IN-VITRO INVESTIGATION OF SELECTED PLANT EXTRACT IN MDA-MB 231 CELL LINE FOR THE TREATMENT OF BREAST CANCER

Dissertation submitted to

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MASTER OF PHARMACY

IN

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Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled **"*In-vitro* investigation of selected plant extract in MDA-MB 231 cell line for the treatment of breast cancer "**submitted by University **Reg no. 261925912** is a bonafide work carried out by the candidate under the guidance of **Dr. S. Muthukrishnan, M.Pharm., Ph D.**, Department of Pharmacology, PSG College of Pharmacy 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 2020-2021.

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DECLARATION

I do hereby declare that the dissertation work entitled **"*In-vitro* investigation of selected plant extract in MDA-MB 231 cell line for the treatment of breast cancer"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology, was done by myself candidate under the guidance of **Dr. S. Muthukrishnan, M.Pharm., PhD**., Associate professor, Department of Pharmacology, PSG College of Pharmacy, during the academic year 2020-2021.

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EVALUATION CERTIFICATE

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ABBREVIATIONS

ACExt	-	Acerola cherry extract
ADAM	-	A disintegrin and metalloproteinase
ANOVA	-	Analysis of Variance
AO	-	Acridine Orange
BRCA	-	Breast cancer gene
BTB	-	Branch target buffer
DCF	-	DA-2′,7′-dichlorofluorescein diacetate
DCIS	-	Ductal Carcinoma *Insitu*
DMEM	-	Dulbecco's Modified Eagle Medium
DMSO	-	Dimethyl sulfoxide
DPPH	-	1,1- diphenyl-2-picrylhydrazyl method
EAAEC	-	Ethyl acetoacetate extract
EB	-	Ethidium Bromide
EDTA	-	Ethylene diaminetetra acetic acid
EEC	-	Ethanolic extracts
EGFR	-	Epidermal Growth Factor Receptor
EMT	-	Epithelial to mesenchymal transitions
ER	-	Estrogen Receptor
FBS	-	Fetal Bovine serum
FRAP	-	Fluorescene recovery after photobleaching
GLOBACAN	-	Global Cancer Program
HER2	-	Human Epithelium receptor 2
HPLC	-	High performance liquid chromatography
IBC	-	Inflammatory Breast Cancer
IHEC	-	Institutional Human Ethical commitee
IL	-	Interlukein
LPS	-	Lipoolysaccharide
MDR	-	Multidrug resistance
MIR	-	Mortality/incidence ratio
MTOR	-	Mammalian Target of Rapamycin
PARP	-	Poly (ADP-Ribose) Polymerase
PBS	-	Phosphate buffer solution

PD-1	-	Programmed death receptor-1
PD-L1	-	Programmed death ligand-1
PI3K	-	Phosphoinositide- 3 kinase
PR	-	Progesterone Receptor
ROS	-	Reactive Oxygen Species
RPMI	-	Roswell Park Memorial institute Medium
SD	-	Standard deviation
TNBC	-	Triple negative breast cancer
TNF-α	-	Tumor necrosis factor-α
VEGF	-	Vascular endothelial growth factor
WHO	-	World Health Organisation

1.1. Breast cancer

Cancer is a group of disease that causes changes in the cell and grow out of control forming lumps or masses called as tumors and can invade the adjacent tissues and causes the destruction of the tissues. When a proto-oncogene mutates, it becomes an oncogene in an uncontrolled manner, this can lead to cancer **(Siddiqui *et al.*, 2013)**

Breast cancer is a most deadly lethal heterogeneous disease occurs in unregulated manner within any component of breast tissue especially ducts and lobules **(Harbeck *et al.*, 2017).** The treatment strategies with higher efficacy and lower toxicity are still to be investigated. It is diagnosed by different types of analysis **(Akram e*t al.*, 2017)** such as clinical examination, radiological examination, immunohistopathological examination and other methods for better prognosis include Mammaprint, Clinical breast examination and Oncotype DX.

1.2. Anatomy and its function

Female Breast is made up of adipose tissue and grandular tissue which are sensitive to normal change in body. It contains 12-20 lobes and smaller lobules which connects via milk ducts. It possess a network of lymph vessels, nerves blood vessels, lymph nodes, fibrous connective tissue and ligaments. It contains 15-25 milk ducts extended at the base of nipple for synthesizing milk sinuses (carrier of milk). The nipple stimulation enhances prolactin secretion and skin contains several apocrine and sebaceous sweat glands. The epidermis of areola is pigmented and contains several smooth muscle fibres of circular radially present in connective tissue and longitudinally lactiferous ducts to nipple. The anatomy of human breast was shown in **Figure 1** (**Akram e*t al.*, 2017).**

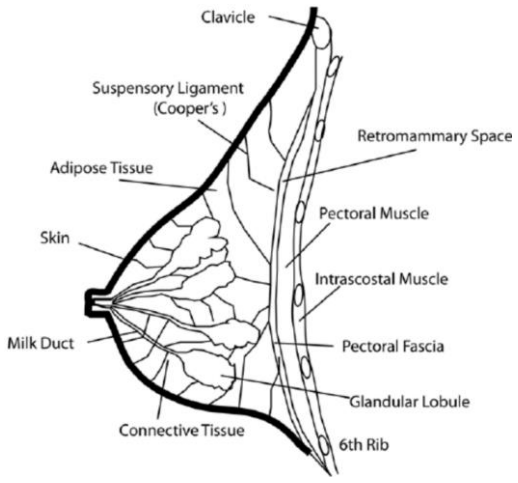


Figure 1 Structure of Human Breast (Hipwell JH *et al.*, 2016)

1.3. Epidemiology

As per WHO, Breast cancer leads to increased mortality of women at the age of 20-59 years **(Siegel *et al.*, 2018).** From this 1.7 million cases of breast cancer are diagnosed every year with nearly fourfold increase in incidence rates. Different types of cancer ratio affecting different organs was represented in **Figure 2,**

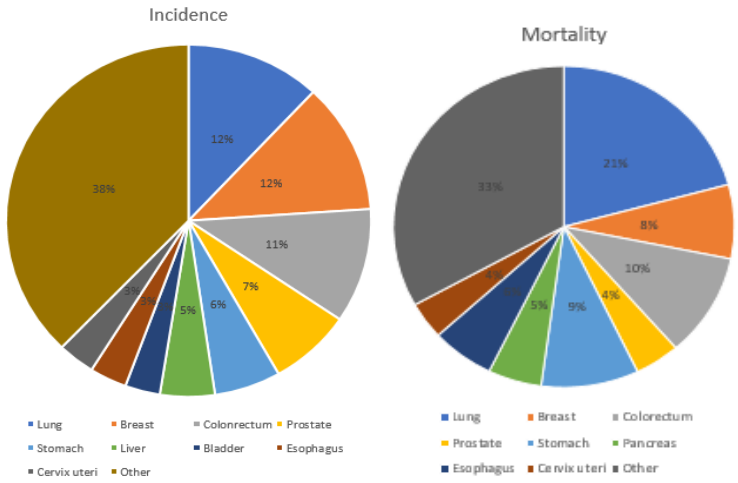


Figure 2: Incidence and mortality ratio of different cancers

Mortality/incidence ratio (MIR) is a measure to evaluate the cancer mortality with incidence in a region having higher mortality than on its incidence. According to Global Cancer Program (GLOBOCAN), breast cancer stands the second most common cancer worldwide trending to rise upward and has been prolonging globally **(Chaffer *et al.*, 2011).**. Fifth most common cause of cancer death is breast cancer. The ratio of different developing countries was shown in **Figure 3 (Madhav *et al.*, 2018),**

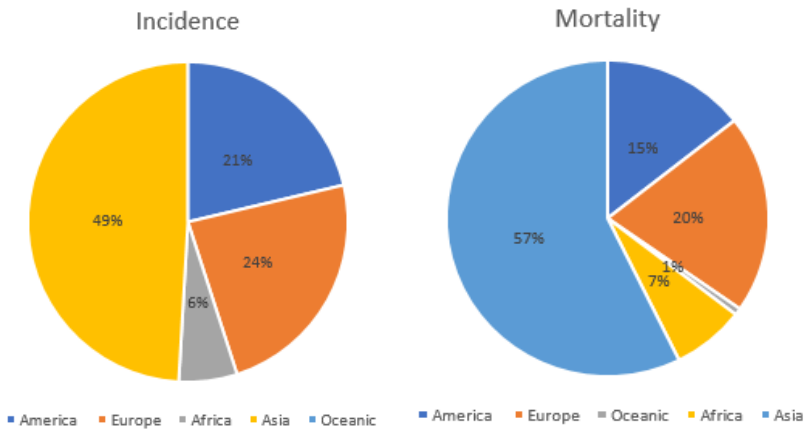


Figure 3: Incidence and mortality ratio in developing countries

In India, breast cancer trend to increase upward, where 1,00,000 women were diagnosed with fatality ratio of 20-43%. Peak age of breast cancer is 40-50 yrs in women compared to 50-70yrs in other countries having incidence of TNBC subtypes was prevalent with almost all races. Breast cancer projection in India during 2020 suggests the number as high as 17,97,900 with its high occurrence of 3yrs and mortality of 5 yrs after diagnosis with relative percentage of 10% among all the cancers and shown in **Figure 3 (Maurya *et al.*, 2020).** The TNBC had been reported to be more (54.-6% and 51.5%) among postmenopausal women compared to premenopausal women.

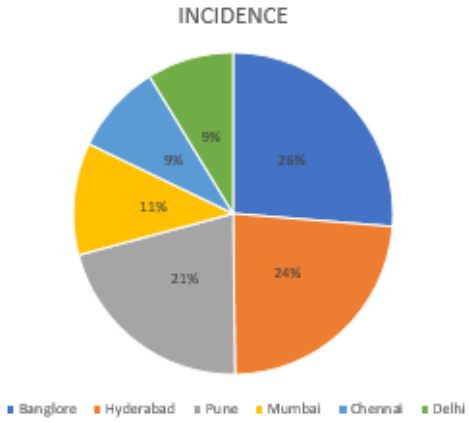


Figure 4: Incidence and mortality ratio in India

The changes in breast cancer incidence to mortality over last decade (2019). In 2018, 62% are diagnosed and such patients have a survival rate for 5 years and estimated new cases were increased upto 38.29% and death rates were increased to 1.95%. **(Madhav *et al.*, 2018).**

1.4. Disease Pathophysiology

Breast tumors starts from the ductal proliferation, and develop into benign tumors or even metastatic carcinomas after various carcinogenic factors. Tumor microenvironments such as stromal influences or macrophages plays vital role in breast cancer initiation and progression. Cancer cells enter into blood, lymph nodes and tissue to produce secondary tumor and they get nutrient and oxygen supply by angiogenesis. The breast carcinoma changes in cell can be either myoepithelial/epithelial cell. When mutation occurs, genes encoding the protecting pathway unable to suicide and leads to cancer **(Anastasiadi *et al.*, 2017).** The main causes include:

- Injury to DNA and genes like P53, BRCA1 and BRCA2.
- Neoplastic cell multiplies and convert into massive tumor.

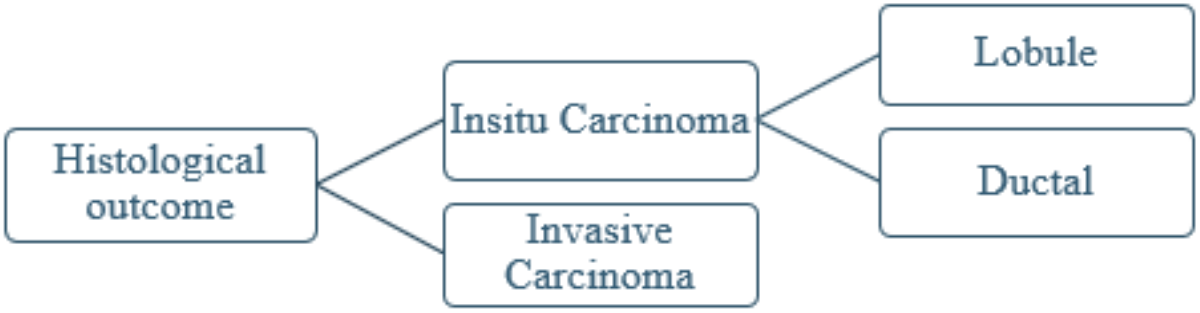
- Growth Factors signalling tumors and over expression of leptin in breast adipose tissue causes and proliferative **(Stucchi *et al.*, 2016).**

1.5. Risk factors affecting breast cancer:

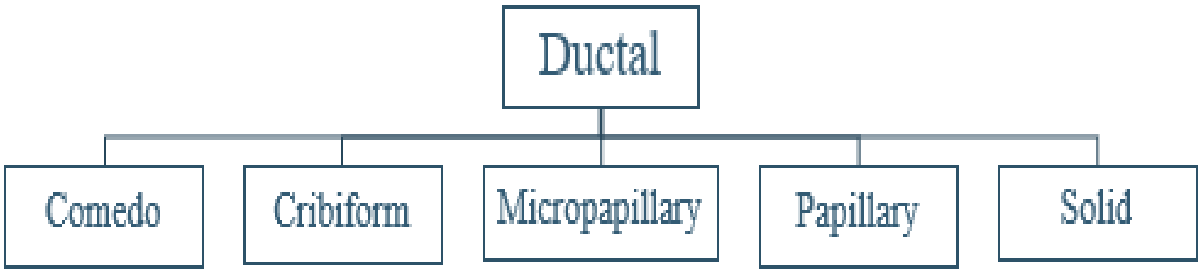
The factors including **e**arly menarche and delayed menopause (after age 55), BRCA1 and BRCA2 mutation, 1st Birth at late age, history of breast or ovarian cancer, breast feeding, hormonal, alcohol intake, long term usage of sex hormones, anthropometric factor **(Kennecke *et al.*, 2010).** Menopause resulting from surgical removal of ovaries (oophorectomy) decreases risk, occurs more common in obese and single women than married, early menarche, nulliparity, pregnancy after 30 years, oral Contraceptive, Hormonal Replacement Therapy increases breast cancer (**Anastasiadi *et al.*, 2017).**

1.6. Classification of breast cancer:

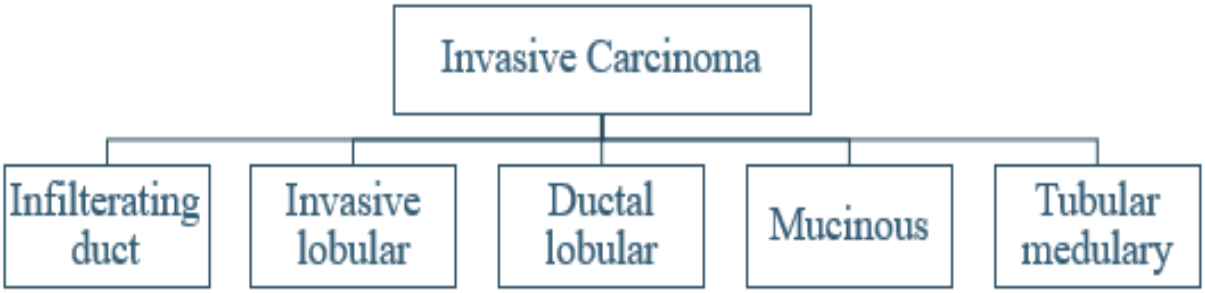
Based on histology, breast cancer is sub classified as **(Pusztai *et al.*, 2006),**



Ductal carcinoma is further classified based on tumors **(Arch *et al.*, 2014), ,**



Invasive carcinoma subtypes **(Nielsen *et al.*, 2004),**



1.6.1. Most Common Breast Cancer Types

Invasive Breast Cancer /Infiltrating Breast Cancer:

It invades and spreads outside the normal ducts and lobules surrounding the breast tissue. It possess two types (Invasive Ductal Carcinoma, Invasive Lobular Carcinoma) **(Mathew *et al.,* 2017).**

Non-Invasive (Insitu breast cancer):

DCIS(Ductal Carcinoma)(Insitu Intraductal) : Non-invasive /preinvasive develops inside the pre-existing normal ducts of breast tissue (**Valenzuela and Julian 2007**).

Metastatic Breast Cancer:

Metastatic Breast Cancer is the late stage/advanced stage which spreads to other organ in the body **(Inoue *et al*. 2017).**

Inflammatory Breast Cancer (IBC):

Inflammatory Breast Cancer is the more aggressive uncommon growing type that differs from other spreads with symptoms of inflammation around breast tissue, red colour pitting, thickening of skin, etc (**Cariati *et al.,* 2005)**

Paget disease of Breast:

It raises in breast ducts and spreads to nipple and extent to areola of skin occurring 3% of total cases that divides rapidly and are diagnosed with biopsy with mammogram, MRI. It possess ER/PR positive and most cells with HER2 negative (**Merrill *et al*., 2017**)**.**

Angiosarcoma of Breast:

Angiosarcoma is a rare form of sarcoma in the epithetial cells that line blood and lymph vessels of breast skin / tissue. It possess rapid spread and treated accordingly and prior radiation is also applicable (**Akram *et al.,* 2017**)**.**

1.6.2. Staging of breast cancer

According to Breast Cancer organisation, stages of breast cancer involves the size, type of tumor, spreading or penetration into breast tissues. Starting from stage 1 to stage 4 tumor, they are described as,

Stage 0: It is non-invasive tumor with cancerous cells that begins to grow within the boundaries of the breast surrounding tissue. Eg: DCIS(Ductal Carcinoma) **(Nozad *et al*., 2017)**.

Stage 1(non-invasive): It is a invasive Breast Carcinoma and invasion is possible. It has two stages 1A and 1B. The 1A stage measures upto 2cm and no length nodes and 1B stage measures small group of cancer cells larger than 0.2mm lymph nodes (**Valenzuela and Julian 2007**).

Stage 2: It also has 2 stages 2A &2B. The 2A stage describes the tumor in lymph node not in breast tissue with smaller/larger size of 2cm but not more than 5cm. The stage 2B describes the tumor can't reach the axillary lymph node and are large than 5cm **(Inoue *et al*., 2017).**

Stage 3: It is categorised into 3A, 3B, and 3C. The 3A stage possess, 4-9 axillary lymph nodes/ sentinel lymph nodes and no tumor in breast. The 3B can be in different sizes but possess 9 axillary lymph nodes and occur as inflammatory breast cancer of warm, red, and swollen skin of breast causing swelling/ ulcer on skin of the breast. The 3C stage describes the tumor of 10 axillary lymph nodes and include the lymph node above and below the clavicle.

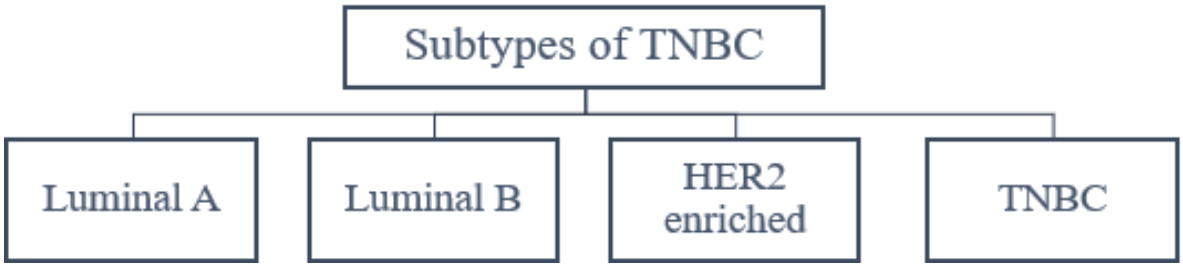
Stage 4 (invasive) : It is the late stage/advanced and metastatic cancer and it spreads to other organs ,brain, bones etc **(Neuman *et al*. 2010).**

Based on molecular markers, breast cancer is classified into three major groups (Nielsen *et al*., 2004),

i. Estrogen Receptor(ER)/Progesterone Receptor positive (PR positive).
ii. HER2 positive (amplification of erbB2) with or without estrogen receptor and progesterone receptor positive.
iii. Triple negative breast cancer with Estrogen Receptor/Progesterone Receptor/HER2 negative.

From this, (i) and (ii) possess therapeutic option and (iii) does not possess standard treatment.

1.6.3. Intrinsic/Molecular Subtypes of TNBCs



I. *Luminal A Breast Cancer:*

The luminal A Breast Cancer is the major subtype of 50-60% in total with the estrogen receptor activated transcription factor. Based on immunohistochemistry (IHC) profile,

the expression of estrogen receptor, progesterone receptor and HER2 negative with lower level of Ki67, based on cell proliferation. This luminal A Breast cancer possess better prognosis with GATA3 marker expression levels and lower relapse rate than others. It includes hormonal therapy i.e the third generation inhibitors like Aromatase Inhibitors (AI), Selective Estrogen Receptors Regulators and Selective Estrogen Receptors Modulators (SERMs**) (Dai *et al.*, 2015).**

II. *Luminal B Breast Cancer:*

The luminal B Breast Cancer occurs with less than 20% of all other types. The luminal B has worse prognosis and aggressive phenotype with proliferative index than luminal A. Based on immunohistochemistry (IHC) profile, estrogen receptor, progesterone positive and HER2 positive with higher level of Ki67/ER. Luminal B has worse prognosis than luminal A than the treatment with AI and SERMs and new inhibitory molecules for the treatment of luminal B are tested in clinical trials **(Dai *et al.*, 2015).**

III. *HER2 enriched(positive) Breast Cancer:*

HER2 Enriched Breast Cancer occurs upto 10% of breast cancer and spreads more faster than luminal cancer with increased expression of HER2 and proliferation cluster and decreased expression of luminal and basal cluster. The treatment option includes targeted therapy like monoclonal antibodies **(Dai *et al.*, 2015).**

IV. *TNBC:*

Among all, TNBC occurs 20% of all other breast cancer types with tumors characterized the lack of hormonal receptors expression by estrogen negative, progesterone negative, HER2 negative. It is more common in BRCA1/2 gene mutation **(Boyle et al., 2010).**

The TNBC possess worst prognosis by the lack of targeted therapy. Compared to other breast cancers, TNBC usually affects younger patients in larger size, higher grade and biologically more aggressive **(Sharma, 2016).**

Based on Immunohistochemical and cDNA microarrays, breast cancer differs in prognosis and therapeutic target and are categories in Table 1 as follows (Medina *et al.*, 2020),

Table 1: Sub-classification of breast cancer

SUB TYPES	ER,PR AND HER2 STATUS	OTHERS IHC FEATURE	CELL OF ORIGIN	PREVALENCE	OTHER CHARACTERISTICS
Luminal A	ER+ or PR+ or both, HER2+	Keratin 8/18+ve	Luminal epithelial cell	40	Younger age Best prognosis Higher survival rate
Luminal B	ER+or PR+or both, HER2+	Keratin 8/18+ve	Luminal epithelial cell	10-20	Higher tumor grade Poor prognosis
Basal like	ER-, PR-, HER2∓	Keratin 5/6/17+ve EGFR+ve	Basal/ myoepithlial cell	-	15%Younger age Associated with hereditary BRCA1 Poor prognosis than other types
HER2+	ER-, PR- ,HER2+		Late luminal progenitor	10	20-25%, Poor prognosis, Poor grade Lymph nodes positive

1.6.4. General characteristic of TNBC

Table 2: Characteristic features of TNBC

S.No	CLINICAL AND PATHOLOGICAL CHARACTERISTICS	MOLECULAR CHARACTERISTICS
1.	More common in black women.	Somatic P_{53} mutation, but clinically actionable
2.	Tumor lymphocyte infiltration than other subtype.	BRCA mutation associated TNBC for defective DNA repair and sensitive to DNA damaging agents.
3.	Highly chemo sensitive	By gene expression analysis, basel-like subtype is most common.
4.	High prevalence of BRCA mutation	BRCA1 or BRCA2 mutation /BRCA ness.
5.	Often seen as internal cancer with early recurrence.	TNBC subtypes are identified mostly by GFA
6.	Increased onset of metastasis to death	P13K activation with lower P13K mutation.

1.7. Key pathways and drug targets for breast cancer

The pathways that cells take become malignant with the development of all types of tumor cells that are highly variable. Some pathways involves,

- Notch Signaling Pathway,
- Hedgehog Signaling Pathway,
- Wnt/-Catenin Pathway,
- Poly (ADP-Ribose) Polymerase (PARP) Inhibitors,
- PI3K/AKT/mTOR) Inhibitors,
- Cancer Stem Cells (CSCs) and Autophagy.

Notch Signaling Pathway

Notch signaling is initiated by ligand binding to Notch receptor, which undergoes a two-step proteolytic cleavage by ADAM family proteases and secretase, releasing the Notch intracellular domain (NICD). This pathway comprises of 4 Notch receptors namely, Notch-

1,2, 3 and 4 that translocates to the nucleus binds to CSL that are converted to an activator of Notch target genes relevant to different types of hematological malignancies. Two major classes of Notch inhibitors: secretase inhibitors and monoclonal antibodies **(Speiser, Erşahin and Osipo, 2013)**

Hedgehog Signaling Pathway:

Hedgehog signaling involves three ligands: Sonic (SHH) expressed during embryogenesis; Indian (IHH) expressed in hematopoietic cells and cartilage; Desert (DHH) expressed in peripheral nervous system and testes. SHH has an important role in breast malignancy because it maintains abnormal proliferation and promotes invasion and metastasis. Thiostrepton, (sonic signaling), suppresses the population of CD44+/CD24-cancer stem cells (CSCs) in TNBC **(Kubo *et al.*, 2004).**

Wnt/-Catenin Pathway:

Wnt/-catenin is the most commonly overexpressed pathway leading to activation of transcriptional factor responsible for epithelial to mesenchymal cell (EMT) transitions. Wnt ligands (WNT5A, WNT11, and WNT3A) involves promoting migration and invasion, and FZD6 receptor is the most important representative in TNBC. OMP-18R5 an antibody targeting Frizzled receptors diminishes proliferation of tumor cells in the lung, breast, colon tumors.**(Geyer *et al*., 2011).**

Poly (ADP-Ribose) Polymerase (PARP) Inhibitors-

The polyadenosine diphosphate-ribose polymerase also called poly (ADP-ribose) polymerase (PARP) is a superfamily of 18 proteins leads to recovery of the cells from DNA damage, gene transcription, and apoptosis. Mostly 70% breast cancers evolving in BRCA1 mutation and 23% of BRCA2, express a triple negative phenotype involving the novel therapeutic target with PARP expression. PARP-1 and PARP-2 proteins are induced by DNA strand breaks and DNA repair processes and synthesised PARP possess BER (excision repair pathway) and single-strand break repair (SSBR) pathways. Olaparib (AZD-2281) and Veliparib (ABT-888) are PARP inhibitors **(Comen and Robson. 2012).**

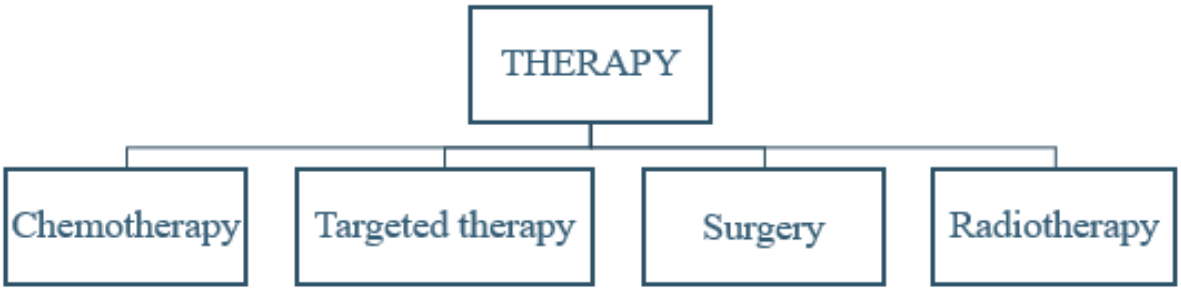
PI3K/AKT/mTOR) Inhibitors:

PI3K/Akt/mTOR pathway has a direct relationship with malignancy and PIK3CA is the second detected mutation. (Wahba and El-hadaad, 2015**).** It is similar for PIK3CA mutations or low PTEN expression. Drugs like ipatasertib and capivasertib improves the

outcomes with high-risk TNBC. Pictilisib is pan-PI3K inhibitor with greater role of α-inhibitor activity than buparlisib **(Gupta *et al.*, 2020).** The mTOR pathway is transformed in TNBC patients with poor prognosis. mTOR is a subunit of two multi-protein complexes, mTORC1 and mTORC2. Both mTORC1 and mTORC2 can be activated by growth-factors stimulation, whereas mTORC2 is a kinase that phosphorylates and activates Akt. Everolimus, an m-TOR inhibitors trials **(Zaytseva *et al.*, 2012).** The 6 classes of inhibitors- Pan-class I blocker, AKT blocker, Pan-PI3K/mTOR blocker, Rapamycin analogs, isoform-selective (PI3K blocker).

1.8. Current drug treatment

Breast cancer management differs depending on the stages of the cancer-its mass, extend to other organs of the body and the physical condition of the individuals. Present management for breast cancer includes chemotherapy, targeted therapies, hormonal treatment, radiation therapy and surgery**.** Breast cancer has limited treatment options where, TNBC is prone to recurrence and metastasis, and has a poor prognosis.



1.8.1. Chemotherapy

The therapeutic strategies for the management of TNBC are targeting DNA repair complex like (platinum compounds), p53 like (taxanes), cell proliferation like (anthracyclines). (Tong *et al.*, 2018) Some drugs include,

Taxol: Taxotere is a side chain analogue of taxol, produced by semi synthesis of 10-deacetyl-baccatin III. The mechanism of action of taxel is mainly through the inhibition of microtubule depolymerization, and cannot form spindles and fibers during mitosis, forcing the cells to stop in prometaphase, thereby inhibiting cell division (e.g., taxel or docetaxel) **(Gupta *et al.*, 2020).**

Anthracyclines: Anthracyclines and anthracycline antibiotics are a class of chemotherapeutic drugs derived from *Streptomyces peucetius var. caesius* used to treat leukemia, lymphoma, breast cancer, uterine cancer and lung cancer. The optimum dose of doxorubicin is 60 mg/m^2 and epirubicin is 100 mg/m **(Yin *et al.*, 2020).**

***Platinum agent*:** The cis-structured platinum compound namely cisplatin, has inhibitory effect on cancer cells. The addition of carboplatin to conventional taxel chemotherapy and anthracycline chemotherapy significantly increased the rates in TNBC patients.

Cyclophosphamide: Cyclophosphamide does not have antitumor activity but after entering the body, it is first converted to aldophosphamide by the microsomal oxidases in the liver and that aldophosphamide is unstable and is activated by cytochrome P450 to produce nitrogen mustard having cytotoxic effect and acrolein with alkylating activity in tumor cells. Currently, TC is commonly used as an neoadjuvant chemotherapy regimen and the adjuvant cyclophosphamide, methotrexate, and fluorouracil chemotherapy **(Yin *et al*., 2020).**

Fluorouracil: 5-Fluorouracil (5-Fu) itself does not have any biological activities, but by the action of orotate phosphoribosyl- transferase, 5-Fu can be converted into active metabolites, fluorouridine monophosphate and fluorodeoxyuridine monophosphate. Capecitabine is a cytotoxic agent that has a selective activity for the treatment of advanced primary or metastatic breast cancer with an in-effective paclitaxel or anthracycline chemotherapy **(Gupta *et al*., 2020).**

***Aromatase inhibitors*:** These are compound designed for decreasing oestrogen formation by targeting aromatase enzyme in the formation of estrogen. The third-generation aromatase inhibitors blocks the estrogen biosynthesis through reversible (nonsteroidal agents such as letrozole and anastrozole) or irreversible (steroidal agents, i.e., exemestane) inhibition of the aromatase enzyme **(Yin *et al*., 2020).**

1.8.2. Targeted therapies

Due to the high heterogeneity of breast cancer, it is particularly difficult to discover new therapeutic targets and perform targeted therapy.

EGFR targeted therapy:

It is detected by approximately 25–50% EGFR inhibition by anti-EGFR monoclonal antibodies like cetuximab, panitumumab, nimotuzumab or EGFR small molecule inhibitors. The EGFR pathway inhibition after receiving cetuximab alone or cetuximab plus carboplatin possess minimal effect **(Gupta *et al*., 2020).**

Immune Checkpoint Blockade Therapies :

Immunotherapy has turn into an emergent treatment in the management of breast cancer. Immune checkpoint inhibitors targeting programmed death receptor-1 (PD-1) and

programmed death ligand-1 (PD-L1) have shown a promise in treating advanced and metastatic TNBC in combination with standard chemotherapy. Existing therapies (such as hormonal or trastuzumab-based therapy), which are based on targeting ER or HER2 with current treatment of Pertuzumab, shows superior breast cancer (**Gupta *et al*., 2020).** Ipilimumab (anti-CTLA-4), pembrolizumab (anti-PD-1, monoclonal IgG4-κ antibody). Atezolizumab helps in TNBC patients with advanced or metastatic cancer at a dose of dose is 840 mg intravenous (IV) infusion over 60 min, followed by 100 mg/m^2 paclitaxel for 28-day cycle

***PARP Inhibitors*:**

PARP is a class of DNA repair enzyme maintains genome stability and participate in cell cycle progression and apoptosis. BRCA1/2 are well-known tumor suppressor genes which possess loss of function mutations, associated with tumor aggression, and poor outcomes in breast cancer. The standard therapy are capecitabine, eribulin, vinorelbine and Olaparib was generally well-tolerated with minimal side effects and acceptable toxicity **(Yin *et al*., 2020).**

VEGF targeted therapy:

Vascular endothelial growth factor (VEGF) is the most important angiogenic factor in breast cancer since it stimulates tumor cell proliferation and growth in new vessel formation in growing tumors. VEGF expression is often higher in TNBC with poor prognosis **(Gupta *et al*., 2020).** Antiangiogenic treatment with bevacizumab, monoclonal antibody to chemotherapy as first-line treatment of HER2-negative metastatic breast cancer that blocks by circulating VEGF-A, preventing its binding to the VEGF receptor 2. HER2 signaling induces VEGF transcription, and inhibition of HER2 with trastuzumab results an antivascular effect **(Boyle *et al*., 2010).** Sunitinib – a tyrosine-kinase inhibitor includes VEGF-1, 2 and 3, platelet-derived growth factors alpha and beta showed anti-tumor activity.

Adjuvant regimen for TNBC: :

According to National Cancer Control Programme adjuvant therapy is followed for treating women most widely in India and were tabulated in **Table 3** as follows

Table 3: Adjuvant regimen for TNBC (Gupta et al., 2020)

S.no	Present adjuvant regimen
1.	Taxel/docetaxel+Adriamycin+cyclophosphamide(TAC)
2.	Docetaxel + cyclophosphamide(TC)
3.	Adriamycin+cyclophosphamide(AC)
4.	Cyclophosphamide + Methotrexate+ Fluorouracil(CMF)
5.	Cyclophosphamide + Adriamycin+ Fluorouracil(CAF)
6.	Cyclophosphamide +Epirubicin+ Fluorouracil+Paclitaxel/ docetaxel(CEF-T)

Conventional treatment of TNBC : The different conventional treatment options were tabulated in **Table 4** as follows **(Medina *et al.*, 2020),**

Table 4 : Conventional treatment of TNBC

S.No	Conventional treatment	Drugs	Mechanism	Dose/scheme
1.	Neoadjuvant treatment Early TNBC , advanced/meta static	Anthracyclines+ Taxanes/ capecitabine+ taxane	Cytotoxicity Stabilization microtubules	Doxorubicin 20mg/m^2 + Cyclophosphamide 600mg/m^2 -4 weeks followed by Paclitaxel 80mg/m^2 12 weeks.
2.	New neoadjuvant agents	Platinums (carboplatin) Bevacizumab	Cytotoxicity VEGF Immunotherapy	Abraxane 125mg/m^2 Carboplatin AUC , Bevacizumab10mg/kg.
3.	Adjuvant agents	Anthracyclines and Taxanes	Cytotoxicity	Cyclophosphamide 600mg/m^2 + Doxorubicin 20mg/m^{2+} Docetaxel 75mg/m^2 for q3 weeks 6 cycles

1.8.3. Surgery

The surgery for breast cancer consists of two main options based on type and stage of tumor. In breast-conserving surgery, only the tumor and an area of normal tissue surrounding it are removed. Breast-conserving surgery includes the following:

- Lumpectomy: (removal of the lump only): A small amount of surrounding normal tissue is removed.
- Quadrantectomy: About one fourth of the breast is removed.
- In mastectomy, entire breast tissue is removed. Standard practice requires the tissue removal, indicating that the cancer has been completely excised. More recently, the technique of sentinel lymph node (SLN) dissection has become popular, as it requires the removal of far fewer lymph nodes, with fewer side effects **(Akram *et al.*, 2017).**

1.8.4 Radiation Therapy

Radiation therapy involves using high-energy X-rays or gamma rays that targets a tumor or post-surgery tumor site in killing cancer cells that may remain after surgery or recur where the tumor was removed. 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 **(Akram *et al.*, 2017).**

1.9. Herbal source

Natural products and their analogs are a prime source of effective conventional drugs for the treatment of many forms of cancer, while the actual compounds were isolated from the plant that provide leads for the development of potential novel agents **(Cragg and Newman, 2005).** They can be developed into a drug candidates by the processes of fractionation, isolation followed by analog synthesis through modern medicinal chemistry-based molecular modification or through different techniques **(Zhang, Lin and Ye, 2018)**

Continual improvements in bioassay technology coupled with the discovery of new biological targets will also benefit the drug discovery process. Thus, medicinal plants have long been appreciated for treating illness, and played an important role as a source of effective anti- cancer agents for thousands of years and it is significant that about 60% of currently used anti-cancer agents are derived in one or another from natural sources, including plants, marine organisms and micro-organisms. Fruits with anti-inflammatory and antioxidative properties can be good

candidate for cancer chemo-preventive agent because oxidative stress and chronic inflammation play important roles in cancer development **(Buyel 2018).**

The new technologies of the anticancer potential of the medicinal plants extracts were isolated and fractionated for treating cancer **(Itokawa *et al.*, 2008)**. Thus, medicinal plants have been appreciated for treating illness, and played an important role as a source of effective anti- cancer agents **(Fouche, 2008**) and about 60% are currently used anti-cancer agents are derived in one or another from natural sources, including plants **(Akhtar *et al.*, 2018),** marine organisms and micro-organisms. Similarly fruits with anti-inflammatory and antioxidative properties can be good candidate for cancer chemo-preventive agent because oxidative stress and chronic inflammation **(Buyel, 2018).**

1.9.1 Acerola fruit description

Acerola, among other fruits, is a food suggested as part of a proper diet for healthy living. **Acerola *(Malpighia emarginata DC*.)** considered a 'super fruit', belongs to Malpighiaceae family. The richest natural sources of ascorbic acid, a natural source of vitamin C, is abundantly present in this fruit and other phytocomponents which possess antioxidant, anti-inflammatory, hepatic activity, antifungal, antiviral, anti-HIV and anticancer activity. The acerola fruit picture were shown in **Figure 5,**

Figure 5 Acerola fruit (**Schreckinger *et al.*, 2010**)

The chemical composition of acerola fruit reported **o**ther than ascorbic acid (vitamin C) includes several phytonutrients like carotenoids, phenolics, flavonoids, polyphenols, procyanidins and anthocyanins (malvidin-3,5-diglucoside) but they are not fully investigated appears to be a promising candidate in combating various diseases associated with the oxidative stress. Glucose, fructose and a small amount of sucrose, malic acid(32%), citric acid and tartaric acid, pro-vitamin A, vitamins B1 and B2, niacin, albumin, iron,

phosphorus and calcium and 4.51% pectin responsible for biological activity **(Vendramini and Trugo, 2000).**

Identification and fractionation of various eluted phytoconstituents were determined through analytical techniques including various composition as **ascorbic acid, volatile** compounds reported in **Pino *et al.*, 2001** like furfural, hexadecanoic acid, limonene, 3-methyl 3-buthenol, **phenolics** reported from **Betta *et al.*, 2018** like cyanidin-3-αo-rhamnoside, cyanidin-3-αo-glucoside, pelargonidin-3-αo-rhamnoside gallic acid.

Other constituents include **anthocyanin** pigments like quercetin-3-α-o-rhamnoside, n = malvidin -3,5-diglucoside, flavonoids including Leucocyanidin-3-o-β-D-glucoside (Aceronidin), catechin, epicatechin, Rutin, Narigenin, vanillin (**Vera *et al.*, 2008**) and **flavanols** includes quercetin, hyperoside, kaemperol glucoside, taxifolin, isoquercitrin, myricetin, luteolin and **carotenoids** includes β- carotene, β-cryptoxanthin, neoxanthin, antherxanthin, neochrome and isoforms, auroxanthin, , β-cryptoxanthin-5,6-epoxide, , β-cryptoxanthin-5,8-epoxide and **stilbenenes** including resveratrol were identified from its qualitative studies **(Porez-gµlvez, 2005).**

Various new novel compounds: Leucocyanidin-3-O-b- D-glucoside, aceronidin, three novel norfriedelanes A-C was shown to have significant acetylcholinesterase inhibitory effects. Tetranorditerpenes, acerolanins with 2H-benz[e]inden-2-one, three polyphenols such as cyaniding-3-α-o-rhamnoside (C3R) and pelargonidin-3- α - o - rhamnoside (P3R) as anthocyanin, and quercetin-3-α-o-rhamnoside (quercitrin; Q3R) possessing cytotoxic activity **(Prakash *et al.*, 2018).**

In fact, the biological activities were demonstrated using different extracts like saline **(Barros *et al.*, 2019)**, methanol, hexane and acetone **(Motohashi *et al.*, 2004)** of acerola fruit and its phytoconstituents were analysed in different parts like flower, fruit, leaves, mature and unripe fruits**(Alvarez-Suarez et al., 2017)**. The range of nutraceutical phytsonutrients present in acerola pulp in edible films and waste utilized for development of valuable by-products.

The *in-vitro* anticancer activity studies were evaluated for its antioxidant and anti-inflammatory properties **(Cabral *et al.*, 2020),** the phytochemical characterization through UPLC-MS chromatography to identify the chemical compounds in saline extract from leaves for its antioxidant, anti-fungal and cytotoxicity potential**, (Barros *et al.*, 2019)** genotoxic and antigenotoxic effects using mice blood cells **(Nunes *et al.*, 2011)** anti-HIV

activity, anti- Helicobacter pylori activity and MDR reversal activity**,** tumor-specific cytotoxic activity through fractionation in HSC-2 and HSG **(Motohashi *et al.*, 2004).**

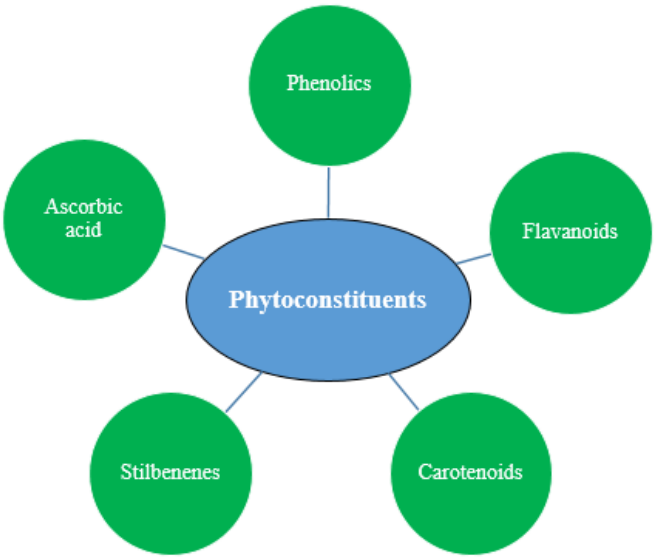


Figure 6: Schematic representation of reported phytoconstituents

The *in-vivo* studies **(Barros *et al.*, 2019)** initiating lung carcinogenesis induced in mice **(Nagamin, 2002)** and hepatoprotective effect through inflammatory responses (acute hepatic injury in rats), viral and alcohol-related liver diseases **(Issue *et al.*, 2004)** cytotoxicity and proliferation were performed using splenocytes from Balb/c mice studies **(Barros *et al.*, 2019)** were performed on these acerola cherry fruit extract for its various biological activity.

The aim of this study is to evaluate the *in-vitro* anti-cancer activity of **Acerola (*Malpighia emarginata)*** fruit extract in preventing TNBC cell line MDA-MB 231 for the treatment of breast cancer.

1. **Alvarez-Suarez *et al.*, 2017**, determined the acerola chemical composition and protective capacity against oxidative damage using an *in-vitro* model of human dermal fibroblast (HDFa) for improving antioxidant enzyme activities and mitochondrial functionality. From the HPLC-DAD/ESI-MSn analyses, high content of vitamin C, total polyphenols, three hydroxycinnamoyl derivatives, β-carotene two anthocyanins and fifteen flavonols and folates were analysed. HDFa were pre-incubated with an acerola crude extract (ACExt) and subjected to oxidative stress induced by AAPH. Apoptosis, intracellular ROS and biomarkers of lipid and protein oxidation increased after inducing stress, while the activities of antioxidant enzyme catalase and superoxide dismutase and mitochondrial functionality were markedly affected. The ACExt, protected oxidative damage through decreasing apoptosis, intracellular ROS levels and lipid and protein damage than antioxidant enzyme activities and mitochondrial functionality. From these studies the acerola fruits as relevant sources of functional compounds with promising effects on human health.

2. **Barros *et al.*, 2019,** evaluated the phytochemical characterization through UPLC-MS chromatography to identify the chemical compounds in saline extract from *Malpighia emarginata DC* leaves for its antioxidant, anti-fungal and cytotoxicity potential. For antioxidant potential, DPPH, ATT and FRAP methods were used. The antibacterial and antifungal tests were performed by evaluating the MIC50, MIC90, CMB and CMF parameters. The cytotoxicity and proliferation were performed using splenocytes from Balb/c mice and were evaluated by flow cytometry and Splenocytes showed greater cell viability (more than 90%) and showed higher proliferate index in 24 and 48 hours of incubation with the extract showed the presence of total phenolic compounds, some flavonoids and phenolic acids. From this study, a list of phenolic compounds among other bioactive compounds in the *M. emarginata* -saline extract possess antioxidant and antifungal agent without promote animal cell damage suggesting saline as an dissolving medium demonstrating the safe use of this plant against normal cells and can stimulate new investigations to use this extract like antifungal and immunostimulant compound in future assays.

3. **Cabral *et al.*, 2020,** studied the antioxidant and anti-inflammatory properties of *Malpighia emarginata D.C* (acerola) and *Camellia sinensis L.*, (green tea) as an *in-vitro* model for

inflammation, using LPS-stimulated RAW-264.7 macrophage cell line. A powder blend formulated with both *Malpighia emarginata D.C* and *Camellia sinensis L*. with higher content of ascorbic acid and epigallatocathechin-3-gallate were developed using different conditions for microencapsulation, through spray-drying process. The co-treatment with blends were modulated for the redox parameters in cells during the *in-vitro* inflammatory response and modulated inflammatory response by altering the secretion of cytokines IL-1β, IL-6, IL-10, and TNF-α. From these the synergistic effects of *Malpighia emarginata* D.C and Camellia sinensis L given in combination had antioxidant and anti-inflammatory using blend powder can be used in the products to heath beneficial and in prevention of chronic diseases.

4. **Chaudhary *et al*., 2020,** identified that the natural plant was an important target to investigate the antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic properties which has been noted for different phytochemical constituents responsible for various therapy. From these, acerola, one among other fruits, is a food suggested as part of a healthy diet and well-being and to reduce the risk of various diseases.

5. **Dai *et al*., 2017**, revealed the effectiveness and better prognosis of different breast cancer cell lines for growth using suitable media. The molecular features of 92 breast cancer cell lines includes MDA-MB231, MBA-MB 456, MDA-MB 463, MCF-7 **(Chavez *et al*., 2012)** categorizes for different culturing medium including RPMI, DMEM, Ham's F12, McCoys etc are critical for growth and maintenance of cell culture. From these studies, MDA-MB 231, an heterogenous TNBC cell line with DMEM media is a selected for treatment for breast cancer.

6. **Gupta *et al*., 2020,** emonstrated the combinatorial approaches that the secondary mutations or compensatory pathways in resistant tumors may markedly improve on the effects of targeted agents with the standard chemotherapy, like anthracyclines, alkylating agents, and taxanes, to treat TNBC alone or in combination.

7. **Higgins et al.,2011,** demonstrated that the TNBC was an aggressive molecular diverse entity with poor prognosis and results in poor survival and lack of targeted therapy.

8. **Issue *et al*., 2004,** evaluated the hepatic inflammatory responses (acute hepatic injury in rats), viral and alcohol-related liver diseases by treating with the water and tropical lemon

juice extract powders from acerola fruit purees and leaves(100 mg/kg). The effect of the water extract from fruit purees (100 mg/kg) was stronger than ascorbic acid (10 mg/kg). From these the hepatoprotective effect exerted from fruit purees is the combination of high vitamin C content, phenolic acids, anthocyanins and flavonoids while the leaves possess the presence of phenolic acids and flavonoids. Polyphenolic compounds were identified and analysed by use of GC/MS-SIM and other constituents were studied for their different activity that significantly increased serum levels of AST, ALT, and GGT in rats subjected for acute -galactosamine (GalN) intoxication.

9. **Johnson *et al*., 2003**, demonstrated that the acerola (*Malpighia emarginata DC*.) was considered as a 'super fruit' also known as **''Barbados cherry,'' ''French cherry,'' or** West Indian cherry, belonging to Malpighiaceae family L., and only one is accepted by the present scientific name by the taxonomists. Taylor, Delva, and Schneider 2013S found that the evergreen shrub of acerola flourishes in warm and tropical climates bearing a small trilobite cherry like fruit. From these studies the biological activities with individual bioactive compounds and the extracts of the fruit were focused on the molecular aspect of the components in various activity.

10. **Kumar *et al*., 2017,** evaluated the apoptotic effects of BTB extracts on human breast cancer cell lines (MCF-7 and MDA-MB-231) through intrinsic and extrinsic pathway. The extracts were prepared by homogenization and centrifugation and the cytotoxic activity of BTB was evaluated by MTT assay and the apoptotic effects were characterized by DNA fragmentation, nuclear staining assay, mitochondrial membrane potential analysis, annexin-V FITC and caspase 3/7 activity assay. The cytotoxicity with IC50 values of 50 µg/ml in MCF-7 and MDA MB231 cells. The mitochondrial membrane potential was decreased by and causing DNA fragmentation and caspase 3/7 was activated and the expression of Bax increased as well as Bcl-2 and Bcl-xL decreased in a dose dependent manner in both cells and induces cell cycle arrest in S and G2/M phase in both cell lines. The cell cycle and gene expression of cell lines were analysed by flow cytometry and qRT-PCR. From these studies, MCF-7 and MDA-MB 231 cell lines were widely used for treating positive and negative breast cancer by evaluating through different assays and staining techniques by inducing apoptosis and exhibiting anti-cancer activity.

11. **Lves *et al.*, 2007**, determined anthocyanin pigments in four tropical fruits and quantified anthocyanin structures using HPLC- ESI-MS/MS with authentic standards and mass spectra for the first time. Major fruits includes acerola (*Malphigia emarginata*), jussara *(Euterpe edulis*), jambolão (*Syzygium cumini*), and guajiru (*Chrysobalanus icaco*). All these fruits contains anthocyanin pigments with their backbone including cyanidin, delphinidin, peonidin, pelargonidin, petunidin, malvidin and acerola contained nonacylated glycosides. From these results, acerola fruit extract exhibited two anthocyanin peaks identified as cyanidin 3-rhamnoside and pelargonidin 3-rhamnoside by NMR and antioxidant capacity attributed not only to vitamin C content but also to phenolic compounds possessing oxygen scavenging and inhibitory activity with total anthocyanin content of acerola depended on the cultivator having hepatoprotective effect with acerola extract powders from fruit purees.

12. **Martins *et al.*, 2013**, described the high levels of vitamin C and rutin showing antioxidant property with biochemical and anti-genotoxic effects in different stages of maturity (unripe, ripe and industrial) for the first time. From HPLC analyses, all types of acerola juice contained vitamin C and rutin and quantified the antioxidant properties using DPPH tests and revealed higher antioxidant potentials compared to pure compound. From animal studies, male mice were treated with standard (STA) or a cafeteria (CAF) diet for 13 weeks that increased the feed efficiency and induced glucose intolerance and DNA damage were observed by comet assays and micronucleus tests. From these reports, food supplementation along with acerola leads to diet-induced DNA damage and also benefits to the blood, kidney, liver and bone marrow but with vitamin C and rutin causes decreased DNA damage in kidney and all tissue samples suggesting that these acerola juice helps to reduce oxidative stress and decreases genotoxicity along with food supplements on CAF diet-induced obesity model in order to repair the damage or prevent chronic disease.

13. **Mezadri *et al.*, 2018,** evaluated the antioxidant property of hydrophilic extracts of acerola pulps and juices by 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ORAC and 1,1- diphenyl-2-picrylhydrazyl (DPPH) methods. From the *in-vitro* studies, the antioxidant activity values were higher than other fruit juices (rich in polyphenols such as strawberry, grape and apple juices). By HPLC, Vitamin C, total phenol index (TPI), total anthocyanins and polyphenolic compounds responsible for antioxidant activity, were determined. By Solid phase extraction (SPE) three soluble polyphenolic fractions (phenolic

acids, anthocyanins and flavonoids) were separated from the different sample extracts. Five different polyphenolic compounds and by diode-array detection: chlorogenic acid, ()-epigallocatechin gallate, epicatechin, procyanidin B1 and rutin contributed activity ranges between 40% and 83% and their respective antioxidant activities were calculated. These results identified that the acerola fruit and derivatives are potent antioxidant foods and might have potential value as functional food ingredients.

14. **Motohashi *et al.*, 2004,** evaluated the acerola fruit by fractionation for tumor-specific cytotoxic activity and MDR reversal activity suggesting its activity in cancer chemotherapy and prevention. It was fractionated using column chromatography with silica gel or ODS column with various organic solvents using two extraction methods**.** From these extraction methods the higher cytotoxic activity was concentrated in acetone extract (A4 and A6), and in hexane extract **(**H3 and HE3). These four fractions showed higher cytotoxic activity against tumor cell lines such as human oral squamous cell carcinoma (HSC-2) and human submandibular gland carcinoma (HSG), when compared with normal cells such as human periodontal ligament fibroblasts (HPLF) and human gingival fibroblasts (HGF). HE2 (hexane extract), AE2 showed higher anti-bacterial activity and are inactive against Helicobacter pylori, and human immunodeficiency virus (HIV). H3, H4 and HE3, which displayed higher tumor-specific cytotoxicity also showed higher multidrug resistance (MDR) reversal activity, than (±) verapamil as standard. Many fractions of barbados cherry extracts showed vitamin C radical peak in by ESR spectroscopy, suggesting its presence and other extracts showed various magnitudes of O_2^- scavenging activity predicting antioxidant and prooxidant. From these studies, various biofunctional properties were investigated viz. radical generation, anti-bacterial,tumor-specific cytotoxic activity, anti-HIV activity, anti- Helicobacter pylori activity and MDR reversal activity showing various biological activities suggested for their future application especially for cancer therapy.

15. **Nagamine . 2002,** proposed that the acerola cherry extract (ACE) pretreatment, inhibited the cell proliferation and the activation of Ras signal pathway at a promotion stage initiating lung carcinogenesis induced in mice by 4-methylnitrosamino-1-3-pyridyl-1-butanone (NNK), with a dose 70mg/kg body weight and 700mg/kg body weight showing both tumor-specific cytotoxic activity and multidrug resistance reversal activity. This treatment suppressed the proliferation and ACE regulates abnormal cell growth that has

anticarcinogenic activity against lung cancer based on the additive and/or synergisticeffects of vitamin C and other food factors in ACE and effective in lung cancer prevention.

16. **Nunes *et al*., 2015**, investigated the genotoxic and antigenotoxic effects of acerola fruit at two stages of ripeness using mice blood cells. The genotoxic potential of no ripeness stage were performed for damage DNA (Comet assay) or cytotoxicity (MTT assay). The activity were analyzed by unripe fruit which possess higher DNA protection than ripe fruit (red color) extract. The antioxidant capacity of substances also showed that unripe samples inhibit the free radical DPPH more significantly than the ripe. The compounds were determined using HPLC showing higher levels of vitamin C as compared to ripe acerola along with the complex mixture of nutrients of *Malpighia glabra L*., in its ripeness stage, influenced the interaction of the fruit extract. It is pointed as a good source of natural antioxidants protecting it against oxidative stress that can be used dietary supplements in protecting the body.

17. **Pereira *et al.*, 2018**, reported the gene expression levels of acerola contents validating the information regarding the molecular and biochemical nature providing the important natural source of vitamin C that was only observed. The first data on acerola transcriptome generating valuable information to identify reference genes for RT-qPCR, identified the most stable expressed genes based on acerola transcriptome data in leaf, flower and fruit at 12, 16 and 20 days. The combination of the most stable reference genes RBL and U3 for leaf/flower group, TGD4, F-box, RCC1, PGAL and RBL (fruit/flower) (total samples) were required for accurate normalization for gene expression profiling.

18. **Pereira *et al*., 2019**, reported the first transcriptome coupled with metabolite analysed the major ascorbate in acerola fruit during ripening, exhibiting high amounts of ascorbate experiencing high respiratory rates and ethylene signalling, cellular respiration, sugar accumulation, and softening key regulatory genes with transcriptome sequencing generated over 600 million reads, 40,830 contigs, and 25,298 unique transcripts. This provides a sequence and transcript expression based on functional investigations to improve fruit quality having extensive major pathway changes required for acerola ripening that can be examined to accumulate AsA in fruit with low levels. These results revealed the main metabolic changes in the acerola during ripening and contributing to the elucidation of molecular mechanisms involved in the regulation of metabolites in fruit.

19. **Pino *et al.*, 2004**, identified and fractionated anthocyanin aglycons and other phenolic compounds by chromatographic and spectral data showing maximum absorbance at 370 nm. The individual peaks were observed by descending paper chromatography from retention time and co-elution with standards showing the presence of quercetin (Rt 8.2 min) and kaempferol (Rt 13.0 min) and different colours of anthocyanin pigments due to their hydroxylation and methoxylation patterns. The identified phenolic pigments were pelargonidin, malvidin 3,5- diglycoside and cyanidin 3-glycoside and other pigments include quercetin, kaempferol and phenolic acids p-coumaric acid, ferulic, caffeic and chlorogenic acid. From these studies, the phenolic compounds were identified as phenolic anthocyanin pigments(3,5-diglycosilated malvidin, pelargonidin) and non-anthocyanin phenolics(p-coumaric acid, ferulic acid, caffeic acid, chlorogenic acid, kaempferol and quercetin) showing anthocyanins, flavonoids and phenolic acids of ripe acerola.

20. **Porez-gulvez *et al.*, 2005**, investigated carotenoid composition in acerola fruits and derived products by means of TLC and HPLC chromatographic techniques and by comparison of UV-visible spectra and their retention times compared with that of standards showing seventeen compounds in ripe fruits with four major carotenoids (β-carotene, β -cryptoxanthin, lutein, and violaxanthin) and minor carotenoids (neoxanthin, antheraxanthin, neochrome, luteoxanthin, auroxanthin, β –cryptoxanthin-5,6-epoxide, β -cryptoxanthin-5,8-epoxide, cis- β -carotene, and cis-lutein) with average composition follows lutein (99.21 mg/100 g fw), b-cryptoxanthin (417.46 mg/100 g fw), β-carotene (536.55 mg/ 100 g fw), violaxanthin (395.33 mg/100 g fw), and total minor carotenoids (197.33 mg/100 g fw) showing modifications affecting the nutritional value antioxidant properties of fruits. The acerola product is combined with other fruit juices or even nutraceuticals and were manufactured into other functional food products.

21. **Pino *et al.*, 2001**, identified volatile components that were isolated from acerola fruit by steam distillation-solvent extraction and analysis were based on GC-MS methods with retention index and founded one hundred fifty constituents including major constituents of furfural, hexadecanoic acid, 3-methyl-3-butenol, and limonene and amounts of esters, 3-methyl-3-butenol gives unique flavour to acerola fruit. From the present studies, one hundred three compounds were identified for first time having aroma flavour having 31% aliphatic esters, 24% terpenoids, 15% aldehydes and ketones, 13% alcohols, 6% acids, 1%

amino compounds with major constituents composing of were furfural (2.19 ppm), hexadecanoic acid (0.58 ppm), 3-methyl-3-butenol (0.72 ppm), and limonene (0.68 ppm) and new findings with the presence of many terpenoids, two pyrazines, 2,5-dimethylpyrazine and 2,5-dimethylpyrazine were identified in the acerola fruit.

22. **Prakash B et al., 2018,** found not only few phytoconstituents, the reported various new novel compounds: Leucocyanidin-3-O-b- D-glucoside, aceronidin, three novel norfriedelanes A-C, tetranorditerpenes acerolanins with 2H-benz[e]inden-2-one. Three polyphenols such as cyaniding-3-α-o-rhamnoside (C3R) and pelargonidin-3- α - o - rhamnoside (P3R) as anthocyanin, and quercetin-3-α-o-rhamnoside (quercitrin; Q3R) possessing cytotoxic activity. These compounds have been reported for its antioxidant properties, inhibition of damage to DNA, cell cycle arrest (especially at the G2/M), induction of apoptosis, inhibition of angiogenesis in tumor cells.

23. **Vendramini *et al.*, 2000,** reported by describing the chemical composition of acerola fruit with one of the richest natural sources of ascorbic acid in the world, a natural source of vitamin C and also contains several phytonutrients like carotenoids, phenolics, flavonoids, polyphenols, procyanidins and anthocyanins (malvidin-3,5-diglucoside) but they are not fully investigated. Glucose, fructose and a small amount of sucrose, malic acid(32%), citric acid and tartaric acid, pro-vitamin A, vitamins B1 and B2, niacin, albumin, iron, phosphorus and calcium and 4.51% pectin responsible for biological activity.

3. AIM AND OBJECTIVES

AIM

To investigate the role of *in-vitro* anticancer activity of selected plant *malpighia emarginata* (acerola fruit) extract for the treatment of breast cancer.

OBJECTIVE

- To evaluate the **Anti-proliferative activity** in MDA-MB 231 cell line.
- To assess the **Apoptotic activity** using AO/EB staining.
- To evaluate the effect of **Cell migration** on MDA-MB 231 using scratch assay.
- To assess the **Reactive Oxygen Species** production by DCFH-DA staining.

4. PLAN OF STUDY

PHASE -1

- Literature review
- Plant extracts selection and collection
- Approval from IHEC
- Protocol design
- Optimization of cell line

PHASE-2

- ***In-vitro* cell culture**

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

- **MTT assay**

  The anti-proliferative activity & IC_{50} values of cell lines was determined.

- **Acridine Orange/Ethidium Bromide Staining (AO/EB)**

  For the detection the morphological changes of apoptosis.

- **Migration Assay/scratch assay**

  The wound healing assay was performed in order to find the migratory ability in cells.

- **DCFH-DA staining**

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

PHASE - 3

- Data interpretation.
- Statistical Analysis.

5.1 CHEMICALS USED FOR THIS STUDY

S.No	Product	Description	Company
1	AL219A	DMEM High Glucose	HIMEDIA
2	Gift sample	Acerola fruit extract	Herba nutra
3	RM9955	Fetal Bovine Serum	HIMEDIA
4	T4049	Trypsin- EDTA	SIGMA – ALDRICH
5	35845	2,7-dichloroflourescein diacetate	SIGMA

5.2 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	Inverted fluorescence microscope	Nikon
8	Micropipettes	Eppendorf

5.3 METHODS

5.3.1 Cell lines and culture condition:

Human breast cancer cells MDA-MB-231 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% 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 before use.

5.3.2 Preparation of complete media and reviving cells:

For the complete media preparation 1% PenStrep (penicillin streptomycin in order to prevent bacterial contamination. Then 10%v/v FBS were added to the culture media, which act as a supplement in *in vitro* cell culture of eukaryotic cells containing very low level of antibodies and high levels of growth factors.

The vials were taken from -80ºC and thawed to room temperature and the contents were removed to 15ml centrifuge tube and 3ml of PBS were added. It was then centrifuged at 1200rpm for 3-5 minutes in Thermo ST 16R Refrigerated Centrifuge. The supernatant was removed and 3ml of complete media was added to the pellet. After resuspending, a cell culture disk (10cm) was taken, to which 6ml of complete 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.

5.3.3 Cell culture and cell counting

For cell culturing, the plate after reaching 70-80% confluency were taken and add 2-3 ml of 0.05% v/v trypsin-EDTA solution and incubate for 2-3 mins for detachment of cells. Once all the cells are detached from the surface, transfer the content to a 15ml tube and centrifuge at 1200rpm for 3-5 mins in Thermo ST 16R Refrigerated Centrifuge. The Supernatant was removed and the pellets was resuspended using fresh media and taken for cell counting & next passage.

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 cell were mixed well and loaded to the haemocytometer and cells were counted under 20x magnification using the equation:

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

5.3.4 MTT assay

The MDA-MB-231 cells were seeded in a 96-well plate at the density of 2×10^6 cells/well and allowed for attachment. The plant extracts was added to the wells in six replicates of different concentrations (31.25, 62.5, 125.250, 500µg/ml) and incubated for 24 hr at 37℃ in 5% CO2. Replace the media and add 20µl of MTT (5mg/ml) in the same well and incubated at 37 °C for 4 hrs. Decant the MTT and add 100µl of DMSO for about 30 minutes. Finally optical density of the formazan crystals was quantified using ELISA reader Multiskan Go at 570 nm (**Guo *et al*., 2019).** Cell viability was calculated using the formula;

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

5.3.5 Acridine Orange/Ethidium Bromide Staining

For the detection the morphological evidence of apoptosis. Dual AO/EB Staining was performed. Cells were seeded at a density of 2×10^6 cells / well in 6 well plate. Following cell adherence, and after extract treatment, cells were washed with 1ml PBS. Then 2-3µl/well of AO/EB solution in PBS (1 part of 100µg /ml EB in PBS) was added and they were examined under Inverted fluorescence microscope (470/440 nm filter) (Nikon Eclipse TS 100). Morphological characteristics of apoptotic cells were observed (**Delphi *et al*, 2015**).

5.3.6 Migration Assay

The MDA MB 231 cells were seeded into a 6-well plate and allowed to grow 80% confluency. Cell monolayers were wounded using 200µl tip at the centre in in perpendicular direction and the wounded monolayers were washed immediately 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. The cells are analysed for migration in the wells using inverted microscope. The relative distance between the cells were further examined using ImageJ Software **(Aumsuwan *et al*., 2016).**

$$\textbf{Cell migration} = \frac{\textbf{0 hrs} - \textbf{24hrs}}{\textbf{0 hrs}} \times \textbf{100}$$

5.3.7 DCFH-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), which is rapidly oxidized by ROS to fluorescent dichlorofluorescein. The MDA MB 231 at a density of 2×10^6 cells/ well were seeded in a 96 well plate. The different concentration of plant extracts were added and incubated for 24 hrs .After removal of media add 50µl of DCF-DA (10mM in DMSO) and observed under inverted fluorescence microscope using blue filter 480-520 nm and fluorescence intensity was measured **(Marvibaigi *et al.*, 2016).**

$$\textbf{Absorbance} = \frac{\textbf{Untreated} - \textbf{Treated}}{\textbf{Untreated}} \times \textbf{100}$$

5.3.8 Statistical Analysis

1) Data will represent mean ±SD
2) Student T-test/ ANOVA for comparing means of 2 groups.

6. RESULTS

6.1 Anti-proliferative activity of *Malphigia emarginata* in MDA-MB 231 cell line

The anti-proliferative effect of *Malphigia emarginata* was studied using MTT assay in MDA MB-231 cell line and were evaluated at concentrations ranging from 31.25µg/ml to 500µg/ml. It was found that there was a dose dependent manner decrease in cell viability. IC_{50} values of *Malphigia emarginata* in MDA-MB 231 cell lines at 24hrs was obtained as 191.27 µg/ml were shown **in Figure 7,**

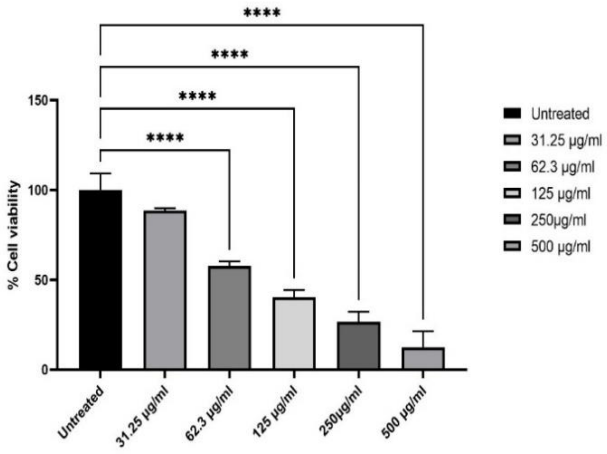


Figure 7: Percentage cell viability of *Malphigia emarginata* in MDA MB-231 cell line

Cells were seeded at a density of $6x10^6$ cells/well and treated with various concentration of drugs. After 24 hours, MTT reagent was added and washed with DMSO and readings were recorded at 570nm. Experiments were performed in triplicates.

The **figure 7**, represents the IC_{50} values determined using MTT assay, and expressed as percentage cell viability at different doses with untreated group as mean ± SD (n=6). P values were calculated using one-way ANOVA ($P<0.0001$)**** indicate that the groups were highly significant when comparing with untreated group. Percentage of cell viability decreased with increase in *Malphigia emarginata* concentration.

6.2 Determination of apoptosis using AO/EB staining:

The morphological and nuclear changes in cells, such as either condensed and fragmented nuclei are considered as early and late apoptosis. For identifying the changes in cell nuclei in MDA MB-231 upon treatment with different concentration of *Malphigia emarginata* were stained with AO/EB. The untreated cells were stained with large green nuclei,

suggesting the presence of live cells. Similarly when cells treated with different concentrations of *Malphigia emarginata* for 24hrs, exhibited a condensed nucleus, cell membrane destruction and apoptotic body formation suggesting an intact nucleus with necrotic cells. Here we have different concentrations of *Malphigia emarginata* to determine the induction of apoptotic changes by visualizing live cells, early apoptotic cells, late apoptotic cells and necrotic cells were shown in **Figure 8**

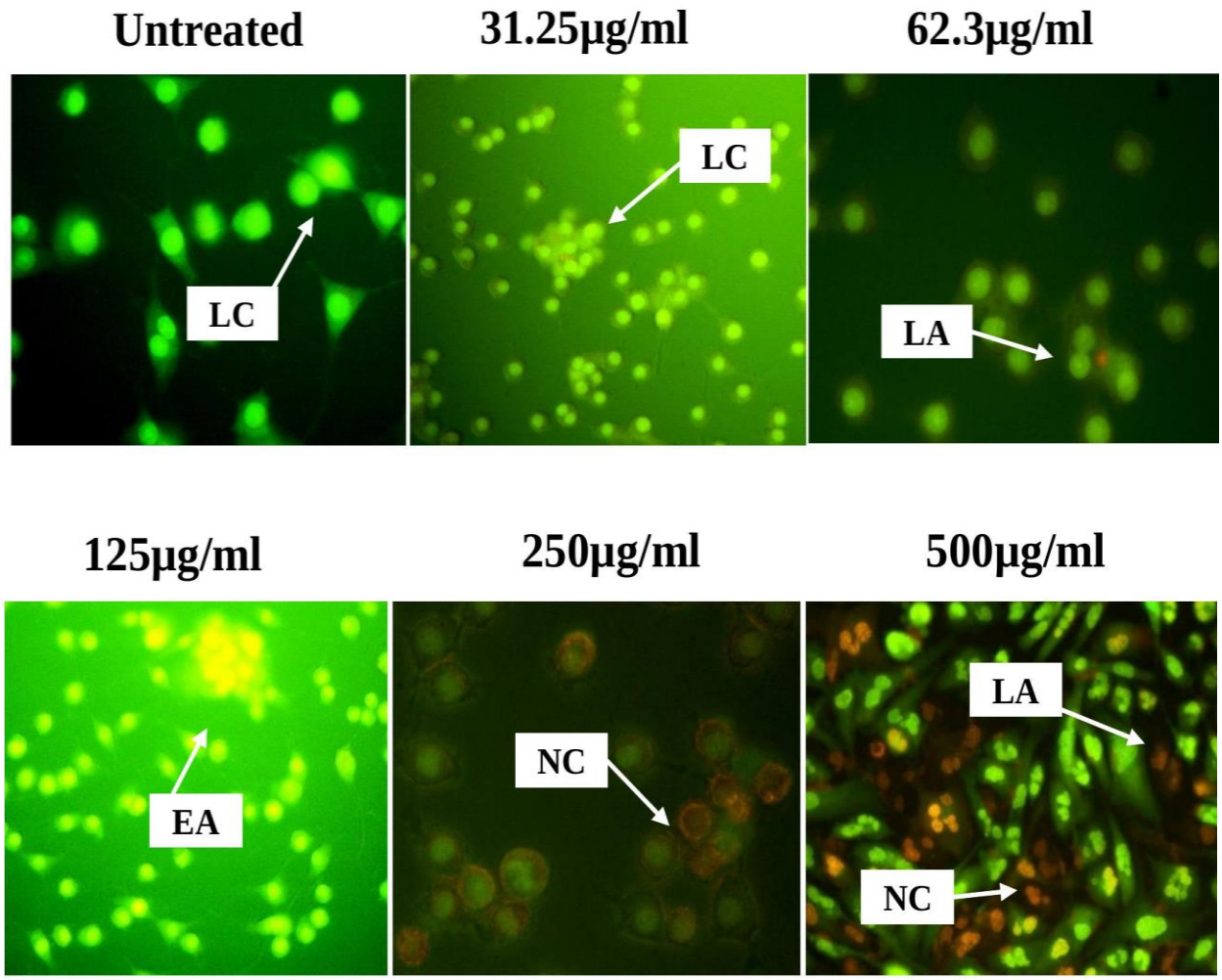


Figure 8: Morphological characteristics of apoptotic cells

The MDA-MB 231 cells were cultured and treated with different concentrations of *Malphigia emarginata* for 24 hrs and 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 Necrotic cells (NC). The results were found to induce apoptosis compared to untreated cells.

6.3 Determination of cell motility by scratch assay:

Observation of live cell motility is an effective method to measure the rate of migration created by the original wound. In the untreated group, cell migration was very dynamic at 24 hours than when compared to 0 hours. The cell migration image were shown in **Figure 9 a,**

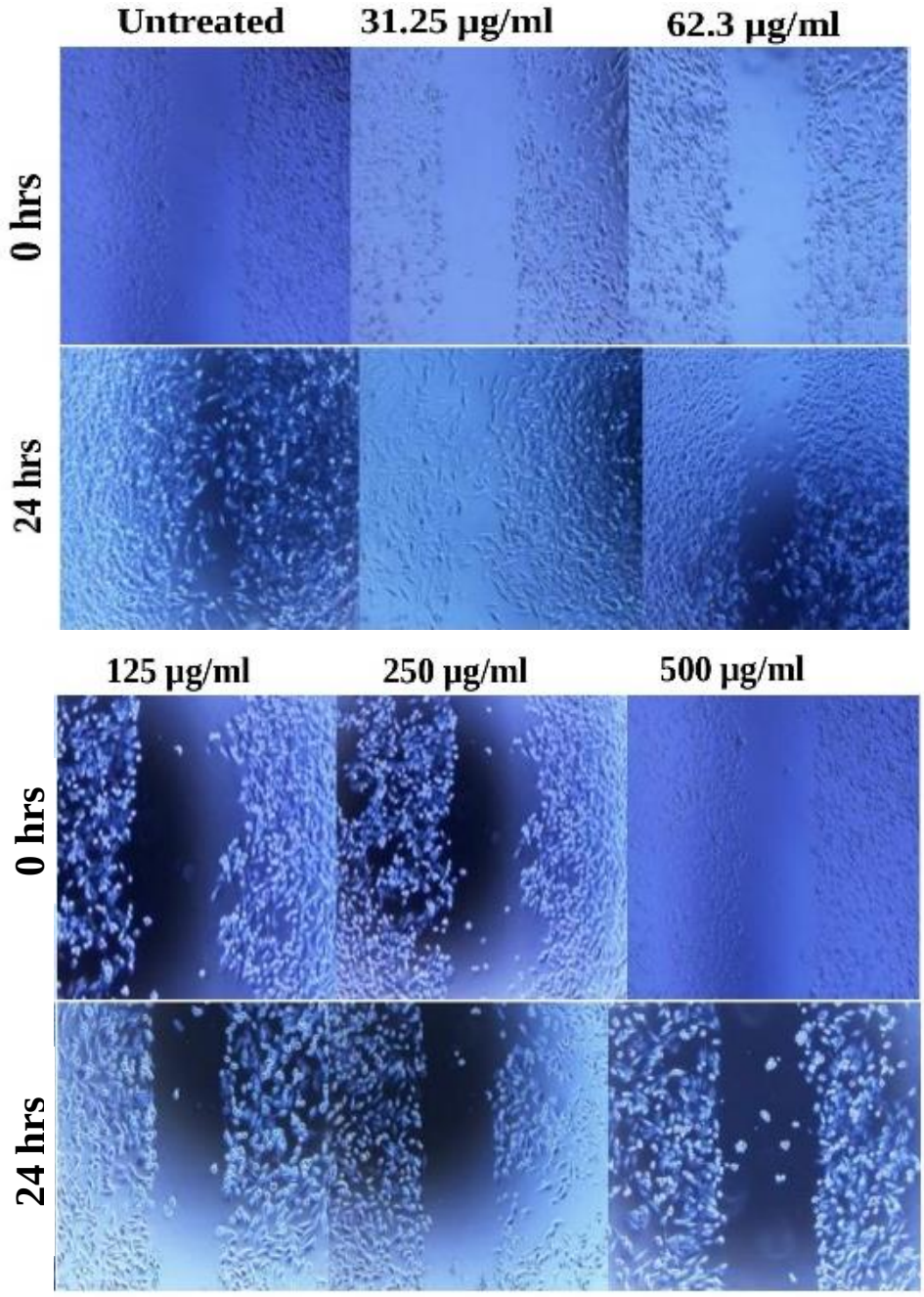


Figure 9a: Photographic image of scratch assay

Inhibition of migratory effect on MDA-MB-231 cells by *Malphigia emarginata*

Comparison of these groups shows the extract has some influence on cell migration inhibition in MDA MB- 231 cells than untreated group. The MDA-MB 231 cells were allowed

to culture in 6 well plate and the cell monolayers were wounded using 100µl tip at the centre. The cells were incubated with different concentrations of *Malphigia emarginata* for 24 hours. The cells were analysed for migration using inverted microscope and the relative distance was further examined using ImageJ Software.

The graphical representation of scratch assay from their time intervals were shown in **Figure 9b:**

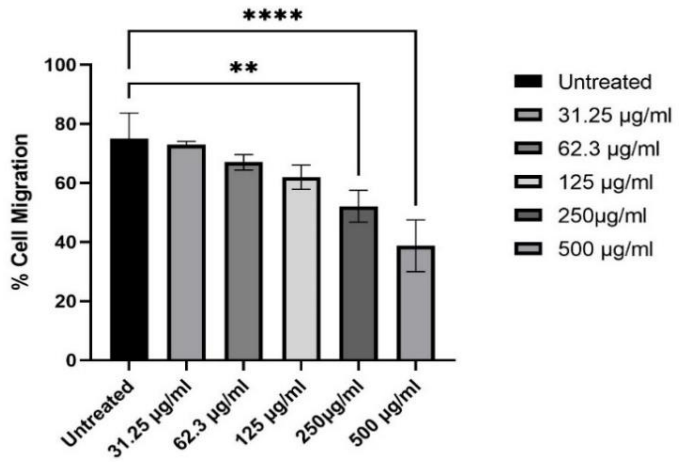


Fig 9b: Graphical representation of migration assay.

The results were presented as the percentage migration in relation to the scratch at 0 hrs and 24 hrs. In graph **** indicates higher significance ($P<0.0001$) compared to untreated and was found to promote more inhibitory migration effects.

6.4 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). This stain 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 converted to fluorescent dichlorofluorescein (DCF).

The MDA-MB 231 were cultured with following *Malphigia emarginata* extracts for 24 hours in 96 well plate and followed the staining of DCFH-DA and allowed for standing upto 30 mins. The mean absorbance at 485nm were performed as triplicates and are their absorbance were graphically represented in **Figure 10,**

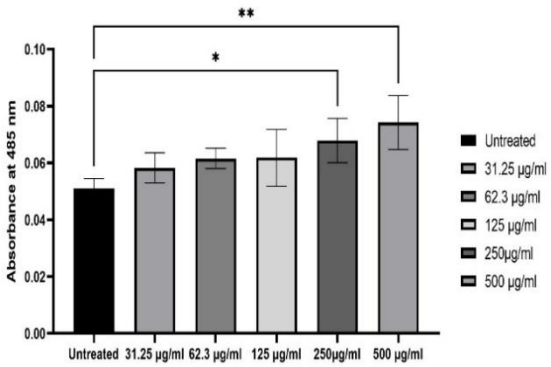


Figure 10: Graphical representation of mean fluorescence intensity at 485nm

The mean fluorescence intensity values confirms the DCF fluorescence in ROS generation that are observed in *Malphigia emarginata* treated cells. Decreased ROS generation was observed in the untreated cells compared to increased *Malphigia emarginata* treated cells. The results were analysed by one way ANOVA indicating ($P<0.001$)** represents significance compared to untreated groups demonstrating the mitochondrial membrane apoptosis by increased ROS generation.

7. DISCUSSION

Breast cancer is the most prominent cancer in women across India **(Crown *et al.*, 2012),** where TNBC in specific lacks the expression of ER, PR, and HER2 receptors remaining the biggest obstacles in cancer treatment **(Kumar *et al.*, 2016).** Current therapeutic management of TNBC are not effective because of poor prognosis and lack of targeted therapies **(Hwang *et al.*, 2019).** The therapeutic properties of plant extract against various diseases were gathered for their beneficial and remedial effect **(Talib et *al.*, 2010; Levitsky et *al.*, 2015).** Many traditional medicine ingredients extracts were proven for all stages of cancer in the development of adjuvant or alternative therapy **(Dixit *et al.*, 2019).**

The present study explored the anticancer activity of *Malphigia emarginata* extract on human breast cancer cell lines. Previously, no study has reported the proliferative effect of *Malphigia emarginata* extract against human breast cancer cell line MDA MD-231, therefore anti-proliferative and apoptotic changes of *Malphigia emarginata* were evaluated by measuring cancer cell proliferation, apoptotic stage and cell migration and ROS production respectively. The extracts added in different concentration from 31.25µg/ml to 500µg/ml induced a strong cytotoxic effect in a dose dependent manner suggesting that the extract inhibited cell proliferation compared with untreated cell growth. Dose dependent increase application of *Malphigia emarginata* inhibited breast cancer cell proliferation shown in the **Figure 7.**

Morphological changes exhibited condensed nucleus, cell shrinkage, cell membrane destruction and apoptotic body formation with more necrosed cells upon increase in concentration showing different stages of apoptosis as shown in **Figure 8.** More than 90% of human cancer-related deaths occurs due to metastatic spread of cancer **(Lin *et al.*, 2019).** Cell migration involved in metastasis developed as a life-threatening stage accompanied by induction of phenotype. Findings showed that the migration potential decreased after treatment after 24h **(Figure 9b).** The cancer treatment has been reported to induce ROS- dependent cell death in cancer cells **(Liao *et al.*, 2009).** ROS have been associated with cancer initiation, progression and metastasis. Results revealed the increased endogenous ROS production leading to the development of oxidative stress at high concentration as shown in **Figure 10** resulting in cell membrane damage and cell lysis with mitochondrial membrane apoptosis.

The *Malphigia emarginata* used to treat disorders such as diabetes, inflammation and gastrointestinal ailments **(Schreckinger *et al.*, 2010).** Recent studies suggest that the *Malphigia emarginata* and its biologically active pytoconstituents such as ascorbic acid, flavonoids,

anthocyanins, phenolics, carotenoids and stilbenenes which may be responsible for its anti-carcinogenic activity(***Chaudhary et al., 2020)***. From previous findings, identification and fractionation of various individual phytoconstituents from different stages of fruit extract at high concentration were quantified by HPLC were reported for its anticancer activity including ascorbic acid **(Mezadri *et al.*, 2008)** and phenolics for its better anti-oxidant, scavenging activity **(Marques *et al.*, 2016),** flavonoids including rutin, epicatechin, quercetin, kaemperol reported for cancer initiation, progression and metastasis **(Nascimento *et al.*, 2018)** and carotenoids including beta-carotene were reported for its beneficial effect **(Porez-gulvez *et al.*, 2005)**. Similarily quantification of volatile compounds by GC-MS including furfural, hexadecanoic acid (**Pino *et al.*, 2001**), anthocyanins reported **(de Rosso *et al.*, 2005)** stilbenenes (resveratrol) have been demonstrated in many types of cancer including those of the breast. These results suggest that the *Malphigia emarginata* possess significant phytochemical properties depending on the chemical structure of the molecule, hence the can be exploited for plant based anti-breast cancer activity in the near future.

In-vitro findings of ABTS and DPPH indicate dose-dependent inhibition of *Malphigia emarginata* extract exhibiting cytotoxicity only at the higher concentrations. *In-vivo* studies **(Barros *et al.*, 2019)** reported for lung carcinogenesis induced in mice and hepatoprotective effect through inflammatory responses **(Issue *et al.*, 2004)** and galactosamine induced liver injury with phenolic compounds suggests the fruit extract reported for its mild biological activity **(Nagamine *et al.*, 2004).** Other studies involving hepatoprotective effect against ethanol-induced stress in mice having antioxidant properties of acerola juice **(Rochette *et al.*, 2013).**

In summary, Many plant species are currently used for the prevention and treatment of cancer, such as *Taxus brevifolia*: paclitaxel and *Catharanthus roseus*: vincristine and vinblastine **(Akhtar and swamy , 2018).** *Malphigia emarginata* extract killed all cancer cells and inhibited cell proliferation, migration at higher concentration with essential bioactive phytoconstituents analytically reported that are responsible for its anticancer activity **(Lim *et al.*, 2012).** Given the long history of *Malphigia emarginata* consumption around the world with no reported side effects and its pharmacological applications have been demonstrated by many studies **(Belwal *et al.*, 2018),** it is suggested that *Malphigia emarginata* should be studied for activity against human breast cancer. In addition, the use of *Malphigia emarginata* has been growing in health and cosmetic areas **(Hanamura *et al.*, 2008).**

Thus from our findings the extract may have moderate therapeutic potential in the management of breast cancer. However further investigation is required for elucidation of molecular mechanisms suggesting for its potent anticancer activity.

8. CONCLUSION

From these studies, we conclude that our findings supported that the *Malphigia emarginata* through the investigation of *in-vitro,* exerted an evidence on Anti-proliferative activity and also confirms the apoptotic stage with the inhibition of migration potential and elevated ROS production in TNBC cells

Based on the reported Phytochemicals analysis, *Malphigia emarginata* extract holds a novel promise strategies in cancer therapy, as a new identical potential treatment for patients with metastatic TNBC.

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