jaikanth, M.Pharm., Ph.D.,*** *Associate Professor, Department of Pharmacology, PSG College of Pharmacy, for this continuous support and his lavishing encouragement during my project.*

It is my immense pleasure to be indebted to my beloved principal ***Dr. M. Ramanathan, M. Pharm, Ph.D.,*** *for providing me the indispensable facilities and ideas to carry out the work successfully.*
Besides my guide, I would like to thank rest of my teachers ***Dr.S.Divakar , Dr.G.Venkatesh, Dr.Karthik Dhananjayan****,* ***Mr.A.Tamil Selvan,Mrs.P. Aruna*** *for their encouragement and constrictive ideas for the successful of my Project work.*

It is my pleasure to thank ***Mr.Abdul Kayum, Research Scholar****, Dept of Pharmacology for his selfless support during my project.*

It's my pleasure to thank all other Staff members, Friends, Lab Technicians, library Persons and Lab Attenders for their help and support during my project work.

I would like to express my gratitude to the PSG Human ethics committee for their cooperation during my project work. I take this opportunity to thank ' PSG Sons' and Charities for all the facilities that were provided to me at the institute enabling me to do the work of this magnitude.

I would also like to extend my special thanks to every single being who rendered their helping hands during my project work and also for being my stepping stones to the success of my project.

Dedicated to

my beloved Parents,

Guide and Almighty

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

ABBREVIATIONS

AMPK	:	Adenosine Monophosphate Activated Protein Kinase
AO	:	Acridine Orange
BRCA	:	Breast Cancer Gene
DCF-DA	:	2′,7′-Dichlorofluorescein Diacetate
EA	:	Early Apoptosis
EB	:	Ethidium Bromide
EMT	:	Epithelial Mesenchymal Transition
ER	:	Estrogen Receptor
HER2	:	Human Epidermal Growth Receptor
HRT	:	Hormonal Replacement Therapy
LA	:	Late Apoptosis
LC	:	Live Cells
LHRH	:	Luteinizing Hormone Releasing Hormone
MDR	:	Multi Drug Resistance
MSMT	:	Metformin Stimulated Mannose Transport
MTT	:	3(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
NC	:	Necrotic Cells
PCR	:	Pathologic Complete Response Rate
PR	:	Progesterone Receptor
RH123	:	Rhodamine 123
SERM	:	Selective Estrogen Receptor Modulator
TGF	:	Transforming Growth Factor
TNBC	:	Triple Negative Breast Cancer
VEGF	:	Vascular Endothelial Growth Factor

1. INTRODUCTION

Breast cancer is complex and heterogeneous disease with abnormal growth of cells that normally lining the ducts and the lobules of breast. Breast cancer is the most frequent cancer among women, impacting 2.1 million women each year, and also causes the greatest number of cancer-related deaths among women. As per WHO in 2018, it is estimated that 627,000 women died from breast cancer – that is approximately 15% of all cancer deaths among women. Resistance/Reoccurrence are the major hurdle in the treatment of breast cancer. Investigation of adjuvant therapeutic strategies are essential and important to enhance quality of life in breast cancer victim**.(DeSantis *et al* ,2014)**

The risk factors for breast cancer includes: Being a woman having an inherited mutation in the BRCA1 or BRCA2 breast cancer genes, having a previous biopsy showing hyperplasia or carcinoma in situ,a family history of breast cancer,having high breast density on a mammogram,being exposed to large amounts of radiation, such as having very frequent spine X-rays for scoliosis or treatment for Hodgkin's disease at a young age,a personal history of breast or ovarian cancer,starting menopause after age 55,getting older — the older you get, the greater your risk of breast cancer,never having children etc. **(Anderson *et al* ,2014)**

Indian women also tend to have western diet which leads to obesity and high alcohol ingestion. Both of these factors contribute immensely in enhancing the risk of breast cancer. these subtypes are characterized on the basis of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Triple negative breast cancer (TNBC) is an aggressive subtype that is defined by lack of expression of ER and PR as well as absence of over expressed or amplified HER2. Heterogenicity of the breast carcinoma might happen from the neoplastic change of either myoepithelial or epithelial cell, or yet from a stem cell that has the ability to develop into myoepithelial or epithelial cells.

Triple negative breast cancer (TNBC) is characterized by an **absence of the Estrogen (ER) and Progesterone (PR) receptors, as well as the Human Epidermal Growth Receptor 2 (HER2/HER neu)** and accounts for about 20% of all breast cancer cases.

Chemotherapeutic regimens are the most common treatment to inhibit tumor growth, but there is great variability in clinical responses of cancer patients; cancer cells often develop resistance to chemotherapeutics which results in tumor recurrence and further progression. Chemotherapeutic drugs exhibit antitumor activities by inhibiting cancer cells via induction of cell cycle arrest and/ or cellular apoptosis. Discovery of new drugs for cancer treatment is an expensive and time-consuming process and the percentage of drugs reaching the clinic remains quite less. Drug repositioning refers to the identification and development of new uses for existing drugs and represents an alternative drug development strategy. (**Rico *et al* ; 2017)**

Neoadjuvant chemotherapy has become an accepted approach for inoperable and operable early-stage breast cancer, allowing breast conservation and assessment of the sensitivity of the tumor to systemic therapy . Metformin,an extensively prescribed and well-tolerated firstline therapeutic drug for type 2 diabetes mellitus, has recently been identified as a potential and attractive anticancer adjuvant drug combined with chemotherapeutic drugs to improve treatment efficacy and lower doses. Diabetics with breast cancer receiving Metformin and neoadjuvant chemotherapy had a better prognosis and higher pathologic complete response rate (PCR) than those not receiving Metformin . Metformin inhibited cell proliferation in breast cancer cell lines, but had no efficacy on breast epithelial cell lines. Specifically, Metformin selectively targeted the chemotherapy-resistant, sub-population of CSCs, reversed EMT, inhibited tumor growth, and improved remission in breast cancer. The combination of Metformin with chemotherapy (e.g., Doxorubicin, Paclitaxel,Carboplatin, 5-FU, Epirubicin, and Cyclophosphamide) was more effective than either drug alone .Metformin combined with rapamycin had a greater effect of apoptosis and proliferation inhibition on breast cancer through PI3K/AKT/mTOR pathways than monotherapy and the same effect was also found in the combination treatment of Metformin with Everolimus, an analog of rapamycin .(**Zhang *et al* ;2016)**

The molecular mechanism underlying anticancer effect of Metformin is that **Metformin inhibits mitochondrial complex I** . which results in the interruption in mitochondrial

respiration resulting to decreasing synthesis of ATP causing cellular energetic stress and elevation of the AMP:ATP ratio which, inRE turn, activates AMP-activated protein kinase – AMPK .(**Zhang *et al;*2016)**

AMPK activation leads to a reduction in mammalian target of rapamycin (mTOR) signalling and decreased protein synthesis and proliferation .

The monosaccharide Mannose has found to cause growth retardation in several tumour types in vitro, and enhances cell death in response to major forms of chemotherapy.It is epimer of glucose. Mechanistically, Mannose is taken up by the same transporter(s) as glucose3 but accumulates as mannose-6-phosphate in cells, and this impairs the further metabolism of glucose in glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway and glycan synthesis. It was reported that the administration of Mannose in combination with conventional chemotherapy affects levels of anti-apoptotic proteins of the Bcl-2 family, leading to sensitization to cell death. Mannose intermediates in glycolysis are reported to inhibit lactic acid production without interfering glycoytic uptake of glucose. (**Shang *et al* ; 2004**).

Metformin and Mannose combination as an adjuvant therapy for breast cancer treatment as Metformin is already reported to posses anti neoplastic activity in wide range of cell line. Mannose is recently reported to inhibit cancer cell proliferation in Ovarian cancer cell lines.Mannose intermediates in glycolysis are reported to inhibit lactic acid production without interfering glycoytic uptake of glucose.**(Gonzalez *et al*;2018)**

In this study we hypothesis that Metformin increases the transport of Mannose and Mannose hinders the formation of lactic acid without affecting glucose metabolism and the combination treatment of Metformin and mannose may increase the anti-neoplastic activity by exploiting the metabolic weakness in breast cancer cells.

2. LITERATURE REVIEW

2.1 POPULATION NEEDS AND OBSTACLES.

Breast cancer is complex and heterogeneous disease with abnormal growth of cells that normally lining the ducts and the lobules of breast. Breast cancer is the most frequent cancer among women, impacting 2.1 million women each year, and also causes the greatest number of cancer-related deaths among women.As per WHO in 2018, it is estimated that 627,000 women died from breast cancer – that is approximately 15% of all cancer deaths among women..**(DeSantis *et al* ,2014)**

The incidence of breast cancer is increasing in the developing world due to increase life expectancy, increase urbanization and adoption of western lifestyles. Although some risk reduction might be achieved with prevention, these strategies cannot eliminate the majority of breast cancers that develop in low- and middle-income countries where breast cancer is diagnosed in very late stages. Therefore, early detection in order to improve breast cancer outcome and survival remains the cornerstone of breast cancer control.limited resource settings with weak health systems where breast cancer incidence is relatively low and the majority of women are diagnosed in late stages have the option to implement early diagnosis programmes based on awareness of early signs and symptoms and prompt referral to diagnosis and treatment**.(Andreson *et al*;2014)**

Population-based cancer screening is a much more complex public health undertaking than early diagnosis and is usually cost-effective when done in the context of high-standard programmes that target all the population at risk in a given geographical area with high specific cancer burden, with everyone who takes part being offered the same level of screening, diagnosis and treatment services. So far the only breast cancer screening method that has proved to be effective is mammography screening. Many low- and middle-income countries that face the double burden of cervical and breast cancer need to implement combined cost-effective and affordable interventions to tackle these highly preventable diseases.Several risk factors for breast cancer have been well documented. However, for the

majority of women presenting with breast cancer it is not possible to identify specific risk factors .(**Anderson *et al* ;2014)** The risk factors for breast cancer includes:

Being a woman

- having an inherited mutation in the BRCA1 or BRCA2 breast cancer genes
- having a previous biopsy showing hyperplasia or carcinoma in situ
- a family history of breast cancer
- having high breast density on a mammogram
- being exposed to large amounts of radiation, such as having very frequent spine X-rays for scoliosis or treatment for Hodgkin's disease at a young age
- a personal history of breast or ovarian cancer
- starting menopause after age 55
- getting older — the older you get, the greater your risk of breast cancer
- never having children
- having your first child after age 35
- high bone density
- being overweight after menopause or gaining weight as an adult
- having more than one drink of alcohol per day
- currently or recently using combined estrogen and progesterone hormone replacement therapy (HRT)
- being younger than 12 at the time of your first period
- current or recent use of birth control pills

Breast cancer is more common in single women than in married women. The breast is an estrogen sensitive organ. Many females who have been on birth control pills or estrogen replacement have found that the medications result in enlarged and often tender breasts. The activity of this medication, combined with the standard western high fat, low fiber diet, which over-stimulates breast tissue, could be a trigger for breast cancer. Epidemiological investigations have also suggested that those women who have many children possess lower risk of breast cancer than those women who have fewer children. Incidence of breast cancer

is 10.04% among all cancers and, most commonly occurs in 40–50 aged women.*(***Akram *etal;*2017***)***

Menopause resulting from surgical removal of ovaries (oophorectomy) decreases the risk. Presence of certain kinds of benign tumours in breast increases the risk of malignancy. The ovaries stop producing the female hormones once the menopause sets in, but in obese women the fatty tissue can provide the estrogen as it is capable of producing it. This increase in hormone production seems to increase the risk of breast cancer in obese post menopausal women. Deficiency of vitamin D and lack of sun exposure is considered to be the important cause of breast cancer. It is found to be more in women than men. The risk of breast cancer increases with age however rarely found before the age of 20 years. Carcinoma in one breast can increase the risk by four times in another breast.While the patients that have the history of ovarian, endometrial or colon cancer have 1–2 times increased risk to develop breast carcinoma. A female who has had breast cancer has an enhanced danger of occurring breast cancer in the other breast.

The minimal role of the gene has been established in the development of breast cancer. BRCA-1 (breast cancer susceptibility gene) is considered to be the cause of 5–10% of breast cancer that is transferred from either father or mother to the next generation. The study indicates that right environmental conditions are required for cancer promoting gene for expression. **(Easton *al*;1995)**

The danger is maximum if the affected family member had breast cancer at a juvenile period, had cancer in both breasts, or if female is a close family member. First-degree family members such as daughter, sister and mother are mainly significant in estimating threat. Numerous second-degree relatives such as an aunt and grandmother with breast cancer might also enhance threat. Breast cancer in a male enhances the danger for the entire close female relatives. Women who have a positive family history of breast carcinoma are 2–4 times more likely to develop the cancer, especially the females who are the carriers of BRCA1 or BRCA2 genes have the significant chance to develop carcinoma of breast.(**Anderson *et al* ;2014**). Breast cancer affects both male and females; though, the prevalence is more in female as compared to male. Generally, females are at 100-fold increased danger of breast cancer than male. Early menarche, nulli parity, pregnancy after the age of 30, oral

contraceptives or hormone replacement therapy all these factors can increase the risk of breast cancer. Steroid hormones include androgens, progesterone and estrogen, which belong to a cluster of structurally connected hormones known as sex hormones that are released into the blood by the gonads and adrenal glands. They are synthesized from single general precursor, cholesterol through a reaction catalyzed by numerous enzymes to make a large diversity of hormones for diverse target organs and tissues. This procedure is well regulated and the discharge of these hormones into the systemic circulation. These hormones cross the plasma membrane to reach the target cells and bind to specific receptors called steroid hormone receptors to exert their activity. Oestrogens have important activities on differentiation, growth and performance of several tissues, including urogenital system of man and women, cardiovascular system, brain, uterus and breast. In accordance with this, Kato et al., reported that the progression of reproductive organ cancer like prostate and breast cancer frequently occurs because of the androgens, progesterone and estrogen, which exert numerous biological activities in normal as well as abnormal cells. The study indicates that the development of normal and abnormal epithelial cells of the breast can be modulated by stromal cells of the breast and can release growth factors after stimulation by the endogenous hormones. An aromatase enzyme is found in adipose tissues, which makes estradiol from the precursor molecule, cholesterol. Fat cells are found in excess amount in breast of aged females; therefore, the quantity of estradiol is higher in breast tissues of post menopausal female than their plasma level. This most likely is responsible for the increasing occurrence of breast cancer in aged female and assists the action of steroid hormones in breast cancer pathogenesis. Benign tumors and proliferative lesions without or with atypia can increase the risk of breast cancer. Breast cancer has been linked with high level of dietary fats and low level of certain nutrients for various years. Animal fat stimulates colonal bacterial to form estrogen from cholesterol found in the diet, thus increasing level of estrogen in the body. The body fat is also involved in synthesis of oestrone, a type of estrogen. Obesity, increased fat consumption, radiation therapy.

Evidence is accumulating that certain environmental pollutants contribute to estrogenic activity and may contribute to the prevalence of breast problems in the industrialized world. Alcohol consumption is linked with breast cancer risk. This association was felt to be

secondary to the fact that consumption of alcohol enhances level of hormones in the blood**.(Akram *et al* ;2017)**

2.2 MAMMARY GLAND ANATOMY AND FUNCTIONS

The mammary gland is a gland located in the breasts of females that is responsible for lactation, or the productionn of milk and in males it is nonfunctional . It is regulated by the endocrine glands and become functional in response to the hormonal changes associated with parturition.The glandular tissue itself is made up of 15–20 lobes composed of solid cords of ductal cells; each lobe is subdivided into many smaller lobules, separated by broad fibrous suspensory bands (Cooper's ligaments), which connect the skin with the fascia, or sheet of connective tissue, that covers the pectoral muscles beneath the breast. Each lobe is drained by a separate excretory duct. These converge beneath the nipple, where they widen into milk reservoirs, before narrowing again to emerge as pinpoint openings at the summit of the nipple. Circular and radiating muscles in the areola, a circular disk of roughened pigmented skin surrounding the nipple, cause the nipple to become firm and erect upon tactile stimulation; this facilitates suckling. The areola also contains sebaceous glands to provide lubrication for the nipple during nursing. Blood is supplied to the breast through the axillary, intercostal, and internal thoracic vessels. The nerve supply is from branches of the fourth, fifth, and sixth intercostal nerves**.(Neville *et al* ;2001**).

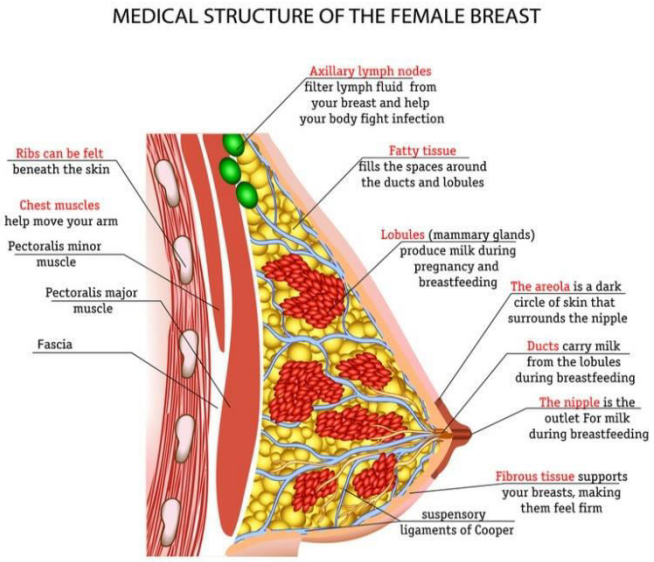


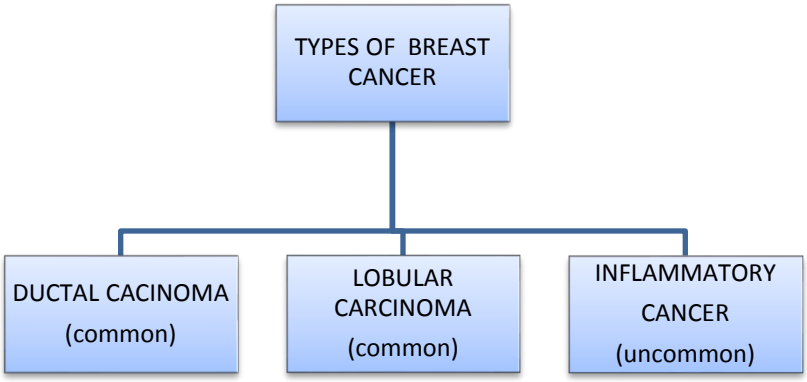
Figure 1 –source: shutterstock images

2.3 NEOPLASAM OF BREAST AND SUBTYPES

The breast is a complex tubulo-alveolar organ fixed within an asymmetrical connective tissue, that go through a chain of alteration from child bearing age to senility. The changes seen with every menstrual cycle and pregnancy guided us to assume the occurrence of precursor cells in the mature tissue that is able of synthesizing novel duct-lobular units. The typical breast architecture contains a stratified epithelium bordered by a basement membrane and fixed in a template of blood vessels, lymphatic and stromal cells. In the usual breast, the stratified epithelium comprised of two dissimilar cell populations, myoepithelial and epithelial, which can be distinguished by way of immunohistochemical staining with antibodies against myosin and CK, correspondingly. It has been postulated that the creation of cellular heterogeneity in breast disorders depends on the primary developmental series of the usual breast. (**Tang *et al*; 2016**)

This heterogenicity of the breast carcinoma might happen from the neoplastic change of either myoepithelial or epithelial cell, or yet from a stem cell that has the ability to develop into myoepithelial or epithelial cells. According to the oncology of breast cancer, neoplastic cells differ from the normal body cells. Normal tissues of the body have limited growth promotion and

regulation which helps to keep the structure and functions of tissues usual. However, cancerous cells have prolonged and chronic proliferation without any external stimuli. Cancer cells overcome the growth suppressor gene. Breast cancer is a malignant disease that initiates in the breast cells. Like other malignant tumors, there are numerous causes that can increase the possibility of developing breast cancer. Injure to the deoxyribonucleic acid (DNA) and hereditary alteration can guide to breast cancer have been associated with the exposure of estrogen. Some patients inherit fault in the deoxyribonucleic acid (DNA) and genes like the P53, BRCA1 and BRCA2 among others. The patients with a family history of breast or ovarian cancer have possibility of developing breast cancer. Neoplastic cells require considerable potential to multiply and convert into a massive tumor. The immune system usually tries to find out cancer cells and cells with injured deoxyribonucleic acid (DNA) and demolish them. Breast cancer might be outcome of malfunction of such an useful immune defence and surveillance. Breast cancer commonly occurs due to an association between genetic and environmental factors. RAS/MEK/ERK pathway and PI3K/AKT pathway defend normal cells from cell suicide. When mutation occurs in genes that are involved in encoding of these protective pathways, the cells become unable of committing suicide when they are no longer required which then leads to development of cancer. These mutations were confirmed to be experimentally associated with estrogen exposure. It was recommended that deformity in the growth factors signaling can assist growth of malignant cells. Over expression of leptinin breast adipose tissue enhances proliferation of cell and cancer. These are numerous growth factors signaling and other factors that interrelate between epithelial cells and stromal cells. Interruption in these might result in development of breast cancer. In cancer cells, enzyme telomerase turns away the chromosomal shortening and allows the extensive replication of cells. Tumor cells get their nutrients and oxygen supply by angiogenesis. Cancer cells break their boundaries and can enter into the blood, lymphatic tissues and other tissues of the body to produce a secondary tumor**.(Villadsen *et al*;2007)**The 2003 World Health Organisation classification recognises 18 distinct histological types of invasive breast cancer ; however the diagnostic criteria for the characterisation of each entity are rather subjective, and information on histological subtype has a limited impact on therapeutic decision making.



Breast cancer includes various clinical, morphological and molecular entities .Histological stratification of breast cancers based primarily on the expression of estrogen receptor (ER), progesterone receptor (PR), and ERBB2 receptor (HER2) has been useful and laid the foundation for the classification of breast cancers. In fact, breast cancer is a prototypic tumor in which the initial molecular subclassification has led to improved outcomes by guiding the administration of targeted therapeutics such as hormonal therapy (e.g.Tamoxifen) and HER2-targeted therapy (e.g.Trastuzumab). While histological stratification is still a common practice, technological advances have unraveled further complexities with the emergence of at least five distinct molecular subtypes [i.e., luminal A, luminal B, HER2-enriched, basal-like, and normal-like] based on gene expression clustering. Building on this, the combined genomic/ transcriptomic analyses of breast cancers have resulted in identification of 10 distinct breast cancer subtypes based on integrated clusters. In line with these advances, efforts to further segregate some of the established histological subtypes have also been carried out for ER negative and triple-negative breast cancers**.(Sandhu *et al* ;2016)**

The prognosis of breast cancer patients has been improved over time. However, further improvements in targeted treatment for breast cancer patients are expecting to solve the problem that why current therapy has effect only on a portion of the patients. A major milestone on the way to this goal is the definition of breast cancer molecular subtypes based on gene expression profiles: Basal-like, LuminalA, LuminalB, HER2-enriched and Normal-like.

Luminal A

The luminal A breast cancer is the most common subtype, representing 50–60% of the total. It is characterized by the expression of genes activated by the ER transcription factor that are typically expressed in the luminal epithelium lining the mammary ducts. It also presents a low expression of genes related to cell proliferation. Based on their molecular profile, all cases of lobular carcinoma in situ are luminal A tumors, as are most of the infiltrating lobular carcinomas. The luminal A immunohistochemistry (IHC)profile is characterized by the expression of ER, PGR, Bcl-2 and cytokeratin CK8/18, an absence of HER2 expression, a low rate of proliferation measured by Ki67 and a low histological grade . Moreover, the GATA3 marker expresses its highest level in the luminal A subgroup. Patients with this subtype of cancer have a good prognosis; the relapse rate is significantly lower than that for other subtypes. In addition, survival from the time of relapse is also longer (median 2.2 years). They have a distinct pattern of recurrence with a higher incidence of bone metastases and with respect to other localizations such as central nervous system, liver and lung which represent less than 10%. The treatment of this subgroup of breast cancer is mainly based on third-generation hormonal aromatase inhibitors (AI) in postmenopausal patients, selective estrogen receptor modulators (SERMs)like Tamoxifen and pure selective regulators of ER like Fulvestrant**.(Shamsi *et al* ;2003)**

LUMINAL B

Tumors with the luminal B molecular profile make up between 10% and 20% of all breast cancers. Compared to the luminal A, they have a more aggressive phenotype, higher histological grade and proliferative index and worse prognosis. The pattern of distant relapse also differs, and although the bone is still the most common site of recurrence , this subtype has a higher recurrence rate in sites such as the liver . Additionally, the survival from time of relapse is lower (1.6 years). Luminal A and B both express ER, but, since luminal B's prognosis is very different, a strong effort to find biomarkers that distinguish between these two subtypes has been made. The main biological difference between the two subtypes is an increased expression of proliferation genes, such as MKI67 and cyclin B1 in the luminal B subtype which also often expresses EGFR and HER2 . From the immunohistochemical point of view, there have been attempts to differentiate between luminal A and B using the protein expression of Ki67 as a

possible marker. The luminal A subtype has been defined as ER+/HER2_ and low Ki67, while the luminal B subtype has tumors with ER+/HER2_ and high Ki67 or ER+/HER2+. However,considering that this marker is the most widely used to measure cell proliferation, efforts are being made to reach a consensus on how to evaluate it. In fact, an international consortium has recently published a set of recommendations for Ki67 assessment in breast cancer.Luminal B tumors have a worse prognosis than do luminal A tumors despite treatment with tamoxifen and AI. Numerous clinical trials are testing inhibitory molecules of thePI3K/AKT/mTOR pathway at different levels, focusing on the treatment of luminal B tumors. **(Evan et al; 2001)**

Table 1 Characterization of four major breast tumor subtypes, population prevalence, and clinical characteristics

Subtypes	Molecular/genetic characteristics	Prevalence (%)	Clinical characteristics
Luminal A	ER+ and/or PR+, HER2−, low Ki67	40	Slow-growing Less aggressive Low recurrence High survival Best prognosis of all subtypes Respond to endocrine therapy
Luminal B	ER+ and/or PR+, HER2+ (or HER2− with high Ki67)	10–20	High proliferation rates Worse prognosis than Luminal A Respond to endocrine therapy
HER2 overexpressing	Positive the human epidermal growth factor receptor 2 (EGFR2) protein, ER, PR−	10	Tend to grow and spread more aggressively More likely to be high grade and node positive Poor short-term survival Targeted therapies exist
TNBC	ER−, PR−, HER2−	10–20	Younger age at diagnosis High histologic grade Higher rates of distant recurrence after surgery Poor short-term prognosis. Lack targeted therapy

Source American Cancer Society. Breast cancer facts and figures 2013–2014 [...]

ER± estrogen receptor positive or negative, *PR*± progesterone receptor positive or negative, *HER2*± human epidermal growth factor positive or negative

2.4 TREATMENT STRATEGIES FOR DIFFERENT TYPES OF BREAST CANCER

Despite the discovery of these new subtypes, the receptor status of a breast cancer is still the most widely used means of assessment because it determines whether it can be treated with targeted therapy, one of the most effective treatments as of now.

Hormone Receptor-Positive Breast Cancer

To date, approximately 85% of Hormone Receptor-Positive (estrogen receptor positive (ER+) breast cancer patients achieve more than five years overall survival after diagnosis, mainly because of the application of modern endocrine therapies. Selective ER antagonists such as tamoxifen can block the binding between estrogen and ER. ER expression modulators, such as

fulvestrant, directly interfere with ER synthesis. Aramatose inhibitors, such as letrozole (Femara), anastrozole (Arimidex), exemestane (Aromasin), inhibit estrogen synthesis. All of these treatment strategies are very effective in treating ER+ breast cancer, and therefore have a better prognosis in general**.(Donnelly *et al;*2013)**

HER2 Positive Breast Cancer

Compared with ER+ cancer cells, HER2+ cancer subtype tends to be much more aggressive and fast growing, and therefore has a worse prognosis due to the fact that this subtype of cancer cells can receive more growth factors through the overexpressed growth factor receptors on the cell surface. On the other hand, HER2+ cancer cells can be effectively treated with HER2 receptor targeting drugs. Monoclonal antibodies, such as trastuzumab (Herceptin), pertuzumab (Perjeta), can arrest cell cycle resulting in reduced proliferation; tyrosine kinase receptor inhibitor, such as lapatinib (Tykerb) can halt phosphorylation, thus interfering with protein synthesis .(**Kelsey *et al* 1996).**

Triple Negative Breast Cancer

Statistically, about 15% of invasive breast cancers lack hormone receptor expression, i.e. the so called "triple negative" cancers. The triple negative cancers have by far the worst prognosis due to the lack of targeted treatments and higher chance of recurrence. Studies are ongoing to find suitable prognostic markers for these cancers for developing targeted therapeutics. For example, androgen receptor is found to be overexpressed in about 40% of triple negative breast cancers and 80-90% of ER+ breast cancers. However, the role of the androgen receptor is different in ER+ and ER- cancer types. In ER+ breast cancer, inhibition of androgen receptor can have a positive stimulating effect on tumor growth whereas in ER- cells (including triple negative) inhibition of androgen receptor can inhibit tumor growth. **(McPherson *et al*; 2000)**

2.5 STAGES OF BREAST CANCER

According to the report of breast cancer.org Stages of the breast cancer depends upon the size and type of tumor and how much the tumor cells have been penetrated in the breast tissues .

Whereas stage 0 describes the non invasive and stage 4 describes the invasive kind of tumor. **(Weinberg *et al*;2007).** Descriptions of those tumor stages are:

Stage 0

This is the non invasive stage of tumour which indicates that both cancerous and non cancerous cells are within the boundaries of that part of the breast in which the tumor begins to grow and no evidence found of their invasion in the surrounding tissues of that part, the example of this tumour stage is ductal cell carcinoma in situ (DCIS).

Stage 1

This stage describes as the invasive breast carcinoma and microscopic invasion is possible in this stage. It has two categories that are 1A and 1B stage. The category 1A describes the tumor which measures up to 2 cm and none of the lymph nodes are involved in it while stage 1B describes that small group of cancer cells larger than 0.2 mm founds in lymph node.

Stage 2

Stage 2 also has two categories 2A and 2B. Stage 2A describes that the tumour is found in axillary lymph nodes or in sentinel lymph nodes but no tumor found in breast. The tumor can be smaller or larger than 2 cm but not more than 5 cm. However stage 2B describes that the tumor could be larger than 5 cm but can't reach to the axillary lymph nodes. **(Eroles *et al*;2012)**

Stage 3

It has been divided into three sub categories that are 3A, 3B and 3C. Amongst which stage 3A describes that no tumor is found in breast but it can be found in 4–9 axillary lymph nodes or in sentinel lymph nodes while stage 3B describes that the tumour can be of any size but have caused swelling or ulcer on the skin of the breast and can have spread up to 9 axillary lymph nodes or to sentinel lymph nodes stage 3B can be considered as inflammatory breast cancer which includes red, warm and swollen skin of the breast. However stage 3C describes the spread of tumor up to 10 or more than 10 axillary lymph nodes and it also have involved the lymph nodes above and below the clavicle.

Stage 4

This is the advanced and metastatic stage of cancer and this stage describes the spread to other organs of the body that is lungs, bones, liver brain etc. **(Eroles *et al*;2012)**

2.6 CURRENT TREATMENT MODALITIES FOR BREAST CANCER.

In the management of breast cancer, aim is to preserve quality of life with prolonged life expectancy. The use of bioflavonoids may inhibit estrogen formation. Effective communication between doctors and patients plays an important role to improve clinical outcome. **(Oshima et al;2011)** reported that effective communication between doctors and patients is effective. A study conducted in Japan indicates that this communication helps the patients to cope with adverse effects. Doctor patient communication enhances the quality of life of breast cancer patients. Previous studies have shown that less exposure from radiations, higher family monthly income, long years after diagnosis, higher education, initial stage cancer and younger age were considerably related with better quality of life (QOL) in patients with breast cancer. Breast cancer is less common in breast feeding women, but the protective effect of this factor is not clearly investigated. Cancer is a fatal disease affecting humankind in every country. Vinblastine and Vincristine was introduced in 1961 as anti cancer drugs. CIPLA has improved the process of isolating Vinblastine and Vincristine in the World, and India is exporting these alkaloids to European countries and the demand is steadily increasing. The main forms of treatment for cancer in humans are surgery, radiation and chemotherapeutic agents. The drugs can often provide temporary relief of symptoms, lengthening of life and occasionally cures the disease. Many hundreds of chemical drugs of known classes of cancer chemotherapeutic agents have been synthesized. The activity of these compounds is based on their capacity for biological alkylation. The effective dose of such alkylating agents was almost the same as the toxic dose. Multi-targeted therapy could be more effective, because the recurrence rate of cancer is high and death occurs due to metastasis**.(Deng *et al*;1996).** reported that Pemetrexed and Lobaplatin is prescribed in metastatic breast cancer. Huang and Cao reported that Cantharidin sodium injection is effective in the management of breast cancer. Cantharidinate sodium injection is herbal origin and is prepared in China for treatment of breast cancer. Breast cancer management strategies

differ depending on the step of the cancer—its mass, place, whether it has extended to other organs of the body and the physical condition of the individual. Present management for breast cancer includes targeted therapies, hormonal treatment, radiation therapy and surgery**.(Weinberg *et al;*2005)**

Psychological adjustment to breast cancer Breast cancer is extremely common and very worrying experience for numerous females every year in developing and developed countries. Psychological research has given an image of the emotional and community impact of breast cancer on females' lives, and of factors linked with better versus worse amendment. Psychosocial mediations have been helpful in reducing patients' grief and improving their life quality. Current study also recommends that psychological aspects might be associated with potentially significant biological ailment linked processes. Additionally, to giving an idea of the psychological aspects in breast cancer, investigation in this vicinity has given a foundation for further studies on adjustment to health-related nervous tension in common.

Surgery

This is the foremost management strategy for individuals whose breast cancer has not extended to further areas of the body and is also a choice for further complex stages of the illness. The kinds of breast cancer surgery vary in the quantity of tissue that is excised with the cancer; this depends on the cancer's characteristics, whether it has extended, and the patient's special feelings. A few of the most familiar kinds of surgery include:

Lumpectomy (breast conserving surgery) Some patients diagnosed with breast cancer undergo some type of surger. According to American cancer society, lumpectomy or partial mastectomy is the procedure of removing the part of the breast that contains malignant tumor along with some healthy tissues and surrounding lymph nodes leaving the major part of the breast intact as possible. This practice generally experienced in women that are in their initial phase of cancer, however the patient also requires another type of treatment such as radiation therapy, chemotherapy or hormone replacement therapy along with this procedure**.(Kato *et al*;2005)**

Mastectomy

Mastectomy is done to decrease the risk of development of breast cancer . Bilateral prophylactic mastectomy decreases the chances of development of breast cancer but does not eliminate the risk of developing cancer completely. Aromatase and tamoxifen decreases the risk of contra-lateral breast cancer and it is considered more effective than contra lateral prophylactic mastectomy.

Reconstructive surgery

Females who have a mastectomy might as well have breast renovation, either immediate reconstruction or delayed reconstruction. It is performed to get better the look of the breast following tumor surgery. All females having a mastectomy must be presented the option to converse reconstructive surgical treatment. Mastectomy is a comparatively simple surgical practice that typically results in stay in hospital for 1–2 days. Deficiency of the breast mass changes the patient's special look and can create wearing a few forms of clothing difficult. The utilization of an exterior prosthesis to tackle these problems can be awkward and scratchy, particularly for females with huge breasts. Breast reconstruction is commonly requested by females with breast cancer who are unable for breast-conserving treatment and females with an increased hereditary danger for breast cancer. Existing breast reconstruction procedures are miscellaneous and might engage the utilization of prosthetic implant or an autologous tissue flap, or both. Researches recommend that breast reconstruction restores body representation, proves vigor, femaleness, and sexuality; and optimistically influences the patient's feelings of comfort and life quality**.(Bonadonna *et al*;2000)**

Ovarian ablation as adjuvant therapy for breast cancer

Ovarian ablation has been employed as management for breast cancer. There are numerous techniques of ovarian ablation such as radiation induced ablation, surgical removal of ovaries and chronic utilization of luteinizing hormone-releasing hormone (LHRH) analogs. Additionally, there are few proposals that cytotoxic chemotherapy might perform by inducing ovarian ablation in premenopausal females with breast cancer. Of the abundant case series and clinical studies of ovarian ablation conducted in the earlier period, numerous have been laden with methodologic issues.

Role of estrogen and progesterone receptors in the management of breast cancer

The estrogen receptor assay has developed into a typical practice in the treatment of complex breast cancer. Tumors missing estrogen receptor react occasionally to endocrine treatment, while improvement proportions of 50–60% are seen in estrogen receptor positive tumors. Current researches demonstrate that the estrogen receptor condition of the principal cancer is a superior interpreter of the endocrine reliance of metastatic cancers at the moment of clinical deterioration. Additionally, the deficiency of estrogen receptor in the primary cancer is an significant self-regulating predictive display of higher incidence of relapse and shorter survival. Quantitative investigation of estrogen receptor and an analysis for progesterone receptor are two procedures for enhancing the precision of selecting or rejecting individuals for hormonal treatment; cancers with a elevated quantitative estrogen receptor amount or those with a positive progesterone receptor show the maximum response. Initial investigation demonstrates that the existence of progesterone receptor might be a improved indicator of tumor hormone dependence than quantitative estrogen receptor**.(Malara *et al*;2006)**

Anti‐ estrogen therapy

It can be used in such types of cancers that are affected by hormones and the tumor has hormone receptors such as estrogen receptors. Clarke et al. stated that the most common category of drugs that are used in breast cancer is anti estrogen, which includes the agents that are (tamoxifen, raloxifene, toremifene etc).(**Anders *et al*;2008**) Tamoxifen inhibits the hormone oestrogen from entering into cells of the breast cancer. This mechanism inhibits the breast cancer cells from developing. Tamoxifen can be suggested to treat female of any age group. However tamoxifen is considered as the drug of choice in women that have positive estrogen receptor breast carcinoma. Tamoxifen is a selective estrogen receptor modulator (SERMS) and acts like estrogen on other parts of the body such as uterus. However, it demonstrates anti estrogen properties of breast tissues and competes with estrogen for binding to the estrogen receptors in the breast. If we have to discuss about the toxic effects of anti estrogen therapy, comparatively there is very least toxicity found in it as compared to other cytotoxic drugs. While some patients withdraw the treatment before completing the course of drug due to the side effects such as hot flushes, gastro intestinal problems and vaginitis etc. Though, the medical indications for discontinuing antiestrogen therapy include adeno carcinoma, sarcoma and thrombo embolic diseases etc. Any

how the American society of clinical oncology recommends Tamoxifen as standard adjuvant therapy for patients with Estrogen positive breast carcinoma. On the other hand Fulvestrant; Faslodex has entirely anti estrogenic action and is considered as estrogen antagonist it demonstrates anti neo plastic activities in breast tissues without having a positive effect on the uterus and bones, which may lead to certain side effects if taken for a long period of time such as osteoporosis. Tamoxifen and raloxifene are selective estrogen receptor modulators (SERMs), a set of medicine that selectively prevents or motivates oestrogen-like activity in different tissues, affecting the estrogen receptors. Tamoxifen exhibits its oestrogen antagonist action in numerous tissues such as uterus, liver, bone and breast. A variety of adverse effects have been reported for females taking tamoxifen, such as venous thrombosis, cataract, endometrial cancer, menstrual disorders and hot flushes. A study indicated that the risk decreasing activity of tamoxifen expands beyond the vigorous management phase of 5 years, and remains for minimum 10 years, whereas the majority of adverse reactions do not carry on behind the 5 year management duration. Raloxifene, has also been revealed to decrease danger of breast cancer, however seems to exert some adverse reactions. A randomized clinical study of Raloxifen and Tamoxifen was planned for comparing the efficacies of raloxifen and tamoxifen on postmenopausal females with an enhanced 5-year threat of breast cancer as expected by the Gail model. **(Foulkes *et al*;2008**) The study demonstrated that raloxifen was comparable to the tamoxifen in decreasing the threat of invasive breast cancer and was linked with a minor danger of cataract and thromboembolism than tamoxifen.

Aromatase inhibitors

These are compound designed for decreasing oestrogen formation by targeting aromatase, the enzyme complex accountable for the last stair in the formation of estrogen. The third-generation aromatase inhibitors including letrozole, exemastane and anastrozole are in present utilization. Randomized clinical trial conducted for investigation of these agents in the treatment of breast cancer has indicated that these compounds contain an outstanding effectiveness in treating females with advanced disorder. Clinical study indicated that females managed with aromatase inhibitors had a superior contra lateral breast cancer threat decline than females managed with tamoxifen.

Radiation therapy

It is useful for reducing the necessity of mastectomies. A combination of a lumpectomy and radiation therapy is being increasingly used over a mastectomy in the early stages of breast cancer. A study was conducted in India. For this study 135 women were selected, most of them had undergone mastectomy. At the time of analysis, there was no local recurrence after hypo fractioned radiation therapy and metastatic disease developed in only four patients. Zhou et al. reported that radiation therapy is effective in early breast cancer patients. This study was conducted on 143 women who underwent either routine or intra operative radiation therapy after breast conserving surgery. At 54 months of follow up, there was a significant local control of the tumour. Highenergy rays from radiation therapy kill cancer cells. This therapy affects only the cells that are treated. Use of radiation therapy may be done after breast cancer surgery to destroy the remaining cells in the chest area**.(Harvey *et al* ;1999)**

Chemotherapy

The process of killing cancer cells by using certain medicines is termed as chemotherapy. It can be given in both situations, before and after surgery, depending upon the condition of the patient. According to the American cancer society the medicines include in chemotherapy are Docetaxel, Paclitaxel, Platinum agents (Cisplatin, Carboplatin), Vinorelbine (Navelbine), Capecitabine (Xeloda), Liposomal Doxorubicin (Doxil), Cyclophosphamide (Cytoxan), Carboplatin (Paraplatin) etc. However it has various side effects. Metastatic or secondary breast can is difficult to treat but it can be controlled and sometime for various years. Chemotherapy can be prescribed to manage metastatic breast cancer to minimize or sluggish its development**.(Wolff *et al*;2007)** It can also be administered to decrease some manifestations. Other treatment option can be initiated prior or alongside chemotherapy.

Taxol

Taxol is used clinically in the treatment of ovarian cancers and is undergoing clinical trials against metastatic breast cancers. It may also have potential value for lung, head and neck cancers. Taxotere is a side chain analogue of taxol, which has also been produced by semi synthesis from 10-deacetyl-baccatin III. It has improved water solubility, and is being clinically

tested against ovarian, and breast cancers. It can be used in those where resistance to cisplatin has been observed .

Anthracyclines

Anthracycline are commonly prescribed in the treatment of breast cancer. They impede with enzymes associated the DNA copying, which is desired for cells to separate to create new cells. Epirubicin and doxorubicin are the most commonly used medicines in breast cancers. There is proof that anthracyclines functions better than various other chemotherapy medicines. However these have adverse reactions such as damage to the heart and loss of hair. Prior to start of medicines, patient should converse with clinician any probable adverse reactions of drugs used and how these medicines might influence life quality.

Anti- oestrogens and prevention of breast cancer

With the accomplishment of anti-oestrogens in breast tumor management, numerous studies evaluated their use as an mediator to avert breast cancer in female at high risk. Tamoxifen is the antiestrogen medicine employed most commonly in the treatment of breast cancer. Administration of Tamoxifen as an adjuvant treatment following surgery, normally for 5 years, decreases the risk of hormone receptor breast cancer recurrence. Metastatic breast cancer is also managed by Tamoxifen. In numerous females, Tamoxifen induce the manifestations of menopause such as mood swings, vaginal discharge and hot flushes. Toremifene is one more medicine strongly related to Tamoxifen. It is used an alternate drug in postmenopausal female for the treatment of metastatic breast cancer. Fulvestrant is another drug that decreases the estrogen receptor numbers. It is usually useful in postmenopausal female, even in Tamoxifen resistant breast cancer. In previous studies, Tamoxifen was evaluated for its efficacy in 13, 388 females at higher risk of breast cancer for the period of 5 years. The study indicated a 49% decrease in risk of increasing invasive breast cancer and as well decreased risk of opposing side breast cancer, reappearance and extended existence in the female who had tamoxifen as accessory after operation. Antioestrogens are currently suggested as chemoprevention for female with atypical hyperplasia, genetic tendency to develop cancer and important family history of breast tumor. They are also prescribed because component of practice post-operative

concomitant management of those with estrogen receptor positive cancers for duration of 5 years following surgery**.(DeCensi *et al*; 2015)**

Human monoclonal antibody

Monoclonal antibodies are prepared in the laboratory. These are used alone or in combination with radiation therapy and chemotherapy to locate and target cancer cells. Usually, the body's immune system attack to foreign antigens such as infectious agents. It will then create antibodies to assist fight it off. The body does not identify cancer cells as a kind of foreign attacker. So, antibodies are then not formed. A randomized clinical trial was conducted to investigate efficacy of denosumab, a completely human monoclonal antibody against receptor activator of nuclear factor κ B (RANK) ligand, in comparison with Zoledronic acid in the prevention of skeletal-related events in breast cancers individuals with bone metastases. Denosumab was found better as compared to Zoledronic acid in preventing or delaying the SREs in breast cancer patients with bone metastasis. It is demonstrated that Denosumab is possible therapeutic alternative for individual with bone metastases. **(Shawky *et al*;2014)**

Immunotherapy

It utilizes the immune system of the body to fight against the cancer cells. Cancer vaccine is one of its examples. Parts of cancer cells or cancer cells are utilized for formation of vaccines. These cells excite the body's immune system to assist assault and destroy cancer cells . Immunotherapy has turn into a significant constituent in the management of breast cancer. HER2 targeted treatment are at the present an important part of HER2 over expressing breast tumor therapy. Trastuzumab, with the new current accompaniments of Pertuzumab and TDM1, encompass considerably superior breast cancer prediction. With various Federal Drug and Administration recommended antibody treatments used in together the adjuvant and metastatic settings, development progresses to be done in the area of immunotherapies. Current achievements in targeted therapies, vigorous particular immunotherapy, grasp assure for continuous success in general endurance within the adjuvant setting. The extremely precise and targeted strategy of vaccine therapy not simply avoids the adverse effects of recent standard of care therapies, active and passive immunotherapies including ipilimumab; however presents remedial strategy beyond now the HER2-overexpressing individuals. Even though vaccines for breast cancers have been

mainly ineffective in precedent clinical studies, the most of these studies conducted in the location of latestage metastatic illness, adverse surroundings for agents intended to stop, as different to manage, disease. With present clinical studies conducted on the adjuvant settings, immunogenicity is at the present indicating association with medical response.

Anti–angiogenesis drugs

Angiogenesis and inflammation are host-dependent manifestations of tumors that can be targeted with impediment strategies long prior to cancer start and develop . Antiangiogenic treatment in breast cancer presents important promise, and numerous continuing investigations are trying to better describe the best management settings and mediator assortment. For patients with estrogen receptor positive aliment, researches recommend a relationship among resistance of endocrine and cancer dependence on angiogenic networks, suggesting a possible curative advantage in mixing endocrine treatment with antiVEGF mediator. Findings from randomized clinical studies emphasize the multiplicity in reaction to antiVEGF treatment and recommend the requirement for better choice of patient subsets further to be expected to advantage from these therapies. The recognition of biomarkers for therapy response is solitary part of deep attention, though mainly study to date has become unsuccessful to discover a relationship linking cancer-associated markers including cancer mutations and EGF expression and scientific response**.(Shawky *et al*; 2014)**

OBSTACLES IN CURRENT TREATMENTS

Chemotherapeutic regimens are the most common treatment to inhibit tumor growth, but there is great variability in clinical responses of cancer patients; cancer cells often develop resistance to chemotherapeutics which results in tumor recurrence and further progression .

Compared to other breast cancers, triple-negative breast cancer (TNBC) usually affects younger patients, is larger in size, of higher grade and is biologically more aggressive. To date, conventional cytotoxic chemotherapy remains the only available treatment for TNBC because it lacks expression of the estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2), and no alternative targetable molecules have been identified so far. Chemotherapeutic drugs exhibit antitumor activities by inhibiting cancer cells via induction of cell cycle arrest and/ or cellular apoptosis. However, these drugs are not specific to tumor cells

and have deleterious effects on normal cells in the intestinal crypt and bone marrow. As a result, they have serious side effects including appetite changes, vomiting, nausea, anemia, hair loss, fatigue, fertility changes, etc. Lowering doses of these drugs may alleviate these side reactions, but could not effectively inhibit tumor growth. Thus, it is of highly clinical significance to identify agents that could be combined with present chemotherapeutic drugs to decrease the doses without reducing their effectiveness as well as to avoid and/or to overcome drug resistance.

2.7 DRUG REPOSITIONING AND EMERGING ADJUVANT THERAPY IN BREAST CANCER

Discovery of new drugs for cancer treatment is an expensive and time-consuming process and the percentage of drugs reaching the clinic remains quite less. Drug repositioning refers to the identification and development of new uses for existing drugs and represents an alternative drug development strategy.In oncology there is an increasing interest in the use of non-cancer drugs for cancer treatmens due to the previous detailed knowledge of pharmacokinetics/dynamics and toxicities, and because most repositioned drugs are available at low cost, normally as generics which provides an opportunity to bypass partially the early costs and time associated to new drugs development. This is a particular issue for low-income countries, where the availability of drugs for cancer treatment is very limited and restricted to some essential cytotoxic drugs and cost is a major factor in influencing access to cancer therapies .

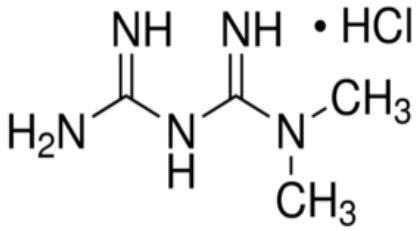
Drug repositioning is frequently combined with metronomic chemotherapy to what has been defined as “metronomics”. Metronomic chemotherapy refers to the regular administration of conventional chemotherapy drugs at low, minimally toxic doses, without long resting periods of time . Importantly, several phase II trials have shown effectiveness of metronomic therapies on different cancer types including TNBC with different drugs . Interestingly, developed and approved drugs that showed an anti-cancer opportunity for therapy can be administered orally, on daily basis, in a metronomic fashion**.(Rico *et al*; 2014)**

The control of distant metastasis and relapse after potentially curative multimodality therapy including a combination of systemic therapy, radiotherapy, and surgery as appropriate, remains a

major challenge. Neoadjuvant chemotherapy has become an accepted approach for inoperable and operable early-stage breast cancer, allowing breast conservation and assessment of the sensitivity of the tumor to systemic therapy .Diabetics with breast cancer receiving metformin and neoadjuvant chemotherapy had a better prognosis and higher pathologic complete response rate (pCR) than those not receiving metformin . Metformin inhibited cell proliferation in breast cancer cell lines, but had no efficacy on breast epithelial cell lines. Specifically, metformin selectively targeted the chemotherapy-resistant, sub-population of CSCs, reversed EMT, inhibited tumor growth, and improved remission in breast cancer. The combination of metformin with chemotherapy (e.g., doxorubicin, paclitaxel,carboplatin, 5-FU, epirubicin, and cyclophosphamide)was more effective than either drug alone .Metformin combined with rapamycin had a greater effect of apoptosis and proliferation inhibition on breast cancer through PI3K/AKT/mTOR pathways than monotherapy and the same effect was also found in the combination treatment of metformin with everolimus, an analog of rapamycin .

2.8 METFORMIN AND MANNOSE FOR BREAST CANCER

2.8a METFORMIN



Metformin,an extensively prescribed and well-tolerated firstline therapeutic drug for type 2 diabetes mellitus, has recently been identified as a potential and attractive anticancer adjuvant drug combined with chemotherapeutic drugs to improve treatment efficacy and lower doses(**Habibollahi *et al*;2013**)

Retrospective studies have reported that patients with diabetes receiving Metformin exhibited decreased cancer incidence and cancer-related mortality . the molecular mechanisms underlying anticancer effects of Metformin, which included insulin- and AMPK-dependent effects,

selectively targetingcancer stem cells, reversing multidrug resistance, inhibition of the tumor metastasis and described the antineoplastic effects of metformin combined with chemotherapeutic agents in digestive system cancers (colorectal gastric, hepatic and pancreatic cancer), reproductive system cancers (ovarian and endometrial cancer), prostate cancer, breast cancer, lung cancer, etc **.(Rico *et al* ;2017)**

The molecular mechanisms underlying anticancer effects of metformin

The increased cancer risk and mortality in diabetes has been associated with obesity, hyperglycemia, and/or high levels of insulin-like growth factor (IGF), insulin and insulin resistance (IR) . Accumulative insulin, IGF, and hyperglycemia have been reported to induce mitosis and tumor growth and/or drug resistance to chemotherapy . Blocking these signaling pathways may reverse the tumor-promoting effects of diabetes and inhibit tumor cell proliferation.. The molecular mechanisms underlying anticancer effects of metformin (Figure2 include insulin- and AMPK-dependent effects, selectively targeting cancer stem cells (CSCs), reversing multidrug resistance (MDR) and inhibition in the metastasis of tumor **.(zhag *et al*;2016)**

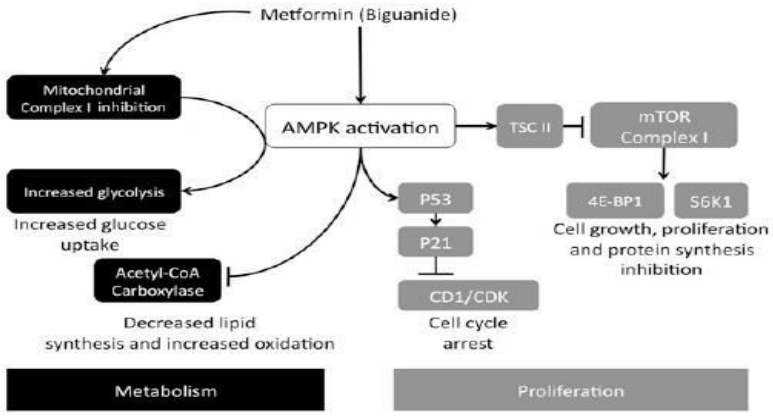


Figure 2

Metformin inhibits mitochondrial complex I . which results in

- interruption in mitochondrial respiration,
- decreasing synthesis of ATP,
- causing cellular energetic stress
- elevation of the AMP:ATP ratio which, in turn,
- activates AMP-activated protein kinase (AMPK)

AMPK activation leads to a reduction in mammalian target of rapamycin (mTOR) signalling and decreased protein synthesis and proliferation . It also could lower gluconeogenesis in the liver byimpairing oxidative phosphorylation, increase glucose uptake in muscle resulting in decreasing circulating glucoseconcentration, reducing insulin level, and increasing insulin sensitivity to overcome insulin resistance. As a result, metformin can potentially counteract the Warburg effect,which increases glycolysis in neoplastic tissue by systemicallyreducing growth factors and resulting in cytostatic action.

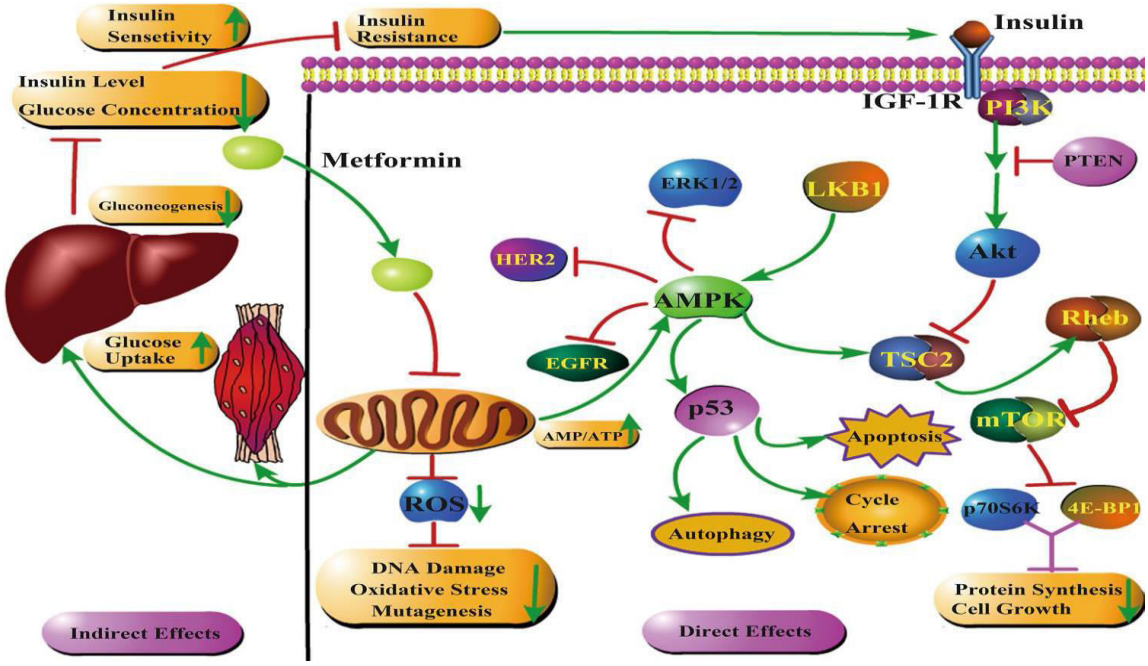
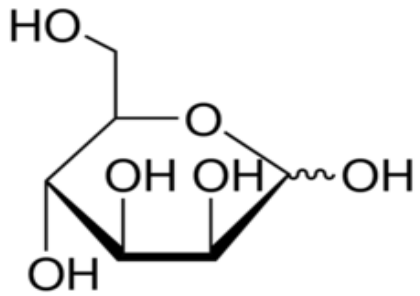


Fig 3:The insulin-dependent and AMPK-dependent molecular mechanism underlying anticancer effects of metformin. (**Erloes *et al*; 2012)**

Metformin shows inhibitory effects on tumor growth through insulin-mediated systemic effects. Alternatively, metformin can also modulate the vital AMPK/mTOR pathway to inhibit the protein synthesis and cell growth. In addition, metformin activation AMPK leads to the phosphorylation of p53, inducing cycle arrest, apoptosis, autophagy, inhibition of activated ERK1/2, a dramatical reduction in EGFRactivation, and other carcinoma associated tyrosine kinase .**Epithelial-mesenchymal transition** (EMT) is a process that epithelial cells acquire the highly invasive and metastatic ability of mesenchymal cells, which is associated with cancer progression and metastasis . Metformin could block transforming growth factor β (TGFβ)

signalling through activating AMPK signal transduction pathwayto reverse EMT phenotype in breast cancer cells**.(Erloes *et al*; 2012)**

2.8b MANNOSE



Mannose is a sugar monomer of aldohexose serious of carbohydrate which is an epimer of glucose . This monosaccharide has found to cause growth retardation in several tumour types in vitro, and enhances cell death in response to major forms of chemotherapy. Mechanistically, mannose is taken up by the same transporters as glucose3 but accumulates as mannose-6-phosphate in cells, and this impairs the further metabolism of glucose in glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway and glycan synthesis.(**Shag *et al*; 2004**)

It was reported that the administration of mannose in combination with conventional chemotherapy affects levels of anti-apoptotic proteins of the Bcl-2 family, leading to sensitization to cell death. Mannose intermediates in glycolysis are reported to inhibit lactic acid production without interfering glycoytic uptake of glucose**.** Mannose impairs the growth of cancer cells and interferes with glucose metabolism by accumulating intracellularly as mannose-6-phosphate. The combination of chemotherapeutic drugs with mannose enhances cell death by potentiating the intrinsic pathway of apoptosis and it was also found that in invivo studies. Mannose impairs tumour growth and induces tumour regression with out altering animal body weight (**Gonzale *et al* ;2018**). Metformin was found to increases the transport of Mannose in dermal Fibroblasts.The increased mannose uptake by1.8-fold and greater affinity for Mannose than basal Mannose transport in presence of metformin was noted as Metformin-stimulated Mannose Transport(MSMT)**.(Jie Shang *et al:*2003)**

3. AIM AND OBJECTIVES

3.1 AIM

- To investigate the role of Metformin in combination with Mannose as an Adjuvant therapy for Breast cancer treatment.

3.2 OBJECTIVES

- To investigate antiproliferative activity of Metformin, Mannose and its combination in MDA MB-231 cell line .
- To assess the apoptotic activity of Metformin and its combination with Mannose using AO/EB staining.
- To determine the Mitochondrial Membrane Potential in Apoptotic cell population by Rhodamine 123 staining.
- To assess the level of Reactive Oxygen Species by DCF-DA staining.
- To evaluate the effect of Metformin and its combination with Mannose on migration potential on MDA-MB 231 using scratch assay
- To evaluvate the effect on caspase activity through ELISA.

4. PLAN OF STUDY

PHASE – 1

- Literature review
- Drug selection and Collection
- Drug identification and validation
- Protocol design

PHASE-2

- *In-vitro* cell culture

The MDA –MB 231 & MDA-MB 468 cell lines were purchased ,cultured and grown .

- Dose fixation -MTT assay

The antiproliferative activity & IC50 values of cell lines treated with Metformin was determined .And also growth inhibitory concentration of Mannose was determined.

- Acridine Orange/Ethidium Bromide Staining

For the detection the morphological evidence of apoptosis Acridine orange/ethidium bromide (AO/EB) dual staining was performed .

- Migration Assay

The wound healing assay was performed in order to find the migratory ability cells in the untreated and drug treated wells.

- DCFH-DA staining

Intracellular ROS levels were measured using a cell-permeable fluorescent probe DCFH-DA.

- Rhodamine-123 staining

Membrane potential of Mitochondria in populations of apoptotic cells were studied using Rhodamine-123.

- Caspase assay

Fold increase in caspase activity was determined

- Isolation of Protein

Protein contents were Isolated and quantified .

PHASE – 3

- Data interpretation.
- Statistical Analysis.

5.MATERIALS AND METHODS

5.1 MATERIALS

5.1.1 CHEMICALS USED FOR THIS STUDY

Sl no	Product	Description	Company
1	AL219A	DMEM High Glucose	HIMEDIA
2	RM9955	Fetal Bovine Serum	HIMEDIA
3	PCT0605	D-Mannose	HIMEDIA
4	317240	Metformin	SIGMA
5	LS001	Hisep™ LSM 1077	HIMEDIA
6	R8004	Rhodamine 123	SIGMA
7	35845	2,7-dichloroflourescein diacetate	SIGMA

5.1.2 INSTRUMENTS USED IN THIS STUDY

S. No	Instruments	Manufacturer
1	Biosafety working hood-class II	Esco(Sentinel Gold)
2	CO2 incubator	Thermo Scientific
3	ELISA reader Multiskan Go	Thermo scientific
4	Autoclave Sterilizer	Everflow autoclave
5	ST16R-Refrigerated centrifuge	Thermo Scientific
6	Deep freezer	Thermo Scientific
7	Invertedfluorescence microscope	Nikon
8	Arthik thermal cycler	Thermo Scientific
9	Micropipettes	Eppendorf

5.2 METHODS

5.2.1 Cell lines and culture condition

Human breast cancer cells MDA-MB-231 and MDA MB 468 were procured from National Centre for Cell Science,(NCCS) Pune, India. The cell lines were grown as a monolayer in DMEM (Dulbecco's Modified Eagle Medium),(Himedia,Mumbai) containing 10% Fetal Bovine serum-FBS (Himedia, Mumbai),1% glutamine, 1% Penstrip.The cells were cultured at 37° C in a humidified atmosphere of 5% CO2 in the incubator(Thermo Steri-cycle CO_2 incubator).Cells were grown confluence for 24 h before use.

5.2.2 Preparation of complete media

For the complete media preparation 1% PenStrep (penicillinstreptomycin in order to prevent bacterial contamination) was added along with 1% Glutamax (for the directsupplement of L-Glutamine, energy production as well as protein and nucleic acidsynthesis). Then 10%v/v FBS were added to the culture media, which act as a supplement in *in vitro* cellculture of eukaryotic cells. They contain very low level of antibodies and high levels of growth factors.

5.2.3 Protocol for reviving the cells

The vials were taken from the liquid nitrogen tank and kept outside for 1 hour to thaw to room temperature.Then the contents were removed to 15ml centrifuge tube and to that 3ml of media or PBSwere added. It was then centrifuged at 1200rpm for 3-5 minutes in Thermo ST 16R Refrigerrated Centrifuge.The supernatant was removed and 1ml of complete media was added to the pellet. After resuspending a cell culture disk (10cm) was taken to which 9ml of media and 1ml of suspended cells were added.The cells were checked under microscope and also for the contamination and kept in CO2 incubator at 37°C with 5% CO2. The cells were periodically checked .

5.2.4 Cell counting:

For cell counting 20µl of cell suspension and 20µl of tryphan blue (Prepare a 0.4% solution of tryphan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i.e., phosphate-buffered saline. Add 0.1 mL of trypan blue stock solution to 0.1 mL of cells.) were mixed well and loaded to the haemocytometer and cells were counted using the equation :

$$\frac{number\ of\ cells\ counted}{4} * dilution\ factor(2) * volume\ in\ tube(5ml) * 10^4$$

Maintaining and storage of cell lines

For cryopreservation freeze medium is Complete growth medium supplemented with 5% (v/v) DMSO and the Storage temperature liquid nitrogen vapor phase.

5.2.5 Dose Fixation study:

MDA-MB-231 and MDA-MB-468 cells were seeded in a 96-well plate at the density of 6×10^3cells/well .Following the cell attachment, in 100 µl of medium, Metformin (Sigma Aldrich), Metformin in combination with Mannose(Himedia) was added to the wells in six replicates and incubated for 24 hr at 37℃ in 5% CO2.After that add 10µl of MTT in the same well without removing media at a concentration of 5mg/ml the cells were incubated at 37 °C for another 4 hrs. Decant the MTT and add 10µl of cell grade DMSO for about 30 minutes. Optical density of the formazan crystals were read at 560 nm using Multiscan Go Thermo UV-VIS spectrophotometer. Cell viability was calculated using the formula;

$$\textbf{Cell viability} = \frac{\textbf{O.D of sample} - \textbf{O.D of blank}}{\textbf{O.D of control} - \textbf{O.D of blank}} \times \textbf{100}$$

5.2.6 Acridine Orange/Ethidium Bromide Staining

For the detection the morphological evidence of apoptosis Acridine orange/ethidium bromide (AO/EB) dual staining was performed using Metformin, Metformin and Mannose combination treated cells. Cells were seeded at a density of 0.6 million cells / well in 6 well plate.After 24 hrs of drug treatment cells were washed with PBS. Then 1 µl AO/EB solution in PBS (1 part of 100µg /ml EB in PBS) was added and the samples were examined under fluorescent microscopy(470/440 nm filter) (Nikon Eclipse TS 100).

5.2.7 Migration Assay

MDA MB 231 cells were seeded into a 6-well plate and allowed to grow to 80% confluency in complete medium. Cell monolayers were wounded using 200µl tip at the centre and 10 µl tip at the two edges of each plate and the Wounded monolayers were then washed with PBS to remove the cell debris. The photograph were taken at 0 hrs and 24 hrs of incubation for the untreated and treated group ie, Metformin (10mM) , Metformin and Mannose (25Mm) combination . The cells are analysed for migration in the wells using inverted microscope. The relative distance between the cells were further examined using ImageJ Software.

5.2.8 DCFH-DA staining:

Intracellular ROS levels were measured using a cell-permeable fluorescent probe DCFH-DA (2′,7′-dichlorofluorescein diacetate)(Sigma Aldrich).DCFH-DA diffuses through the cell membrane and is hydrolyzed by an intracellular esterase to the non-fluorescent dichlorofluorescein (DCFH), which is rapidly oxidized by ROS to fluorescent dichlorofluorescein.The MDA MB 231 (0.6 million cells / well) were seeded in a 6 well plate in a media which contains no FBS .The IC50 concentration of Metformin (10 Mm) and in combination with mannose(25mM) were added and incubated for 48 hrs .DCF-DA of concentration of 10mM in DMSO was prepared and 3 µl was added to each well after the removal of media and observed under fluorescence microscope and fluorescence intensity was measured.

5.2.9 Rhodamine-123 staining

Rhodamine 123(Sigma Aldrich) a cell permeant,cationic fluorescent dye used to monitor the Membrane potential of Mitochondria in populations of apoptotic cells. Mitochondrial energization induces quenching of RH123 fluoresence and is directly propotional to Mitochondrial Membrane potential. MDA MB 231 (0.6 million cells / well) were seeded in a 6 well plate in a media which contains no FBS .The IC50 concentration of Metformin (10 Mm) and in combination with mannose(25mM) were added and incubated for 48 hrs . To each well after the removal of media 3 µl of 5mM of Rhodamine 123 was added and observed under fluorescence microscope and fluorescence intensity was measured.

5.2.10 Caspase assay

Caspase 3, 8, 9 enzyme activity will be assayed by using a calorimetric caspase assay kit (Biovision). Pretreated cells with drugs should be used for this assay. Cells will be

harvested and lysed in 160 µl lysis buffer. Centrifugation should be done to remove cell debris at 10000 rpm for 20 min. The systolic extract should be taken and protein estimation was done using Nano Drop measuring Apparatus(Molecular Devices). Dilute 50-200 µg protein to 50 µl cell lysis buffer for each assay. Add 50 µl of 2X reaction buffer that contains 10 mM DTT to each sample. Add 5 µl of 4 mM DEVD-pNA (caspase 3), IETD-pNA (caspase 8), LEHD-pNA (caspase 9) substrate and incubate at 37°C for 1-2 hour. Read samples at 400 or 405 nm in a multiscan plate reader. Fold increase in caspase activity can be determined by comparing these results with the level in the untreated .

5.2.11 Statistical Analysis

1) Data will represent mean+/-S.D
2) Student T-test/ ANOVA for comparing means of 2 groups(SPSS version 21)

6.RESULTS

1. ANTIPROLIFERATIVE ACTIVITY OF METFORMIN IN MDA-MB 231 & MDA-MB 468 CELL LINES

The anti-proliferative effect of Metformin was studied using MTT assay in MDA MB 231 and MDA MB 468 cell lines. It was found that there was a dose dependent alleviation in cell viability. IC50 values of Metformin in both the cell lines was obtained as 9.906mM and 30.12mM respectively**.(Fig1a&1b)**

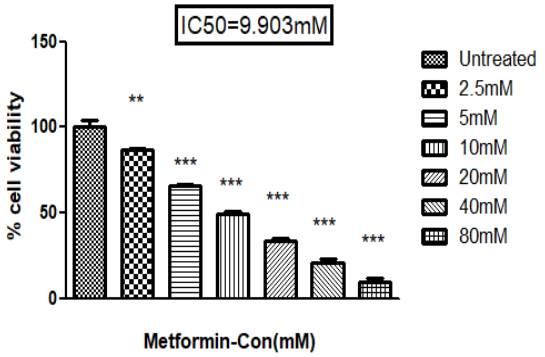


Fig 1a:

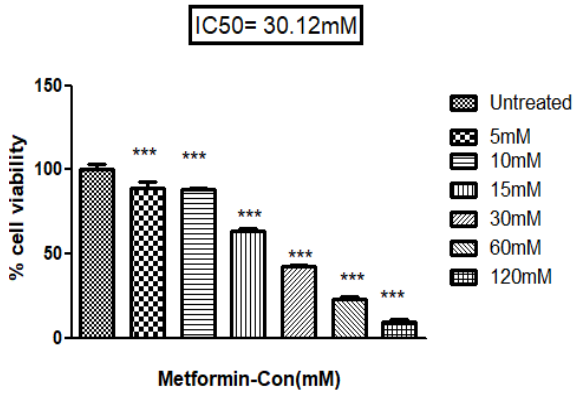


Fig 1b:

Fig 1: Cell viability of Metformin in MDAMB- 231 and MDA MB 468 cell lines. TNBC cells were seeded at a density of 6000cells/well/100µl and treated with various concentration of drugs. After 48 hours, MTT reagent was added and readings were recorded at 560nm. All experiments were performed in triplicates.

Fig:1a: Anti-proliferative activity of Metformin on MDA-MB-231 cells were investigated at a concentration range of 0-80mM.At the concentration of 9.903 mM Metformin exhibited 50% cell death in MDA-MB-231 cells.

Fig: 1b: In MDA-MB-468 the growth inhibitory activity was done in a concentration range of 0-120 mM and the IC50 was obtained as 30.12 mM. Therefore the MDA-MB-468 cell line is less sensitive to Metformin when compared to MDA-MB-231 . The graph represents the IC50 values determined using MTT assay, and expressed as perdcentage cell viability at different doses with untreated group as mean ± SD (n=3). P values were calculated using one-way ANOVA.(***P<0.001) P value : *** indicate that the group shows higher significance when comparing with control group and ** indicates significance P<0.01.

2. GROWTH INHIBITORY ACTIVITY OF MANNOSE IN MDA-MB-231

The cell growth inhibitory effect of Mannose in MDA-MB-231 cells lines were obtained using MTT assay.Mannose from concentration range of 0-50mM was used for finding the growth inhibitory activity. The antiproliferative activity of Mannose was seen in MDA-MB231 cells. 25mM concentration of Mannose was selected for further experiments.

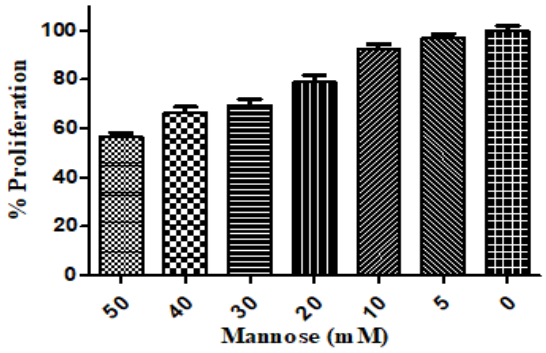


Fig 2

Fig 2: cell growth inhibitory effect of Mannose in MDA-MB-231 cells lines

3.COMPARISON OF GROWTH INHIBITORY ACTIVITY OF MANNOSE,GLUCOSE IN COMBINATION WITH METFORMIN

The graph represent combinatory effect of Mannose with metformin and of Glucose with Meformin in cell growth inhibition. The viability was measured using MTT assay.One way ANOVA was performed and found significant(P<0.001)indicated by*** .When compared with untreated group,it was found that there was no significant difference in selectivity of mannose and glucose when combined with Metformin in MDA MB231 cell lines.

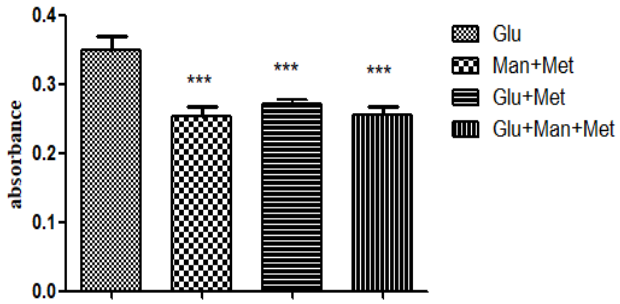


FIG 3:Comparison of Growth Inhibitory activity of Metformin in Presence of Glucose & Mannose

4.DETERMINATION OF APOPTOSIS USING AO/EB STAINING:

The morphological and nuclear variations in cells,such as condensed and fragmented nuclei are regarded as late events of apoptosis. For identifying the changes in cell nuclei in MDAMB- 231 upon treatment with Metformin(10Mm) alone and in combination with Mannose (25Mm) were stained with AO/EB and visualized by fluorescence microscopy.The control cells that stained evenly with lightly green and evenly with AO, but stained negative for EB, suggesting the presence of live cells. But MDA-MB 231 cells when treated with Metformin and in combination with Mannose for 48hrs, exhibited a condensed orange nucleus, whereas the necroticcells showed a structurally intact nucleus with an evenly distributed orange colour by AO/EB staining.Here we have compared Metformin, Mannose and their combination to determine the apoptotic changes by counting live cells, early apoptotic cells, late apoptotic cells and necrotic cells.

Fig 4:

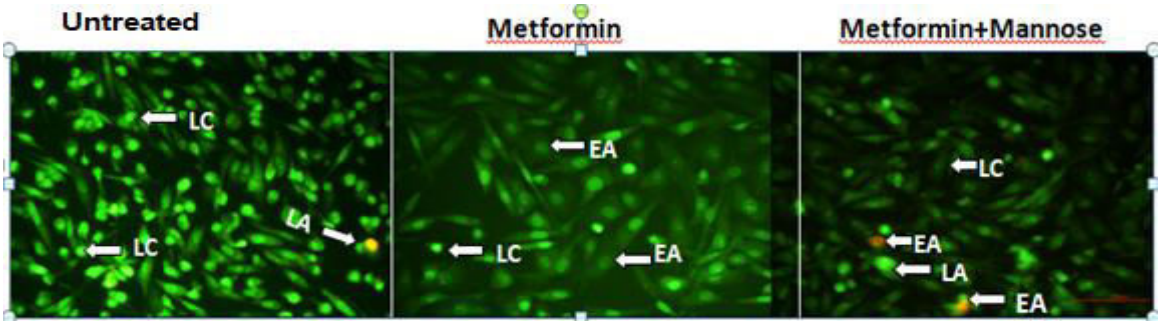


Fig 4a:

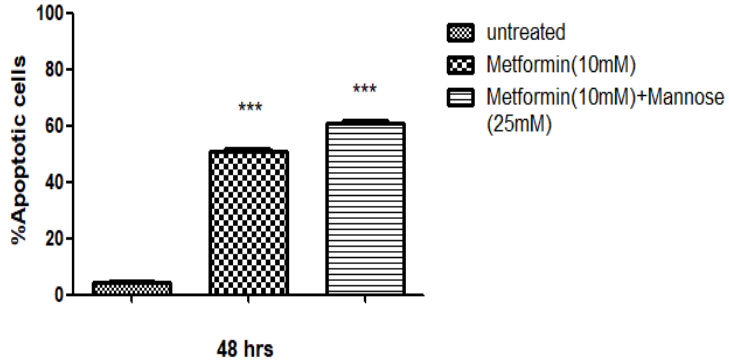


Fig 4: Photographic image of Apoptotic effect of Metformin alone and in combination

with Mannose determined by Acridine Orange-Ethidium Bromide dual staining in MDA MB231 cell llines.

MDA-MB 231 cells were cultured and treated with indicated concentrations of Metformin (10 mM) and Mannose(25mM) combination for 24 hrs following which the cells were stained with Acridine Orange-Ethidium Bromide and visualised under a fluorescence microscope. Arrows indicate cells in early apoptosis (EA), late apoptosis (LA), live cells (LC) and convoluted nuclei.

.

Fig 4a: Graph represents the significance of drug treatment using Metormin (10mM) and in combination with Mannose (25mM). 300 cells were further counted and graph was plotted against % apoptotic cells and at time interval of 48 hours. The data was analysed by statistical evaluation through one way ANOVA as ***(P<0.001) (*** represents significance showed when comparing Metformin alone & combination with Mannose with the untreated)On comparing Metformin alone and in combination with Mannose ,the combinatory effect of Metformin & Mannose was found to induce more apoptosis .

5. DETERMINATION OF CELL MOTILITY BY MIGRATION ASSAY

Observation of live cells motility is an effective method to measure the rate of migration into the space created by the original wound. In the untreated group, cell migration was very dynamic at 48 hours than when compared to 0 hours. The MDA-MB 231 cells were allowed to culture in 6 well plate and the cell monolayers were wounded using 200µl tip at the centre and 10 µl tip at the two edges of each plate. The cells were incubated with corresponding concentrations of drugs ie, Metformin (10mM) and combination with Mannose(25mM)for 48 hours. The cells are analysed for migration in the wells using inverted microscope. The relative distance measured at 100 random points between gap was examined using ImageJ Software.

Fig:5

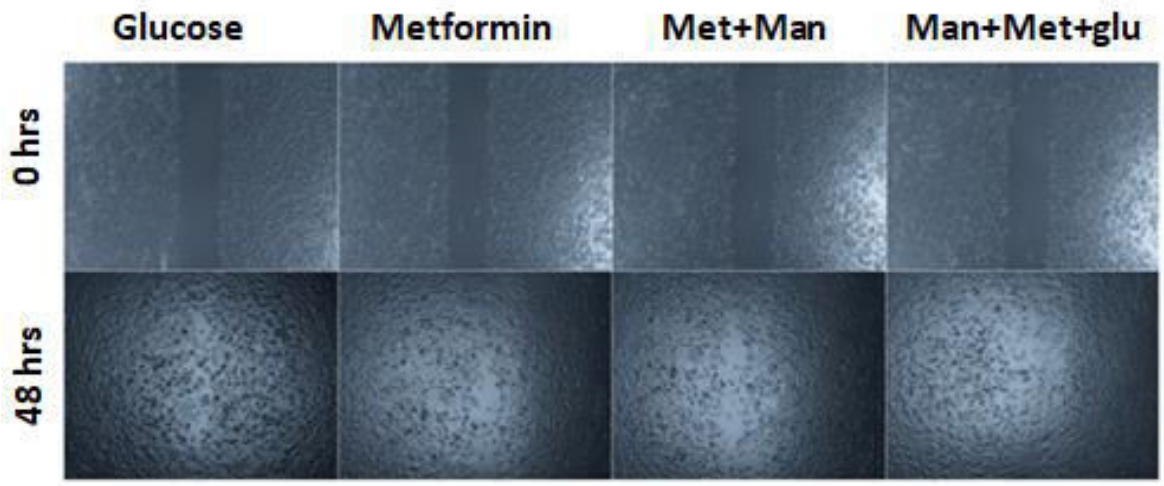


Fig 5a:

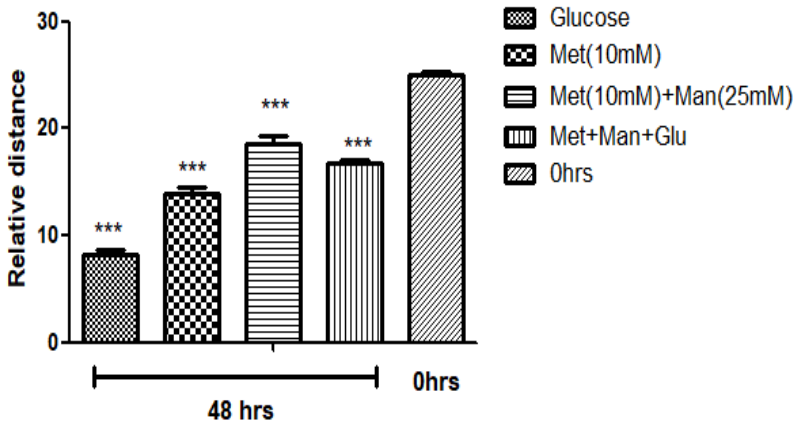


Fig 5: Inhibition of migratiory effect on MDA-MB-231 cells by Metformin alone and in combination with Mannose &Glucose .

The gap did not reached complete closure for either agent. Comparison of these groups shows drugs has some influence on cell migration inhibition in MDAMB- 231 cells than untreated group. **Fig5a:** Graphical representation of migration assay .The results were presented as the distance in relation to the relative distance of the initial scratch at 0 hrs and 48 hrs for Metformin and combinations**.** In graph *** indicates higher significance (P<0.001) Metformin at a concentration of 10 mM alone and in combination with Mannose (25 Mm) found significant & it was found that the combination of Metformin and Mannose promote more inhibitory migration effect on MDA-MB-231 cells than other groups.

6. MODULATION OF MITOCHONDRIAL MEMBRANE POTENTIAL IN MDA-MB 231 CELL LINES ON DRUG TREATMENT USING RHODAMINE 123.

Rh 123 is a cell permeable fluorescent dye used to monitor the Membrane potential of Mitochondria in populations of apoptotic cells . Mitochondrial energization induces quenching of Rh123 fluoresence and is directly propotional to Mitochondrial Membrane potential.

Fig 6:

Untreated **Metformin** **Metformin+Mannose**

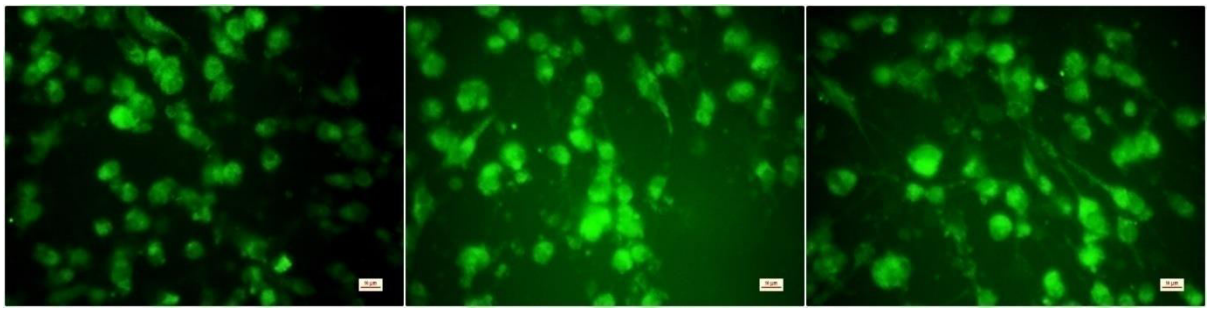


Fig6a:

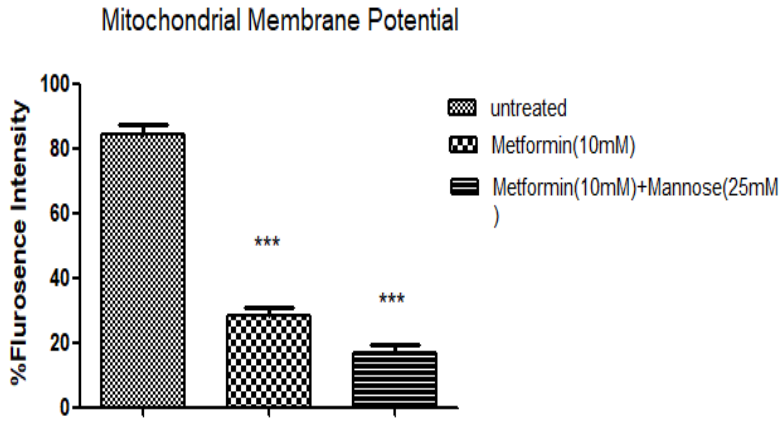


Fig 6: Fluorescence photomicrographs showing the effect of Metformin and in combination with Mannose.

Fluorescence microscopic images showed accumulation of Rh123 dye in the Untreated group is more prominent .Comparitively very less Rh123 accumulation was found in Metformin – Mannose treated cells as the membrane potential decreased.

FIG 6a : The data was analysed by statistical evaluation through one way ANOVA as ***(P<0.001) (*** represents significance showed when comparing Metformin alone & combination with Mannose with the untreated).The percentage of mean fluorescence intensity values confirms the mitochondrial membrane potential was found to be reduced in the Combination treated cells when compared with Metformin alone .

7.INTRACELLULAR ROS GENERATION ASSESSMENT BY DCF-DA STAINING.

Intracellular ROS levels were measured using a cell-permeable fluorescent probe DCFH-DA (2′,7′-dichlorofluorescein diacetate).DCFH-DA diffuses through the cell membrane and is hydrolyzed by an intracellular esterase to the non-fluorescent dichlorofluorescein (DCFH), and is rapidly oxidized by ROS which is highly synthesized in apoptotic cell populations and converted to fluorescent dichlorofluorescein (DCF) .

Fig 7:

Untreated **Metformin** **Metformin+Mannose**

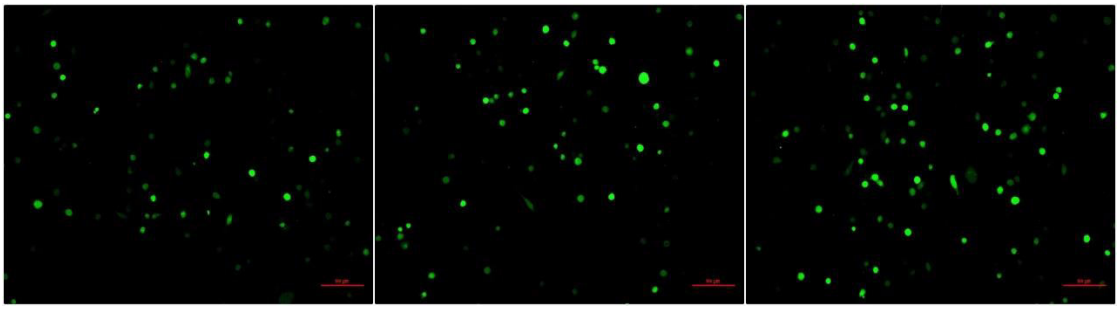


Fig 7a:

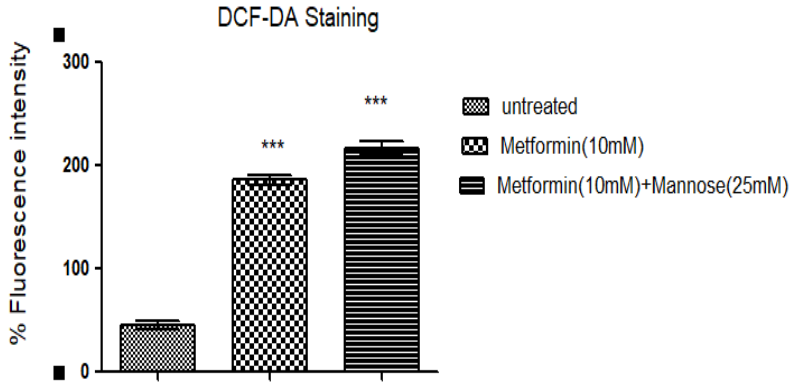


Fig 7 :Intracellular ROS generation by DCFH-DA staining

Fig 7a: Graphical representation of mean florescence intensity ROS (%) in MDA-MB 231 cell lines . The percentage of mean fluorescence intensity values confirms the DCF fluorescence in different treatment groups .ROS generation were observed in Metformin(10mM) alone and in combination with Mannose (20mM) treated cells. While weak ROS generation was observed in the untreated MDAMB 231 cells. Metformin alone treated cells showed significant ROS production. But enhanced ROS generation was found in combination of Metformin and Mannose treated cells.The data was analysed by statistical evaluation through one way ANOVA as ***(P<0.001) .(***represents significance showed when comparing Metformin alone & combination with Mannose with the untreated.

8. CASPASE ASSAY

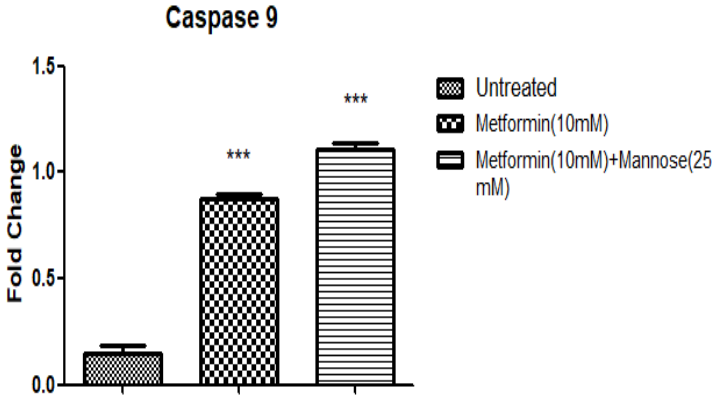


Fig 8a:

Graph showing significance in the caspase-9 activity Metformin alone and Metformin ,Mannose treated groups

Caspase-9 enzyme activity assay: caspase 9 is an inducible enzyme that promotes cell death upon activation via intrinsic pathway. Metormin (10mM) and the combination with Mannose significantly induced caspase-9 activation that was measured by a perceptible increase in activity. shows significant (p<0.001)effect on caspase-9 levels (***represents significance showed when comparing treated group with untreated group)

CASPASE 8

Fig 8:

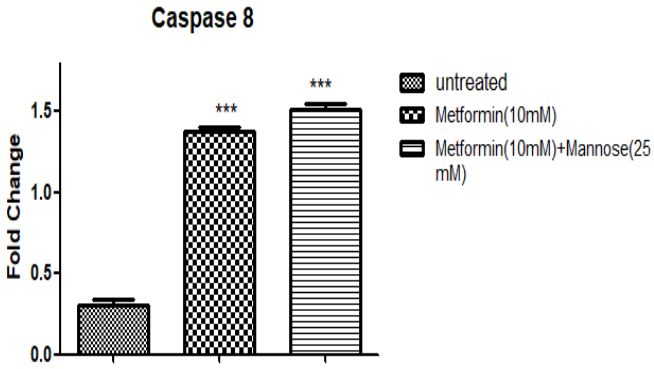


Fig 8:Graph showing significance in the caspase-8 activity Metformin alone and Metformin ,Mannose treated groups

Caspase-8 enzyme activity assay: caspase is an inducible enzyme that promotes cell death up on activation via extrinsic pathway. Metormin (10mM) and the combination with Mannose significantly induced caspase-8 activation that was measured by a perceptible increase in activity. shows significant ($p<0.001$)effect on caspase-8 levels (***represents significance showed when comparing treated group with untreated group)

CASPASE 3

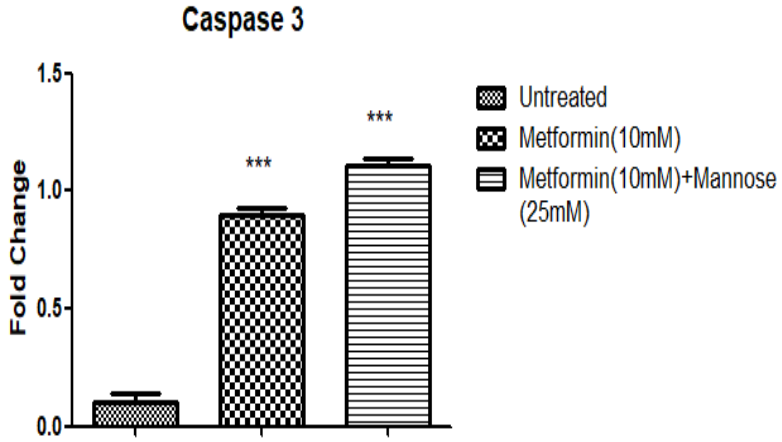


Fig 8b:

Graph showing significance in the caspase-9 activity Metformin alone and Metformin ,Mannose treated groups

Caspase-3 enzyme activity assay: caspase 3 is a protease enzyme that is associated with formation of apoptotic bodies which is formed via both intrinsic and extrinsic pathway . Metormin (10mm) and the combination with Mannose significantly induced caspase-3 activation that was measured by a perceptible increase in activity. shows significant ($p<0.001$)effect on caspase-3 levels (***represents significance showed when comparing treated group with untreated group)

7.DISCUSSION

The present study we have investigated the antiproliferative role of Metformin in combination with Mannose on TNBC cells as an Adjuvant therapy for breast cancer treatment. Investigation of adjuvant therapeutic strategies are essential and important to enhance quality of life in breast cancer victims(**Zhang H *etal*;2016**). Anti-diabetic drug Metformin showed significant and dose-dependent antiproliferative activity. Retrospective studies have reported that patients with diabetes receiving Metformin exhibited decreased cancer incidence and cancer-related mortality**.(Rico *et al* ;2017).**Mannose impairs the growth of cancer cells by interfering with glucose metabolism by accumulating intracellularly as mannose- 6-phosphate**.(Gonzalez PS et al;2018)** Mannose which targets the key source of cellular energy in the breast cancer cells may represent a major advantage over classic chemotherapies.

We have demonstrated in the present study that Metformin (10mM) significantly inhibited the proliferation in a dose-depended manner and Mannose at concentration of (25mM) was able to significantly inhibit the proliferation of of MDA-MB231 cells.The proliferative capacity of tumor cells is deemed vital for the growth and development of tumors(**Lu Z et al.,**).The present study demonstrated that a series of concentrations of Metformin dose-dependently reduced the proliferation and growth of MDA-MB 231 cell lines and Metformin combination with Mannose led to a significant higher reduction in cell viability . The anti-proliferative effect of Metformin and Mannose was examined using MTT assay. IC50 values of Metformin in MDA-MB 231 and MDA-MB 468 was identified to be 9.903mM and 30.12mM respectively.

Cellular migration is known to be associated with an EMT phenotype. Thus we compared the migratory ability of TNBC cells by performing wound healing assays. In a typical scratch wound healing assay, a “wound gap” in a cell monolayer is created by scratching, and the “healing” of this gap by cell migration and growth towards the center of the gap is monitored and often quantitated(**Ravi M et al.,**). Migration and invasion are both key components of EMT in TNBC cells . Since the MDA-MB 231cell line is a metastatic cell line that invades through the basement membrane, we tested the hypothesis that the treatment with Metformin and combination with Mannose would decrease migration and invasion. The cells were analysed for migration in the wells using an inverted microscope. The relative distance between the cells was further analysed using ImageJ Software at random points. It was found to be statistically significant at 48 hrs. After the scratch Metformin and combination with

Mannose treated cells showed lower migration ability compared to that of control cells for MDAMB-231. These results indicate that cell migration reduces upon addition of Metformin and combination with Mannose which is an important factor to consider in TNBC.

Treatment of the MDA-MB 231 with Metformin and Mannose combination resulted in a strong induction of apoptosis. Co-treatment of Mannose with metformin induced a significantly higher reduction in cell viability and a significant higher increase in apoptosis than treatment with the respective single substances.Apoptosis serves a central role in regulating normal tissue equilibrium, and dysregulation of apoptosis presents a key factor in the growth of cancer. Therefore, strategies that target the apoptotic process may inhibit TNBC development(**Oba T, Ito KI**). Apoptotic cells exhibit characteristics, including cell shrinkage, and chromatin and nuclear condensation. To determine whether the inhibition of TNBC cellular proliferation induced by Metformin and its combination with Mannose was associated with apoptosis, morphological alterations were detected by AO-EB staining following treatment for 48 hrs. The control cells that stained evenly with lightly green and evenly with AO but stained negative for EB, suggesting the presence of live cells. On the other hand, MDA-MB 231 cells after 48 h of treatment with drugs, illustrated a condensed orange nucleus, while the necrotic cells displayed a structurally intact nucleus with an evenly distributed orange staining.

Mitochondrial Membrane Potential ($\Delta\Psi m$) is critical for maintaining the physiological function of the respiratory chain to generate ATP; the opening the mitochondrial permeability transition pore (MPTP) leads to the collapse of the $\Delta\Psi m$ and subsequent release of cytochrome C into the cytosol.Mitochondrial membrane potential is commonly detected using cationic (positively-charged) fluorescent dyes that accumulate in the negatively-charged mitochondrial matrix. The dye accumulates in inverse proportion to$\Delta\Psi m$ *i.e*. the more negative the $\Delta\Psi m$, the more dye accumulates. This means that a healthy cell will contain more dye while an apoptotic cell will contain less.Here we have used Rhodamine 123 a cell permeant,cationic fluorescent dye used to monitor the Membrane potential of Mitochondria in populations of apoptotic cells. The IC50 concentration of Metformin (10 Mm) and in combination with mannose(25mM) were added to the MDA-MB 231 cells and incubated for 48 hrs. 3 µl of Rhodamine 123 in a molar concentration of 5mM was added and observed under fluorescence microscope and fluorescence intensity was measured. Fluorescence microscopic images showed accumulation of Rh123 dye in the Untreated group.Less Rh123 accumulation was found in Metformin –Mannose treated cells as the membrane potential

decreased .The percentage of mean fluorescence intensity values confirms the mitochondrial membrane potential was found to be reduced in the Combination treated cells when compared with Metformin alone

DCFH-DA) is one of the most widely used techniques for directly measuring the redox state of a cell. Intracellular ROS levels were measured using a cell-permeable fluorescent probe DCFH-DA ROS and the oxidative damage are thought to play an important role in many human diseases including cancer. In our study the ROS generation was observed in Metformin(10mM) alone and in combination with Mannose (20mM) treated cells. While weak ROS generation was observed in the untreated MDAMB 231 cells. Enhanced ROS generation was found in combination of Metformin and Mannose treated cells than in Metformin alone treated cells .

Results of Caspase assay shows that treatment with Metformin and combination with Mannose in MDA MB 231 cell line activates Caspase-9, caspase-8, caspase-3. Results obtained with Metformin and combination with Mannose are proving it's extrinsic as well as the intrinsic apoptosis pathway inducing property. Apoptosis was confirmed by ethidium bromide and acridine orange staining assay.From our study Metformin and combination with Mannose as anti-MDA MB 231 adjuvant would add new therapeutic dimensions to the current limited approach in the management of this most deadly malignancy in human.

8. CONCLUSION

Discovery of new drugs for cancer treatment is an expensive and time-consuming process and the percentage of drugs reaching the clinic remains quite less. Drug repositioning refers to the identification and development of new uses for existing drugs and represents an alternative drug development strategy.In oncology there is an increasing interest in the use of non-cancer drugs for cancer treatmens due to the previous detailed knowledge of pharmacokinetics/dynamics and toxicities, and because most repositioned drugs are available at low cost, normally as generics which provides an opportunity to bypass partially the early costs and time associated to new drugs development .The antitumour activity of Metformin appears to be valuable as an effective strategy for the treatment of all inoperable, chemotherapy-resistant or recurring breast cancers. Mannose represents an attractive option since it targets the key source of cellular energy in the breast cancer cells may represent a major advantage over classic chemotherapies.

In our study we found that the co-treatment of Metformin and Mannose shows significant increase in growth inhibitory activity in MDA-MB 231 cell lines using MTT assay.The apoptosis of TNBC cells induced by Metformin and Mannose via increase of upregulation of caspase-3, caspase-8 and procaspase-9, which may be associated with both extrinsic & intrinsic mitochondrial pathway. We compared the motility of the Metformin treated cells and the combination of Mannose in MDA MB-231 cells using Wound healing assays. The percentage of wound closure was significantly decreased in Metformin & Mannose treated cells compared to that of Metformin alone treated cells. These results suggest that Metformin& Mannose combination have association EMT process reversal indicating less motility of the cells thus reducing the risk of distant metastatis. Gene expression studies should be done for further confirmation.

Mitochondrial Membrane Potential ($\Delta\Psi m$) is critical for maintaining the physiological function of the respiratory chain to generate ATP; the opening the mitochondrial permeability transition pore (MPTP) leads to the collapse of the $\Delta\Psi m$ and subsequent release of cytochrome C into the cytosol. We assessed the mitochondrial membrane potential using Rhodamine 123 staining showed that there was a reduction in flouresence intensity ie Mitochondrial Membrane Potential ($\Delta\Psi m$) in treated group than the untreated and thus confirmed apoptotic activity was more in Metformin and Mannose treated groups. Reactive Oxygen specices was found to be more prominent in treated cells when compared to

untreated using DCF-DA staining method. Apoptosis was confirmed by Ethidium Bromide and Acridine Orange staining assay.

In conclusion, the present study demonstrated that the combination of Metformin and Mannose enhance the antiproliferative activity on MDA-MB 231cells via the intrinsic and extrinsic apoptotic pathways, indicated by Caspase assay. These findings suggested that Metformin and Mannose combination as adjuvant therapy may be a beneficial option to enhance the current chemotherapy in breast cancer .

.

9. REFERENCES

- DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. Cancer treatment and survivorship statistics, 2014. CA: a cancer journal for clinicians. 2014 Jul;64(4):252-71.
- Anderson KN, Schwab RB, Martinez ME. Reproductive risk factors and breast cancer subtypes: a review of the literature. Breast cancer research and treatment. 2014 Feb 1;144(1):1-0.
- Akram M, Iqbal M, Daniyal M, Khan AU. Awareness and current knowledge of breast cancer. Biological research. 2017 Dec;50(1):33.
- Easton DF, Ford D, Bishop DT. Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. American journal of human genetics. 1995 Jan;56(1):265.
- Neville MC. Anatomy and physiology of lactation. Pediatric Clinics of North America. 2001 Feb 1;48(1):13-34**.**
- Tang Y, Wang Y, Kiani MF, Wang B. Classification, treatment strategy, and associated drug resistance in breast cancer. Clinical breast cancer. 2016 Oct 1;16(5):335-43.
- Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 1994 Oct 7;266(5182):66-71.
- Sandhu GS, Erqou S, Patterson H, Mathew A. Prevalence of triple-negative breast cancer in India: systematic review and meta-analysis. Journal of global oncology. 2016 Jun 29;2(6):412-21.
- Thakur KK, Bordoloi D, Kunnumakkara AB. Alarming Burden of Triple-negative Breast Cancer in India. Clinical breast cancer. 2017 Jul 20.
- Villadsen R, Fridriksdottir AJ, Rønnov-Jessen L, et al. Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol. 2007;177(1):87–101
- Evan G, Vousden K. Proliferation, cell cycle and apoptosis in cancer. Nature. 2001;411:342
- Shamsi U, Khan S, Usman S, Soomro S, Azam I. A multicenter matched case control study of breast cancer risk factors among women in Karachi, Pakistan. Asian Pac J Cancer Prev. 2013;14:183–8

- Donnelly T, Khater A, Al-Bader S. Arab women's breast cancer screening practices: a literature review. Asian Pac J Cancer Prev. 2013;14(8):4519–28
- Kelsey J, Bernstein L. Epidemiology and prevention of breast cancer. Ann Rev Public Health. 1996;17:47–67
- McPherson K, Steel C, Dixon J. Breast cancer-epidemiology, risk factors, and genetics. Brit Med J. 2000;321:624–8
- Weinberg O, Marquez-Garban D, Pietras R. New approaches to reverse resistance to hormonal therapy in human breast cancer. Drug Resist Updat. 2005;8:219–33. 90.
- Kato S, Sato T, Watanabe T, Takemasa S, Masuhiro Y, Ohtake F. Function of nuclear sex hormone receptors in gene regulation. Cancer Chemother Pharmacol. 2005;56:4–9. 92.
- Malara N, Leotta A, Sidoti A, Lio S, D'Angelo R, Caparello B, et al. Ageing, hormonal behaviour and cyclin D1 in ductal breast carcinomas. Breast. 2006;15:81–9
- Anders, C. and L.A. Carey, Understanding and treating triple-negative breast cancer.
    - Oncology (Williston Park), 2008. 22(11): p. 1233-9; discussion 1239-40, 1243.
- Foulkes, W.D., I.E. Smith, and J.S. Reis-Filho, Triple-negative breast cancer. N Engl J Med, 2010. 363(20): p. 1938-48.
- Kumar P, Aggarwal R. An overview of triple-negative breast cancer. Archives of gynecology and obstetrics. 2016 Feb 1;293(2):247-69.
- Harvey JM, Clark GM, Osborne CK, Allred DC (1999) Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 17:1474–1481 26.
- Wolff AC, Hammond ME, Schwartz JN et al (2007) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med 131:18–43
- DeCensi A, Thorat MA, Bonanni B, Smith SG, Cuzick J. Barriers to preventive therapy for breast and other major cancers and strategies to improve uptake. Ecancermedicalscience. 2015;9.
- Shang J, Lehrman MA. Metformin-stimulated mannose transport in dermal fibroblasts. Journal of Biological Chemistry. 2004 Mar 12;279(11):9703-12.

- Shawky H, Galal S. Preliminary results of capecitabine metronomic chemotherapy in operable triple-negative breast cancer after standard adjuvant therapy–A single-arm phase II study. Journal of the Egyptian National Cancer Institute. 2014 Dec 1;26(4):195-202.
- Bonadonna G, Brusamolino E, Valagussa P, Rossi A, Brugnatelli L, Brambilla C, De Lena M, Tancini G, Bajetta E, Musumeci R, Veronesi U. Combination chemotherapy as an adjuvant treatment in operable breast cancer. New England Journal of Medicine. 1976 Feb 19;294(8):405-10.
- Rico M, Baglioni M, Bondarenko M, Laluce NC, Rozados V, Nicolas A, Carré M, Scharovsky OG, Márquez MM. Metformin and propranolol combination prevents cancer progression and metastasis in different breast cancer models. Oncotarget. 2017 Jan 10;8(2):2874.
- Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science. 1996 Dec 20;274(5295):2057-9.
- Oshima T, Takenoshita S, Akaike M, Kunisaki C, Fujii S, Nozaki A, Numata K, Shiozawa M, Rino Y, Tanaka K, Masuda M. Expression of circadian genes correlates with liver metastasis and outcomes in colorectal cancer. Oncology reports. 2011 May 1;25(5):1439-46.
- Zhang HH, Guo XL. Combinational strategies of metformin and chemotherapy in cancers. Cancer chemotherapy and pharmacology. 2016 Jul 1;78(1):13-
- Eroles P, Bosch A, Pérez-Fidalgo JA, Lluch A. Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. Cancer treatment reviews. 2012 Oct 1;38(6):698-707
- Gonzalez PS, O'Prey J, Cardaci S, Barthet VJ, Sakamaki JI, Beaumatin F, Roseweir A, Gay DM, Mackay G, Malviya G, Kania E. Mannose impairs tumour growth and enhances chemotherapy. Nature. 2018 Nov;563(7733):719
- Oba T, Ito KI. Combination of two anti-tubulin agents, eribulin and paclitaxel, enhancesanti-tumor effects on triple-negative breast cancer through mesenchymal-epithelial transition. Oncotarget. 2018 May 1;9(33):22986.
- Ravi M, Tentu S, Baskar G, Prasad SR, Raghavan S, Jayaprakash P, Jeyakanthan J,Rayala SK, Venkatraman G. Molecular mechanism of anti-cancer activity ofphycocyanin in triple-negative breast cancer cells. BMC cancer. 2015 Dec;15(1):768.

Institutional Human Ethics Committee
PSG Institute of Medical Sciences & Research

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER, WHO)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : +91 422 - 4345818, Fax : +91 422 - 2594400, Email : ihec@psgimsr.ac.in
EC-CT-2018-0055

Ref. No.: PSG/IHEC/2018/Appr/Exe/004 May 02, 2019

To
Ms Athira Shaji
II Year M Pharm
Department of Pharmacology
Guide: Dr Jaikanth C
PSG College of Pharmacy
Coimbatore

Dear Ms Athira,

Sub.: Approval for study titled *"In-vitro investigation of metformin in combination with mannose as an adjuvant therapy for breast cancer treatment*

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has exempted the above study from ethical review.

The following documents were received for review:

1. Duly filled application form
2. Proposal
3. Confidentiality statement
4. Application for waiver of consent
5. CV
6. Budget

The validity of this letter is for one year from the date of issue.

Kindly notify amendments, if any, in the study proposal to IHEC.

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

Dr D Vijaya
Member - Secretary
Institutional Human Ethics Committee

SECRETARY PSG IMS&R COIMBATORE-641004 INSTITUTIONAL HUMAN ETHICS COMMITTEE

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

PSG ANIMAL FACILITY

COIMBATORE - 641 004

TOUCHING LIVES WITH CARE
LASA

Certificate of Participation

This Certificate is Awarded to

Ms. / Mr. / Dr. Athira Shaji

For Contribution to the

FIRST NATIONAL SYMPOSIUM ON ANIMAL RESEARCH AND ETHICS

Organized by PSG Institute of Medical Sciences & Research, Coimbatore-641 004

as a participant in Poster Presentation / Delegate / ~~Resource Person~~

12 - 13th, September 2019

This CME is accredited by The Tamil Nadu Dr. M.G.R. Medical University, Chennai

20 *Credit points under Category* ***III***

Coordinator ***PSG Animal Facility***	***Organizing Secretary***	***Principal*** ***PSG College of Pharmacy***	***Dean*** ***PSG IMS&R***

National Seminar

on

"Research Advances and Therapeutic Interventions in Neurodegenerative Disorders"

6th&7th July, 2018

NEUROGEN-2018

JSS ACADEMY OF HIGHER EDUCATION & RESEARCH
(DEEMED TO BE UNIVERSITY) MYSURU

JSS Academy of Higher Education & Research, Mysuru
(Deemed to be University)
(Accredited 'A' Grade by NAAC)

JSS College of Pharmacy
(An ISO 9001 : 2015 Certified Institution)
Udhagamandalam (Ooty), The Nilgiris, Tamil Nadu, India

Certificate

This is to certify that

Dr./Mr./Ms. ATHIRA SHAJI

has participated as a ***Delegate*** *in* **NEUROGEN - 2018**

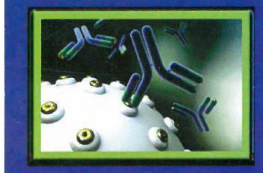
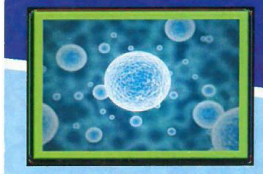
Dr. S.P. Dhanabal
Patron

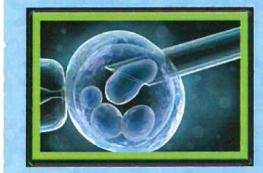
Dr. Praveen T.K
Convenor

Dr. A. Justin
Organizing Secretary

Organized by
Department of Pharmacology
JSS College of Pharmacy
(An ISO 9001 : 2015 Certified Institution)
Ooty, The Nilgiris, Tamil Nadu

Sponsored by
Indian Council of Medical Research
New Delhi



सत्यमेव जयते
Department of Science and Technology
Government of India

सत्यमेव जयते
DEPARTMENT OF BIOTECHNOLOGY
Government of India

Department of Pharmacology
PSG COLLEGE OF PHARMACY
Coimbatore

National Seminar on Future Aspects of Pharmacotherapeutic Approaches in Disease Management

5th & 6th October 2018

Certificate

This is to certify that ~~Prof./Dr./Mr./Ms./Mrs.~~ ATHIRA SHAJI

has actively participated as delegate and presented a paper titled.....

.....

..... *in Oral / Poster session of the said seminar*

Organised by Department of Pharmacology, PSG College of Pharmacy, Coimbatore

Dr. G. Venkatesh
Organising Secretary

Dr. M. Ramanathan
Principal / Chairman