Chapter 5

Chapter 5
To analyse the effect on breast cancer cell migration, invasion and TNF-α regulated cell growth in response to active extract/ fraction/ isolated phytocomponent/s of *Bauhinia variegata* L.

5.1 Introduction

In chapter 4, the potential of different extracts of *Bauhinia variegata* leaves in inhibition of cell proliferation of both the cell lines ER/PR positive MCF-7 and ER/PR negative MDA-MB-231 (MCF-7 and MDA-MB-231) at different time points has been demonstrated. Of all the extracts, aqueous extract was most effective for both the cell lines (MCF-7 and MDA-MB-231) at different concentrations. Apart from cytotoxicity, no molecular level investigation with this plant had been reported with any breast cancer cell lines in literature. For a drug to be acceptable as an anti-cancer drug, its efficacy to stop cancer cell migration and invasion is vital. Therefore, the effect of aqueous extract on cell migration pattern of breast cancer cell lines ER/PR positive MCF-7 and ER/PR negative MDA-MB-231 was further analysed.

During normal physiological process, cell migration is observed either in embryonic morphogenesis, immune- surveillance or during tissue regeneration and repair (Peter Friedl & Bröcker, 2000) Tumor tissue cells however gain the property to migrate through circulatory and lymphatic systems from the primary site to colonize distant secondary sites after invading basement membranes and endothelial walls (P. Friedl & Alexander, 2011; P. Friedl & Wolf, 2003). In malignancy, the neoplastic cells facilitate the tumor progression via invasion and metastasis (Condeelis & Segall, 2003). Depending on the dynamic nature of cell protrusive structures with regards to morphology, structure and function, they are termed as filopodia, lamellipodia, podosomes and invadopodia (Adams, 2001). Cell projections that initiate cell migration and invasion contains filamentous actin and various structural and signalling proteins which responds to chemokines and growth factors (Yamaguchi & Condeelis, 2007) . Cancer cells transit from the primary tumour either as individual cells (amoeboid or

mesenchymal movement) or collective migration (cell sheets, strands and clusters) (P. Friedl & Wolf, 2003). Cancer cells show plasticity in nature and can show epithelial to mesenchymal transition, mesenchymal-amoeboid transition and individual-collective transitions as a part of their migration strategies during tumor progression (J. S. Wu et al., 2021). Migration of cancer cells spread, further posing a challenge to anti-metastasis therapies. The advanced stages of cancer are associated with metastasis and consequential tissue destruction, a main reason of cancer-associated morbidity and mortality (Kashyap et al., 2022). Hence there is a dire need of a potential therapeutic options affecting cancer cell migration and invasion.

In vitro assays are one of the easiest modes to study live cell mechanisms but it cannot mimic the 3D environment and hence has some limitations. Many experiments are difficult to perform in animal models due to ethical issues. To overcome these restrictions, 3D spherical cell aggregates or spheroids model emerge as an excellent approach as it has an advantage over conventional 2D model (*in vitro* cell culture) and animal models to study tumor progression events. 3D spheroids are able to mimic architecture and share the same characteristics as tumor cells such as their spatial architecture, gene expression patterns, physiological responses and drug resistance mechanisms(Costa et al., 2016). 3D structure of the spheroid represents close cell–cell contacts with more tissue-like morphology and with different cellular statuses with reference to nutrient and oxygen. 3D models also resemble the tumor along with their tumor microenvironment (TME) providing us valuable insights of the tumor mechanism. Hence 3D spheroids, aptly known as in-vitro tumours have the potential to screen novel anti-cancer therapeutics and can provide significant information. It is with this intent, studies on spheroids (3D tumor) were performed.

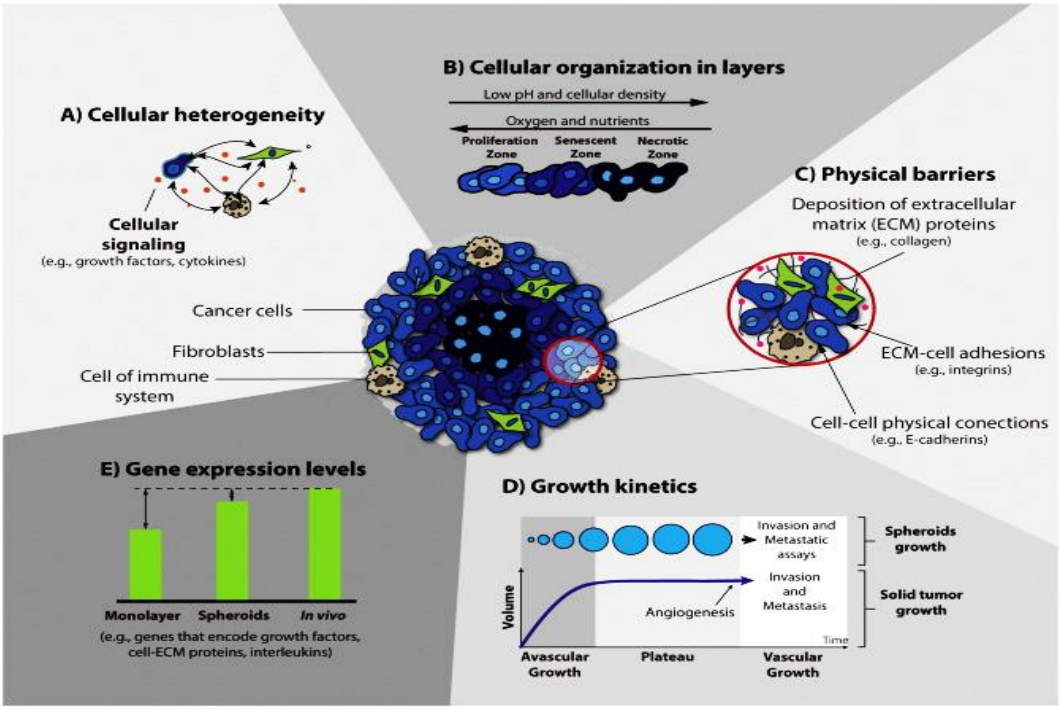


Figure 5.1: schematic diagram of 3D spheroids adapted from (Costa et al., 2016)

5.2 Effect of TNF-α and Estradiol on breast cancer cell proliferation and apoptosis.

A pivotal role is played by the tumour microenvironment (TME) for the dissemination of invasive tumor cells in a complex and coordinated manner, starting from the initiation of metastasis, controlling cancer cell movement, till its invasion, and migration to neighbouring or distant tissues. Multifunctional cytokine, Tumour necrosis factor-α is a major pro-inflammatory cytokine shown to be highly expressed in breast carcinomas (Leek et al., 1998). TNF- α was later shown to be tumorigenic in both *in vitro* studies and *in vivo* studies (Komori et al., 1993; Suganuma et al., 1999).

Cytokines, such as IL-6 and tumor necrosis factor (TNF-α), have an important role in regulating estrogen synthesis in peripheral tissues, including normal and malignant breast tissues (Purohit, Newman, & Reed, 2002). TNF-α has an important role in regulating estrogen synthesis in normal and malignant breast tissues (Mikhaylova, Kuulasmaa, Jääskeläinen, & Voutilainen, 2007).Estradiol is a major naturally occurring estrogen steroid hormone responsible for the development of female secondary sexual characteristics and in the regulation of the estrous and menstrual female reproductive cycle. Estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. Estradiol promotes breast cancer

cell migration via recruitment and activation of neutrophils (Vazquez Rodriguez, Abrahamsson, Jensen, & Dabrosin, 2017). Estradiol signalling happens through its receptor Estrogen receptor (ER). There are two isoforms; ERα and ERβ. Erα is expressed in approximately 15–30% of luminal epithelial cells and not present in any of the other cell types within the human breast(Clarke, Howell, Potten, & Anderson, 1997). The ERα is the key mediator of estradiol action in the normal mammary gland(Couse & Korach, 1999). It has been suggested that TNF-α increases the local estrogen bio-synthesis in human endometrial glandular epithelial cells and directs estrogen metabolic enzymes to produce more hormonally active and carcinogenic metabolites (Salama et al., 2009). There are two major biologically active estrogens in nonpregnant humans; estrone (E1) and estradiol (E2). A third estrogen, estriol (E3), is the main pregnancy estrogen, but plays no significant role in nonpregnant women. TNF-α significantly decreased the E1/E2 ratio by a decrease in the level of estrogen as E1 with a concomitant increase in E2 concentration. The interconversion of weakly active estrone (E1) into highly potent estradiol (E2) and their relative abundance dictate the estrogenic environment and may be contributing to the development of breast cancer(Kamel et al., 2012). TNF-α, has been shown to act as a tumor-derived factor, expressed in ER+ tumour epithelial cells and regulated by 17-β-estradiol (E2)(To, Cheung, Lazarus, Knower, & Clyne, 2014) TNF-α enhances estrogen-induced cell proliferation of estrogen-dependent breast tumor cells through a complex containing nuclear factor-kappa B (M. F. Rubio et al., 2006).

Several reports have demonstrated that estrogen suppresses the apoptosis induced by TNF-α and chemotherapeutic drugs in MCF-7 cells (Burow et al., 1998). TNF-α is down-regulated in MCF-7 cells when treated by E2 for different time periods (Frasor et al, 2003). TNF-α has also been shown to induce a transient increase in Akt phosphorylation in MCF-7 cells, which results in protection of breast cancer cells from TNFα-induced cell death (Lu, Huang, & Basu, 2006). TNF-α may regulate the growth of MCF-7 breast cancer cells through the down-regulation of Er-α expression (Lee et al., 2008). Resistance of the estrogen receptor alpha (ER)-positive, chemosensitive MCF-7 breast cancer cell line to tumor necrosis factor (TNF) was associated with loss of ER expression and a multi-drug resistant phenotype (Antoon et al., 2012). It has been reported that TNF-α treatment in certain breast cancer cell lines inhibits proliferation and induces apoptosis(Burow et al., 1998). In addition to its role as a mediator of the

apoptotic process, TNF-α also exerts selective cytotoxicity against malignant breast cancer cells, promoting an apoptotic cell death (V. K. Gupta et al., 2002). On the other hand, it has also been shown that most breast cancer cell lines are resistant to TNF-α induced apoptosis, and that their proliferation, survival, and progression are mediated by said cytokine (Zhou et al., 2000). The role of TNF-α is controversial; some investigations have proved apoptotic or necrotic effects of TNF-α, while others furnished evidence that endogenous TNF-α activates cellular growth and tumor progression(P. S. Kumar et al., 2007). This difference could be due to the differential expression of TNFRs, of the Bcl-2 family, of caspases activation or of ceramide expression (Burow et al., 1998).Therefore, depending on the cell type and cell context, TNF-α can induce cell survival or death signals. TNF-α is one of the essential pro-inflammatory cytokines found in breast cancer patients, being secreted both by stromal cells, mainly by tumor-associated macrophages, and by the cancer cells themselves. In view of this, the role of TNF-α was explored on estrogen-positive and estrogen - negative breast cancer cells in this study.

5.3 Effect of TNF-α and estradiol on cell migration in breast cancer.

Together with estradiol and epidermal growth factor (EGF), TNF-α potently induced metastasis-related properties and functions in luminal breast tumor cells, representing the most common type of breast cancer (Weitzenfeld, Meron, Leibovich-Rivkin, Meshel, & Ben-Baruch, 2013). Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF- α dependent up-regulation of matrix metalloproteases (Hagemann et al., 2004). Hence, TNF-α can enhance the invasive ability of MCF-7 cells, partly by regulating a series of metastasis related genes (Xiaofeng Chen, Shu, Li, & Yin, 2008). There is evidence that indicates that TNF-α is involved in the transformation, proliferation, angiogenesis, invasion and metastasis of many cancers (Y. Wu & Zhou, 2010). Soria *et al* proposed that TNF-α was a more potent inducer of EMT than IL-1, and that its effects were more prominent on MCF-7 cells (Soria et al., 2011).

High plasma TNF-α levels in cancer patients are associated with a poor disease outcome (Nakashima et al., 1998). Cells expressing TNF-α in inflammatory breast carcinoma show correlation with tumor grade and nodal metastasis, and TNF-α play a role in the metastatic behavior of breast carcinomas (Miles et al., 1994).These studies indicate that

TNF-α may implicate a new possible explanation for inflammation associated breast cancer. Immunohistochemistry, sequencing and serum-profiling studies in TNBC patients showed high expression of TNF-α leading to metastasis (Bilir et al., 2015; H. H. Li et al., 2015). Also, TNF-α gene knockdown is linked with inhibition of cell proliferation and apoptosis in TNBC (Pileczki, Braicu, Gherman, & Berindan-Neagoe, 2012). Therefore, it would be of great importance to find out effect of aqueous extract on breast cancer cell-line proliferation and migration in presence of TNF-α.

Results:

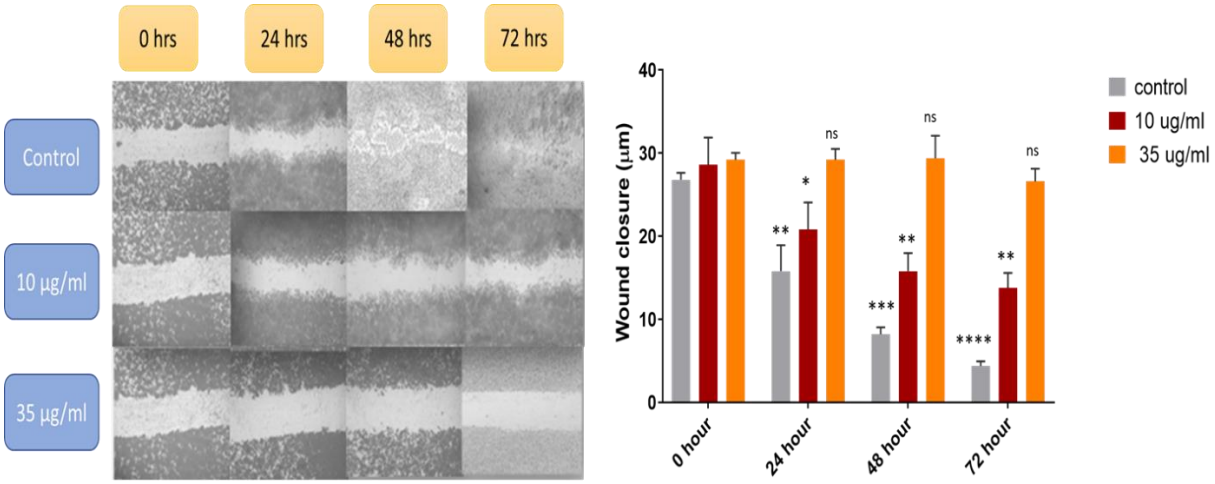
5.4 To assess the effect of aqueous extract on cell migration in breast cancer cell-lines.

Developing inhibitor of cancer cell migration and metastasis, is important to prevent metastasis and decrease the mortality rate. Since the aqueous extract of *Bauhinia variegata* leaves showed promising anti-cancer activity, its effect on the breast cancer cell lines migration was investigated. *In vitro* assays like wound healing assay are used (mentioned in chapter 3) to measure the migratory potential of cells. Also, efforts have been made to developed an experimental setup for cell migration study. Tumorigenic potential of breast cancer cells exposed to aqueous extract was also studied. Cell proliferation assay was done to check the effect of TNF- α and estradiol on breast cancer cell lines. Gel invasion assay was performed to analyze the invasive property of the MCF-7 spheroids in presence and absence of TNF- α and estradiol.

5.4.1 To assess the effect of aqueous extract on cell migration in breast cancer cell-lines by wound healing assay.

Cells migrating from the border of the intact regions into the scratched region were microscopically monitored for the migration of the cells at different time points until the wound is closed. It is difficult to differentiate the difference between the wound closure caused by cell proliferation or cell migration for long term wound healing assays, mostly greater than 24 hours. Hence to overcome this hurdle serum concentration was standardized (2% serum) which was sufficient to sustain the growth of the cells and would ensure the wound healing due to cell motility and not cell proliferation. Cells either migrate individually or as collective sheets of cells (Figure 5.2).

a)



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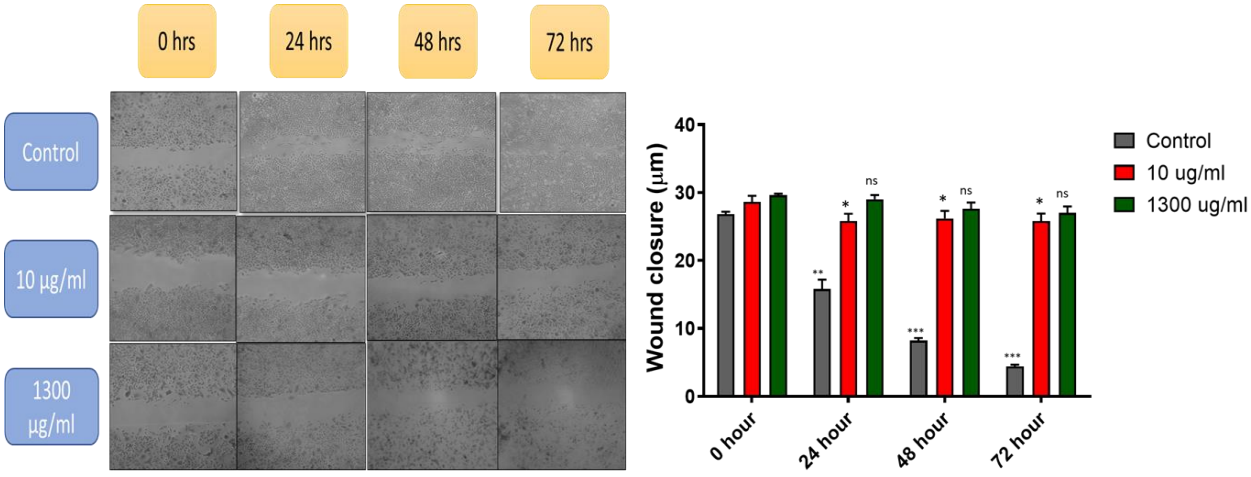

Figure 5.2: The effect of aqueous extract on wound healing ability in breast cancer cell lines a) MCF-7 and b) MDA-MB-231 cells. Wound measurement upon extract exposure for different time periods was done in breast cancer cells. Length between two edges of wound was measured by Image J and plotted at different time periods. All the experiments were repeated thrice. p value is represented as * : p < 0.05, **:p< 0.01, *** : p < 0.001, **** : p < 0.0001.

Images of the wound created were taken at zero hours and cell migration was monitored till 72 hours. It was observed that in the control cells the wound was healed within 72 hours by cell migration in both the cell-lines MCF-7 and MDA-MB-231.

In the treatment group, MCF-7 cells were exposed to different concentration of aqueous extract of *Bauhinia variegata* leaves (10 µg/ml and 35 µg/ml). At 10 µg/ml, a decrease in the wound was observed from 24 to 72 hours. There was no significant decrease at

72 hours as MCF-7 cells were not able to survive after 72 hrs. The graphical representation (Fig. 14a) depicts the same that in untreated MCF-7, wound area was decreased with respect to time which indicates healing of scratch in control cells because of cell migration. Cells treated with different concentrations (10 μg/ml and 35 μg/ml) of aqueous extract showed no wound healing between 0 hrs and 72 hrs.

In the MDA-MB-231 cells, treated with different concentration of aqueous extract of *Bauhinia variegata* leaves (10 μg/ml and 1300 μg/ml), no significant wound healing activity was observed from 24 to 72 hours. MDA-MB-231 cells were also not able survive after 72 hours. The graphical representation (Fig. 14b) depicts the same that in untreated MDA-MB-231, wound area decreased with time indicating healing of scratch in control cells because of cell migration. Whereas upon treatment with different concentrations (10 μg/ml and 1300 μg/ml) of aqueous extract there was no wound healing between 0 hrs and 72 hrs. At 72 hours for MDA-MB-231, treatment at 1300 μg/ml results in cell death may be due to low concentration serum used for the assay. This suggests that aqueous extract of *Bauhinia variegata* leaves possess phytocomponents that have ability to impair migratory property of MCf-7 and MDA-MB-231 cancerous cells.

5.4.2 Development of in-house assay for cell migration study in breast cancer cell lines.

In this method, attempts were being made to mimic Boyden chamber assay, also known as the transwell cell migration assay that measures the ability of cell movement towards a chemo-attractant gradient (High serum). It is a highly accessible procedure that can be performed in research laboratories even just with basic cell biology setup (Lis, Kuzawińska, & Bałkowiec-Iskra, 2014). The detailed description is mention in the materials and methods chapter.

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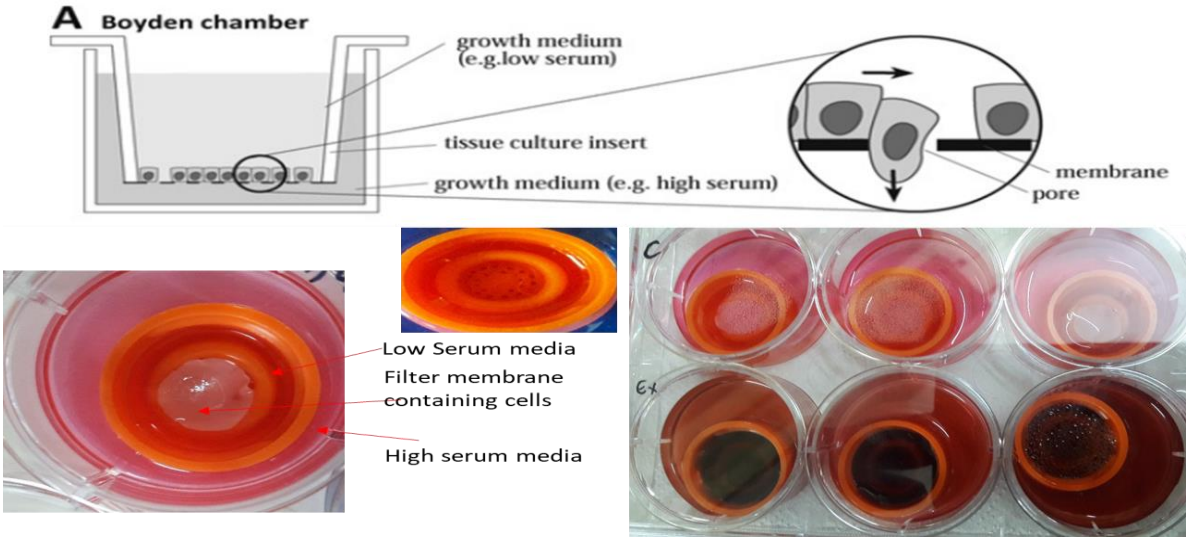
A Boyden chamber
growth medium
(e.g.low serum)
tissue culture insert
growth medium (e.g. high serum)
membrane
pore
Low Serum media
Filter membrane
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High serum media
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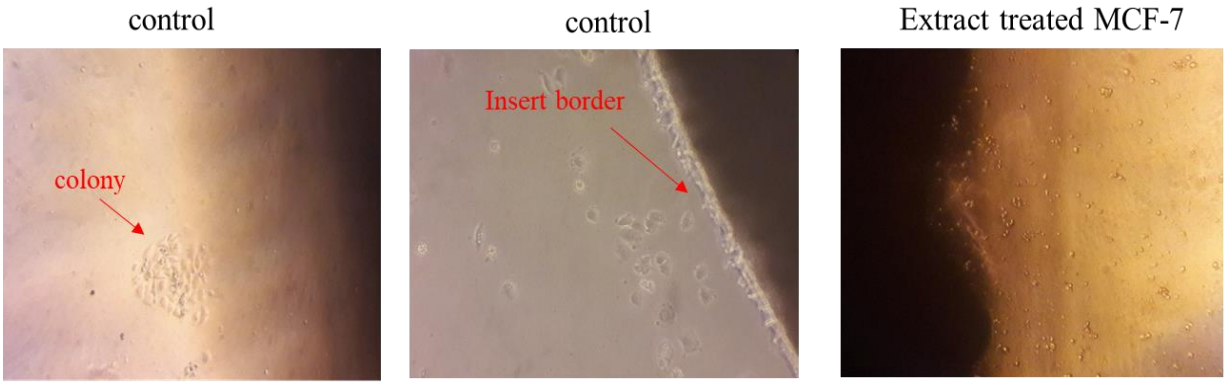
b)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control
Extract treated MCF-7
colony
Insert border

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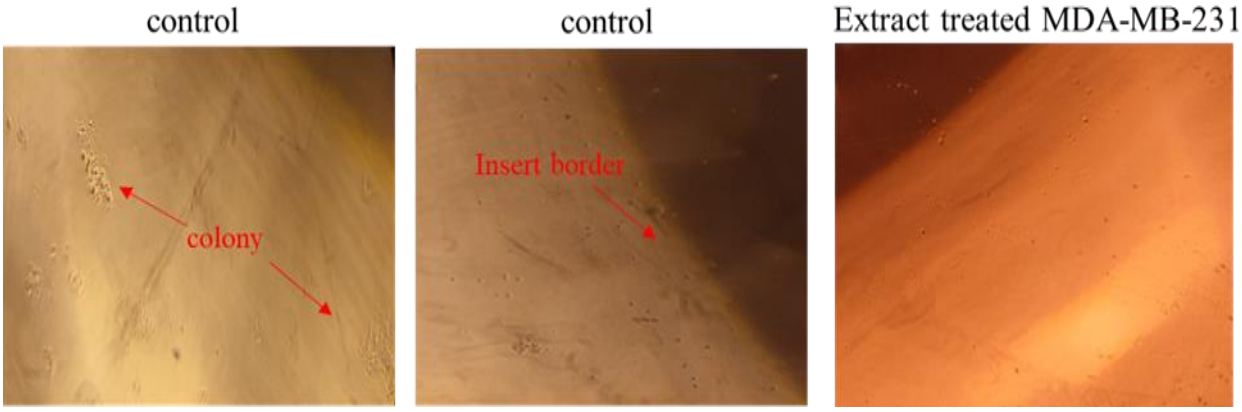
control
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Extract treated MDA-MB-231
colony
Insert border

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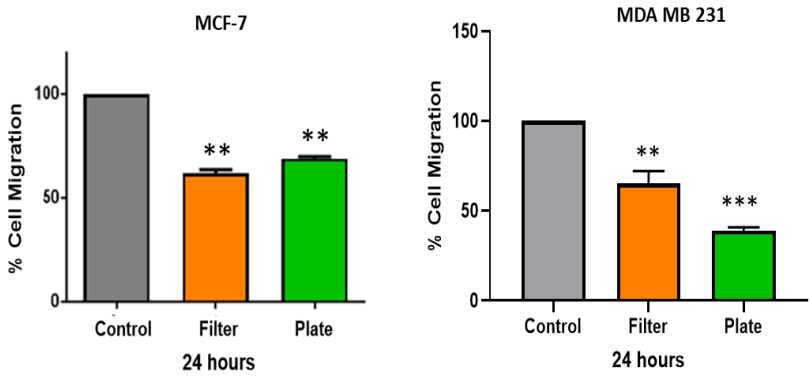


Figure 5.3: Cell migration assay in breast cancer cell lines a) In house development of cell migration assay where a) image adapted from *Kramer et al., 2013.* b) MCF-7 and c) MDA-MB-231 cells at 24 hours. Images are captured using inverted microscope at 10x. Graph represents cells remaining on the filter and migrated on the plate stained by crystal violet. Experiments were repeated thrice.

The cells are seeded on the sterile porous membrane that was placed on the cap with pores. Then low serum medium is added to the upper part. In the lower compartment, high serum media is added in case of control and extract is added in test wells. The detailed protocol can be found in materials and method chapter. Cell migrates through the pores of the membrane into the lower compartment with high serum media (with extract in case of sample wells). Migration was monitored with the intense care by ensuring that the chambers are not moved from their original position to avoid any ambiguity in the results. Images were captured at 24 hours for both the cell-lines using inverted microscope with 10x lens. It was observed that in case of control cells, both the cell lines MCF-7 and MDA-MB-231 were able to pass through the membrane and form colonies after 24- 48 hours. In contrast, in the wells with high serum media along with aqueous extract, the migrated cells were seen as round dead cells implying that phytocomponents present in the aqueous extract of *Bauhinia variegata* leaves have capability to hinder cell migration of breast cancer cells (Figure 5.3).

5.5 To study the effect of *Bauhinia variegata* aqueous extract on cell proliferation on MCF-7 spheroids.

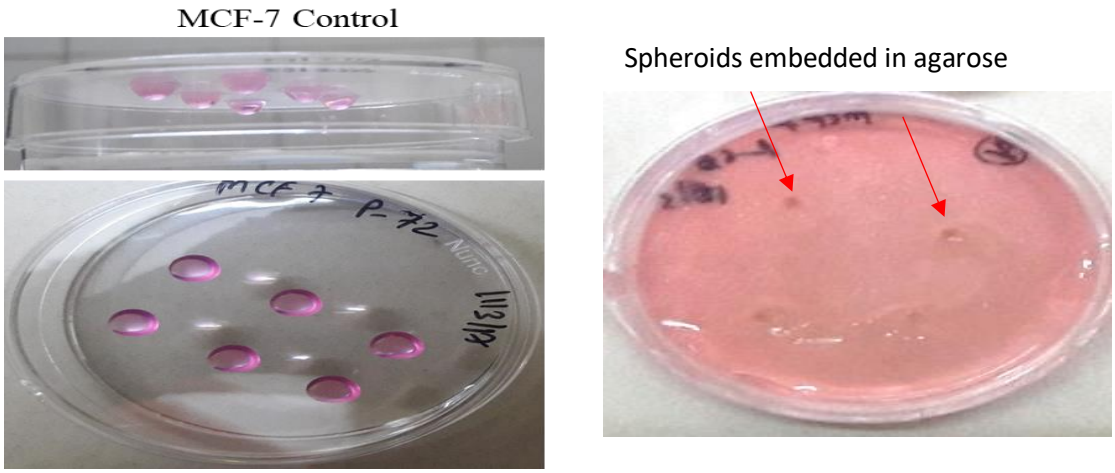
From the cell proliferation experiments (2D culture), it was found that the aqueous extract of *Bauhinia variegata* leaves was able to inhibit cell proliferation of the breast

cancer cell-lines MCF-7 and MDA-MB-231 both at different concentrations. Cell migration assay also points to the efficacy of the aqueous extract of *Bauhinia variegata* leaves towards the cell migration potential of the breast cancer cell lines. Thus, it was interesting to determine the effectiveness of the aqueous extract of *Bauhinia variegata* leaves on the invitro 3D cultures (Spheroids).

5.5.1: Formation of spheroids by hanging drop method in breast cancer cell lines.

The spheroids were generated from the MCF-7 cells by hanging drop method (as explained in chapter 3). Later these spheroids were overlayed with agarose. 1% agarose could embed the spheroids properly without disrupting its structure. 0.36% and 0.5% agarose caused the disruption of the spheroids.

a)



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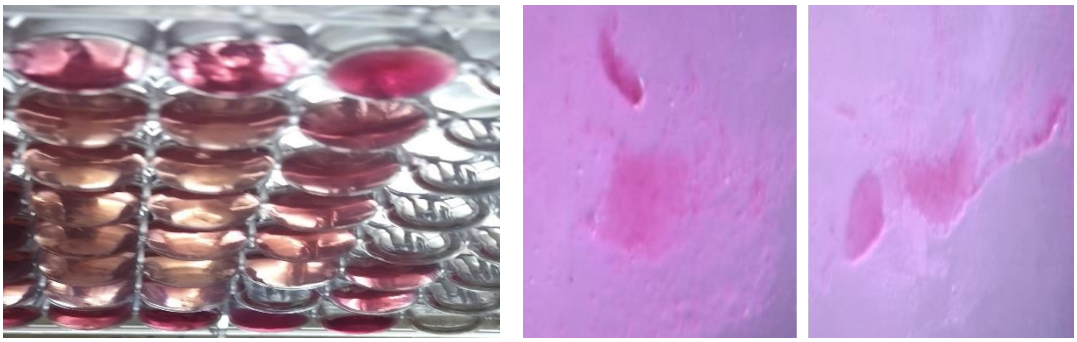


Figure 5.4*:* Formation of Spheroids by Hanging Drop method in breast cancer cells. a) Spheroids generation in MCF-7 cell lines with hanging drop method, and spheroids embedded in agarose for further experiments. b) Spheroids generation

in MDA-MB-231 cell lines by hanging drop method and with u-bottom 96 well plate was not successful. Agarose embedded spheroid Image is taken in Stereo Microscope at 10x (24 hours)

The spheroids generation of MDA-MB-231 cells was tried using U bottom 96 well plate by two methods. In the first method, the cells from the fresh media were seeded into the plate. Spheroid formation was visible by first method in round bottom plate initially. Spheroid when embedded in agar, the cells dispersed. In the second method, pre embedding cells in agar and then seeding into the U bottom 96 well plate was tried. The media was gently replaced in first method and top seeded on the second method every 2 days. The plates were monitored for 50 days and no signs of spheroid formation was observed in second method. Hence problems were encountered while forming spheroids for MDA-MB-231cell lines. Spheroid like structure were observed but were very fragile to be embedded in agarose for further studies, and hence it was difficult to generate spheroids by hanging drop method for MDA-MB-231. This may be accredited to high invasive cell line property of the cells (Figure 5.4).

5.5.2: Effect of aqueous extract of *Bauhinia variegata* leaves on cell proliferation of MCF-7 spheroids.

The cell proliferation of MCF-7 spheroids in response to the aqueous extract of *Bauhinia variegata* leaves was noted. Major difference was observed in the spheroid structure as well as size of untreated and treated spheroids.

Increased luminal space were found in the untreated MCF-7 cells and the peripheral cells were proliferating whereas in the extract treated spheroids, there was lack of luminal space, the spheroid size was small compared to untreated spheroids and the proliferating peripheral cells were not seen. Area and perimeter of treated and untreated tumours were measured using NSI software. The graphical representation of the images showed significant decrease in both the area and perimeter of the extracted treated spheroids compared to control spheroids (Figure 5.5).

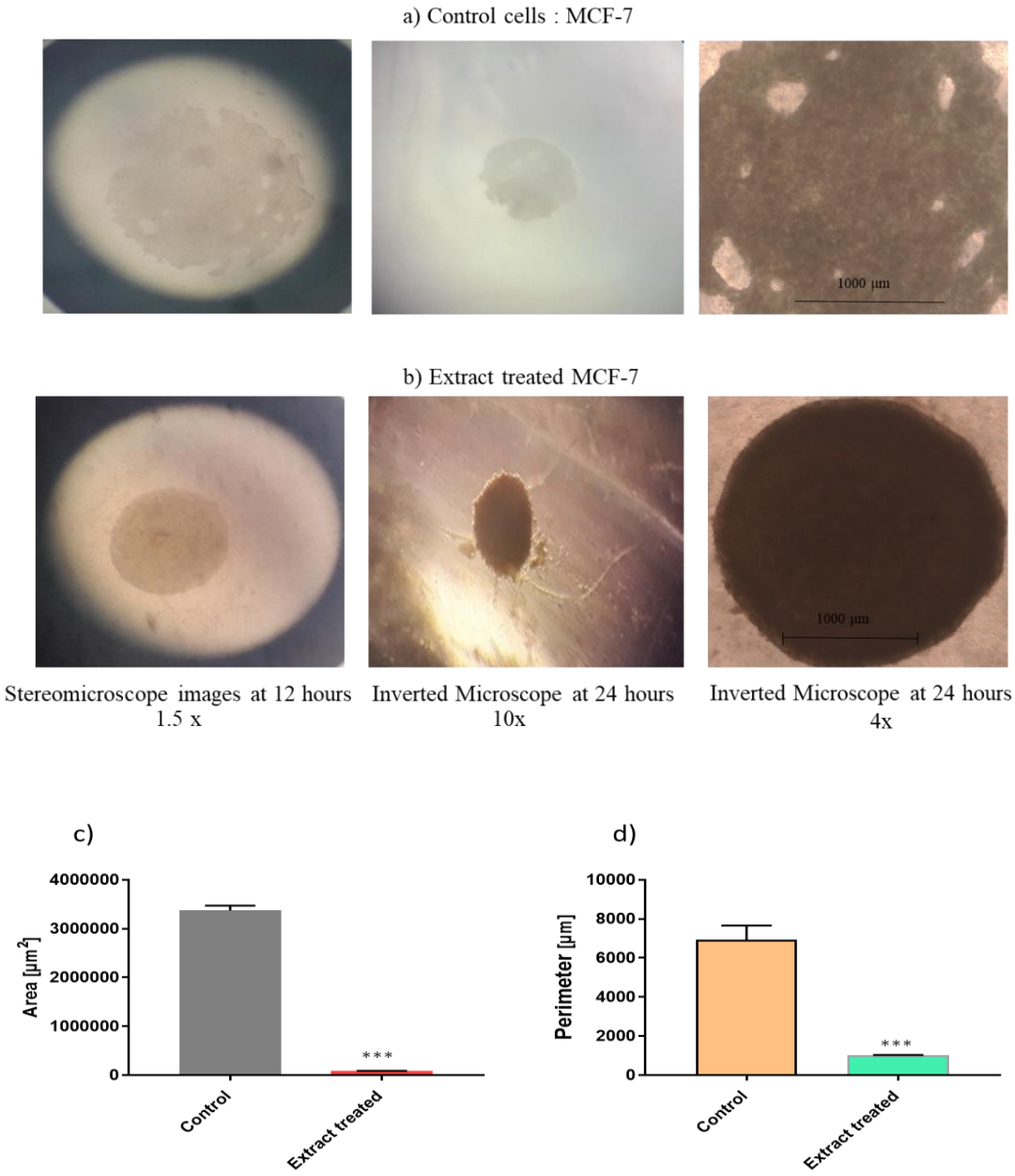
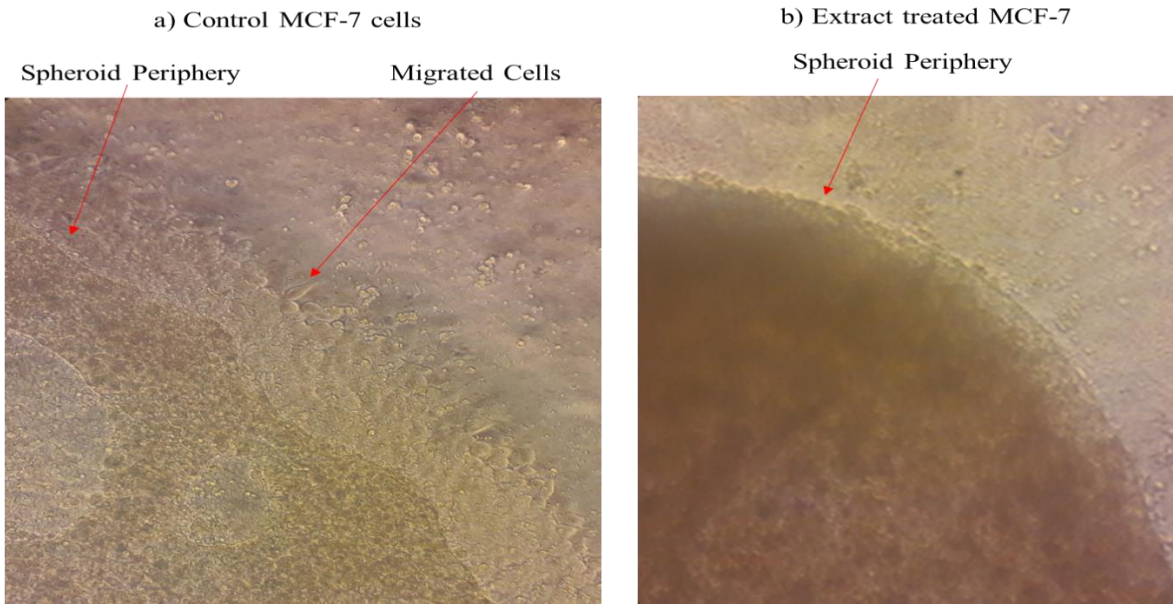


Figure 5.5: The effect of *Bauhinia variegata* aqueous extract on MCF-7 Spheroids. a) and b) shows control and extract treated MCF-7 cells respectively. c) and d) represents treatment with aqueous extract that decreases area and perimeter of MCF-7 spheroids. Images were taken with inverted microscope. Area and perimeter of treated and untreated tumours were measured. Experiments were performed in triplicates. p value shows significance difference (*: $p < 0.001$).**

5.5.3: To assess the effect of aqueous extract on cell invasion in MCF-7 spheroids.

Cell invasion is defined as cell movement where the cell modifies its shape, passes through a 3D matrix, interacts with extracellular matrix and restructures the 3D environment. ‘‘3D migration’’ refers to non-proteolytic and non-destructive movement

in 3D matrices. With the purpose of understanding effect of aqueous extract of *Bauhinia variegata* leaves on cell invasive properties, the agarose embedded spheroids were further monitored for their invasiveness and comparative analysis was done with reference to migration in untreated spheroids (Figure 5.6 a).



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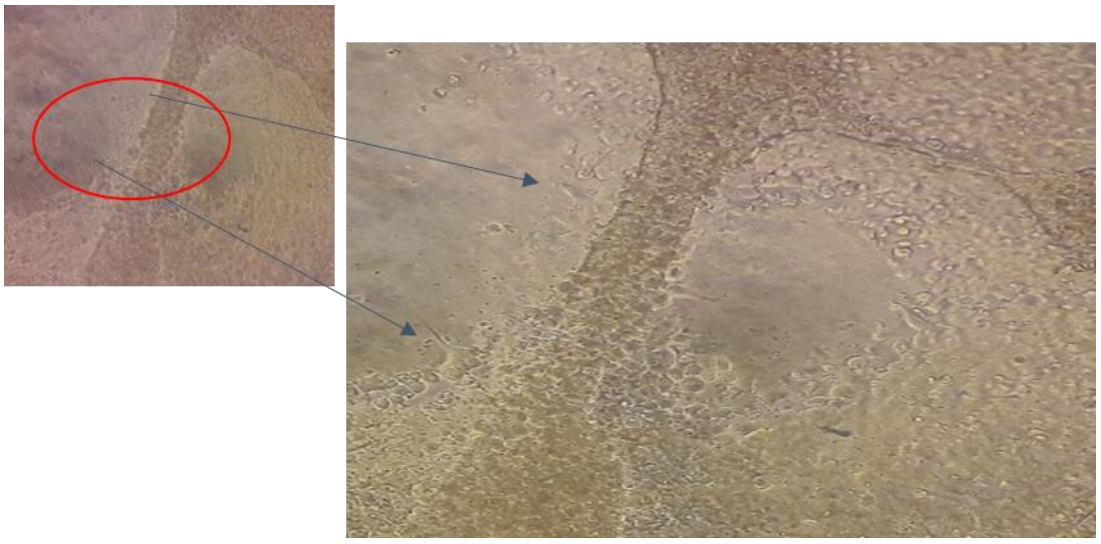
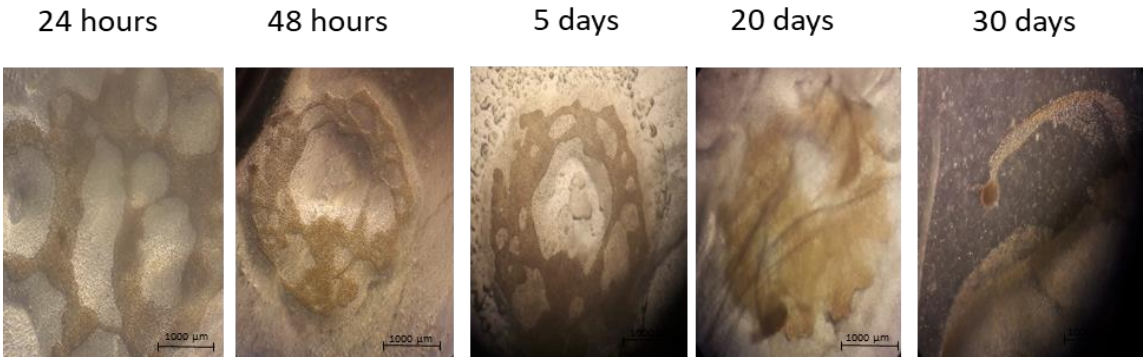


Figure 5.6: Cell invasion in MCF-7 spheroid. a) control cell migration involving cell polarization, lamellipodia extension, and trailing edge retraction. b) cell protrusions for motility were absent in extract treated cells. Images of control MCF-7 cells as seen under inverted Microscope 40x at 48 hours. Experiments were done in triplicates.

In untreated spheroids, the tumor cells migrate from the periphery of the sphere in a concentric manner. Cell movement was measured microscopically (Fig. 5.6). The leading cells showed the formation of invadosomes, which may be important for the cell–cell junctions. The lagging cells followed the leading cells. It can be concluded that the control cells have sustained its aggressiveness at 48 hours and migrates via formation of invadosomes. Whereas the extract treated MCF-7 spheroids showed distinct border with no traces of migration even after 48 hours of formation.

Control



Extract treated:

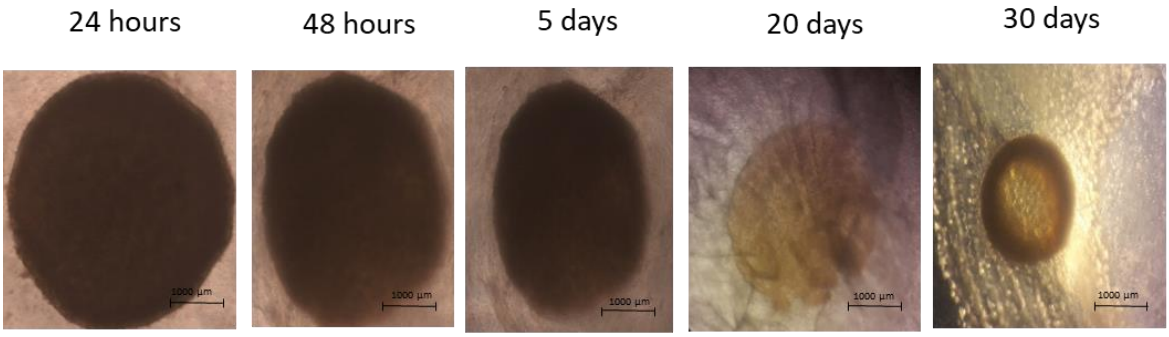


Figure 5.7: Timeline for the spheroids subjected to aqueous extract of *Bauhinia variegata* leaves. Images taken by inverted microscope at 4x, 20x magnification, 30 days 40x magnification).

To further investigate the spread of the cancer cells from the periphery of the spheroids over the period of time, spheroids were monitored regularly and images were taken till 30 days. In the untreated spheroids, it was observed that the cell invasion was progressive till 30 days. Along with the cell progression in the medullar area, cortical buds also started appearing which elongated with the advent of time. As the spheroids get larger, the increased hypoxia and nutrient deficiency might lead it to cell death.

Image at 30th day showed structure far away from the embedding place, somewhat similar to acinar spheroids. The extract treated spheroids appeared to be shrinking slowly and at 30th day it was totally seen empty within which is indicative of necrotic type of cell death (Figure 5.7).

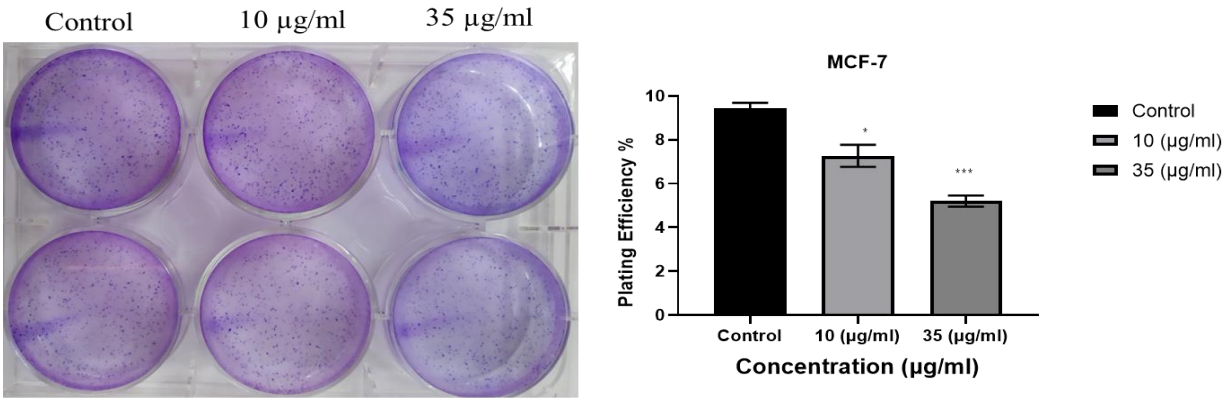
5.6: Aqueous extract regulates tumorigenic potential of breast cancer cells.

From the above experiments, it can be confirmed that the aqueous extract of *Bauhinia variegata* leaves has phytocomponents that are responsible for its anti-cell proliferative, anti-migratory and anti-invasive activity. So, it was hypothesized that extract may also regulate the tumorigenic potential in breast cancer cell-lines. To prove this hypothesis, MCF-7 and MDA-MB-231 cells were treated with extract and clonogenic ability was monitored using colony forming assay.

The clonogenic cell survival assay (described in chapter 3) determines the ability of a cell to proliferate indefinitely, thereby retaining its tumorigenic ability to form a large colony or a clone. Aqueous extract of *Bauhinia variegata* leaves showed a significant decrease in colony forming units in concentration dependent manner in both the cell lines MCF-7 and MDA-MB-231cells as compared to control. There is significant percentage decreases in colony formation in aqueous treated cells (Figure 5.8). Thus, these results suggest that the aqueous extract of *Bauhinia variegata* leaves inhibits the tumorigenic potential and migration ability of breast cancer cell lines.

Literature suggests that the interplay of various molecules drives the breast cancer progression and the main cytokine involved is TNF-α. In addition, estradiol also plays a vital role in the cell proliferation of breast cancer cells, specifically hormonal responsive cell lines. So it would be important to explore if aqueous extract of *Bauhinia variegata* leaves is able to sustain its anti-proliferative and anti-invasive ability in presence of these two important molecules which have pivotal role to play in breast tumorigenesis.

a) b)



c) d)

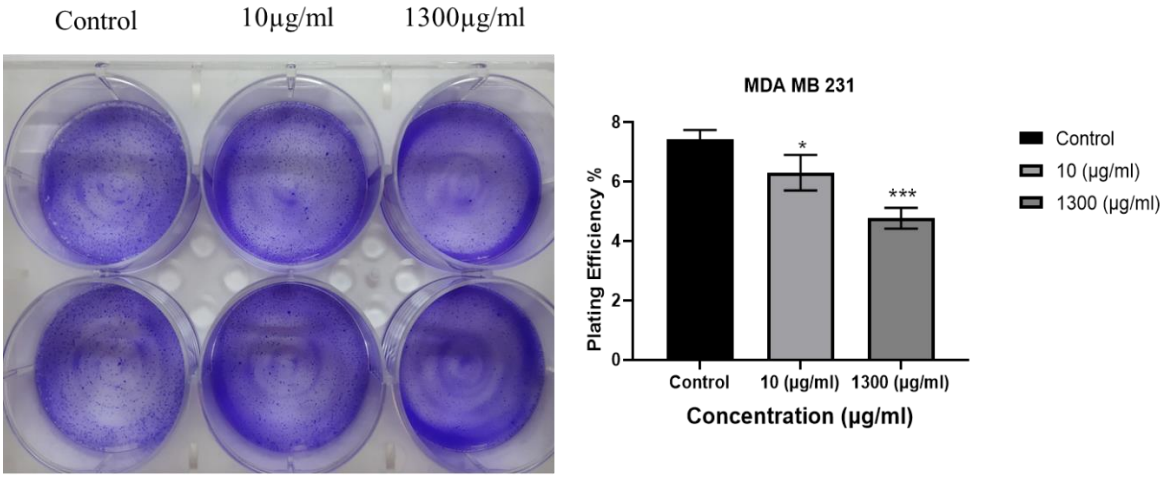


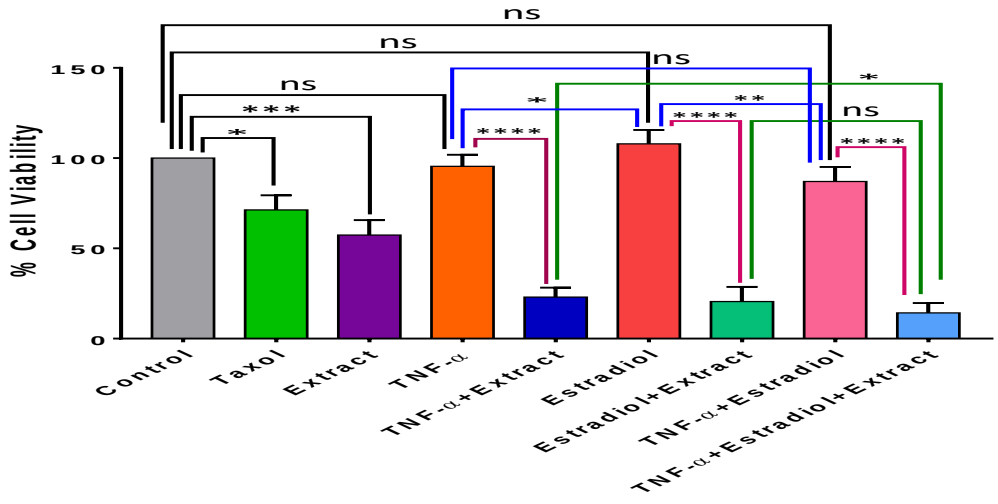
Figure 5.8: The aqueous extract affects clonogenicity of breast cancer cell lines a) shows colony forming ability and b) % Plating efficiency of the MCF-7 cells whereas c)shows colony forming ability and d) % Plating efficiency of in MDA-MB-231 cells. The experiment was repeated thrice.

5.7 To evaluate the effect of aqueous extract of *Bauhinia variegata* leaves on cell proliferation in absence and presence of TNF-α in breast cancer cell lines

The anti-proliferative ability of MCF-7 and MDA-MB-231 cell lines in presence of TNF-α were assessed. Here different combination of TNF-α, Estradiol and aqueous extract of *Bauhinia variegata* on MCF-7 and combination of TNF-α, and aqueous

extract of *Bauhinia variegata* on MDA-MB-231 were used. Taxol was used as a standard drug.

a)



b)

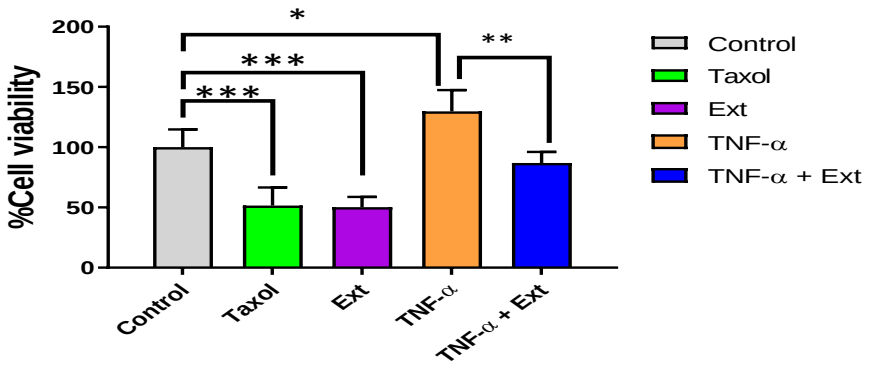


Figure 5.9: The effect of aqueous extract on cell proliferation in absence and presence of TNF-α in MCF-7 for 24 hours (TNF- α : 10 ng/ml, Estradiol: 10 ng/ml, Ext: 35 µg/ml). To evaluate the effect of TNF-α on cell proliferation in absence and presence of aqueous extract in MDA-MB-231 for 24 hours. (TNF- α : 10 ng/ml Ext: 1300 µg/ml) * :p < 0.05, ** : p < 0.01, *** : p < 0.001, ****:p<0.0001

In MCF-7, enhanced cell proliferation of the cells was observed in presence of TNF-α and Estradiol. The cell proliferation was not significant in estradiol only group compared to TNF-α only group. There was no significant growth in MCF-7 exposed to TNF-α alone as compared to the control group. MCF-7 exposed to the combination of TNF-α and estradiol, significant decrease in the cell growth was seen compared to only

estradiol group. Upon treatment with aqueous extract of *Bauhinia variegata* leaves to TNF- α alone, estradiol alone, and combination group (TNF- α and estradiol) significant reduction in cell proliferation was observed.

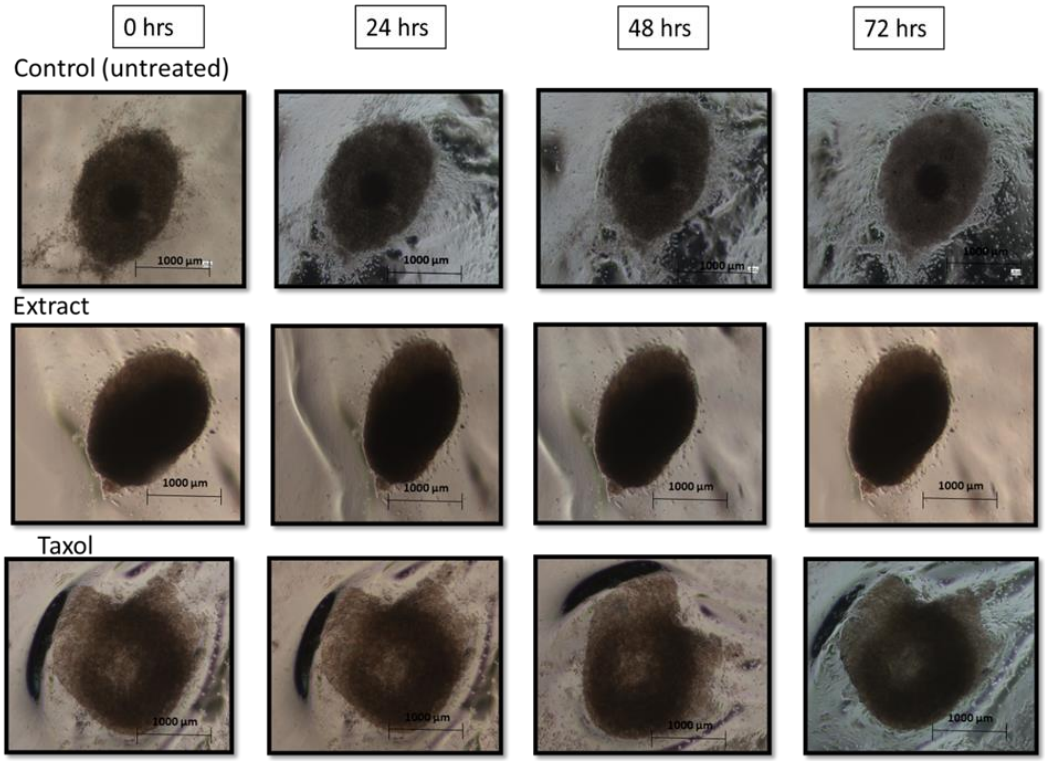
In triple negative cell line, it was observed that there was notable increase in the MDA-MB-231 cells with TNF-α compared to control and significant growth reduction was observed in the group where TNF-α and the aqueous extract of *Bauhinia variegata* leaves was together. These results suggest that TNF-α modulating property of the phytocomponents present in the aqueous extract of *Bauhinia variegata* leaves (Figure 5.9).

5.8 To evaluate the effect of aqueous extract of *Bauhinia variegata* leaves on cell invasion in absence and presence of TNF-α and estradiol in MCF-7 spheroids.

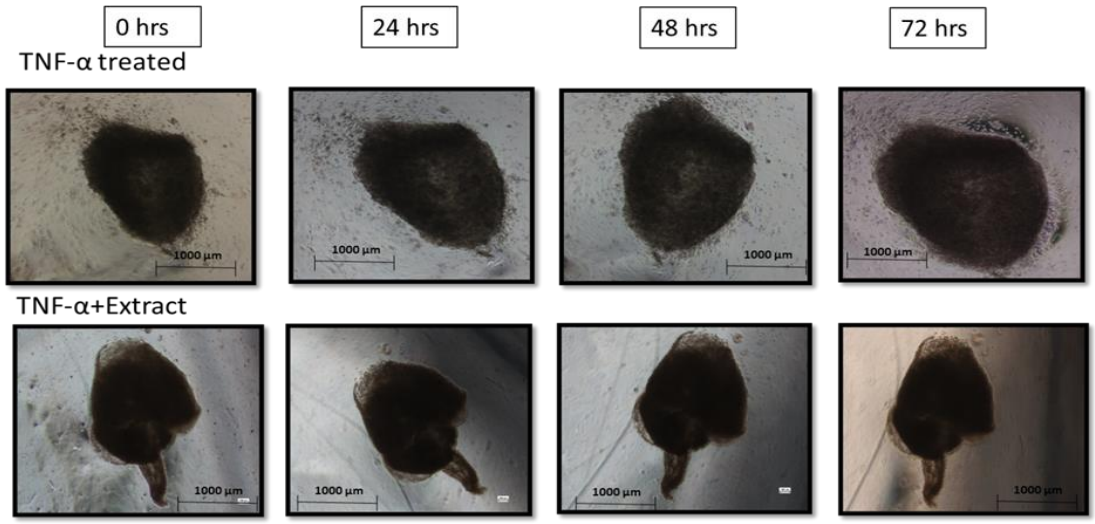
From the cell proliferation assay it was found that aqueous extract of *Bauhinia variegata* leaves was quite effective in reducing the cell proliferation in the presence of TNF-α, estradiol and its combination. To investigate this further, anti-invasiveness of the aqueous extract of *Bauhinia variegata* leaves was checked on the MCF-7 spheroids in presence of both, estradiol and TNF-α for different time interval (24, 48 and 72 hours).

It was observed that the size of the untreated spheroid increased with time (24 to 72 hrs). The size of the spheroid treated with aqueous extract of *Bauhinia variegata* leaves showed slight decrease with time (24 to 72 hrs) whereas the size of Taxol treated spheroid there is no significant increase. The spheroid with only TNF-α showed increase in cell invasiveness at 72 hours compared to 24 hours, but aqueous extract of *Bauhinia variegata* leaves decreases the cell invasiveness was seen. Increase in the cell invasiveness was observed in the spheroids with estradiol group from 24 to 72 hours, but in presence of aqueous extract of *Bauhinia variegata* leaves decrease in the cell invasiveness was seen. The spheroid with TNF-α and estradiol combination showed increase in cell invasiveness at 72 hours compared to 24 hours. but in presence of aqueous extract of *Bauhinia variegata* leaves slight decrease in the cell invasiveness was seen (Figure 5.10).

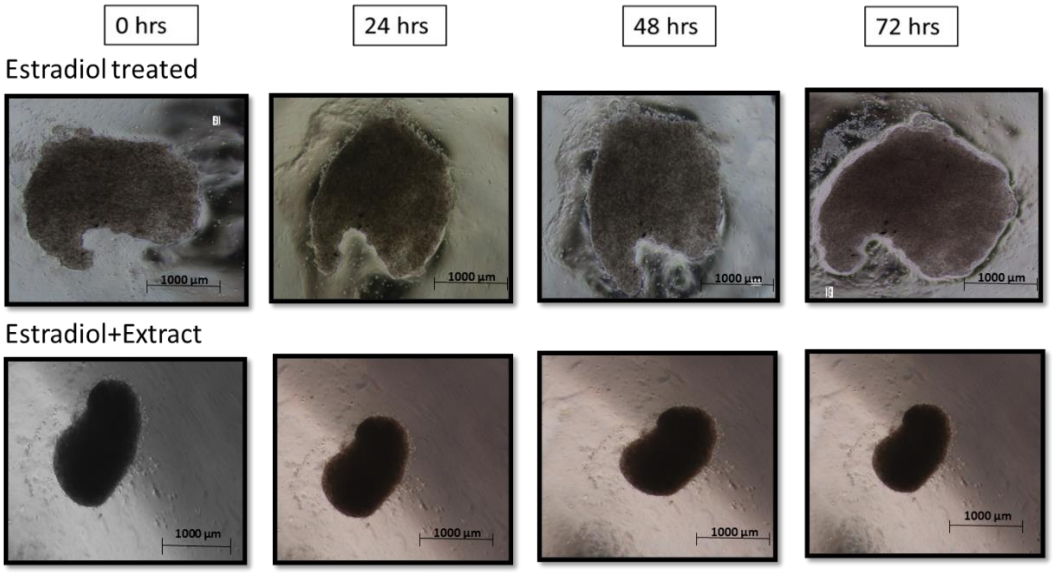
a)


0 hrs
24 hrs
48 hrs
72 hrs
Control (untreated)
Extract
Taxol
1000 µm

b)


0 hrs
24 hrs
48 hrs
72 hrs
TNF-α treated
TNF-α+Extract
1000 µm

c)



d)

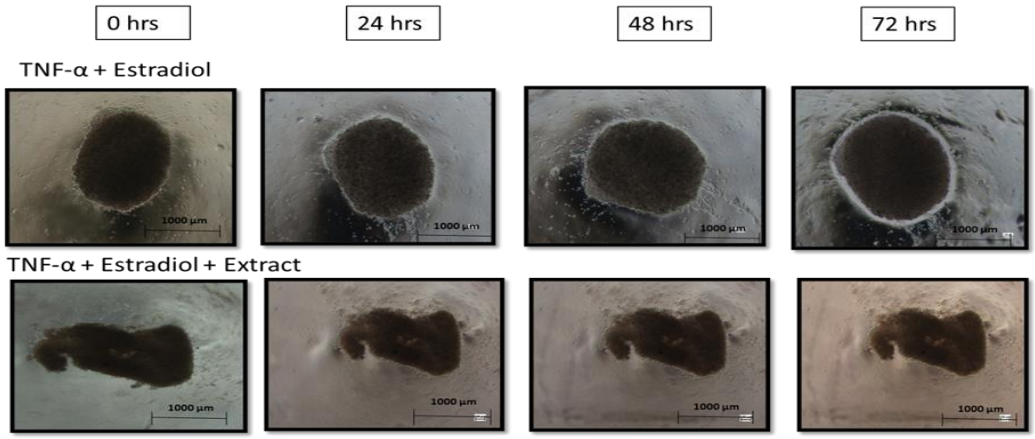
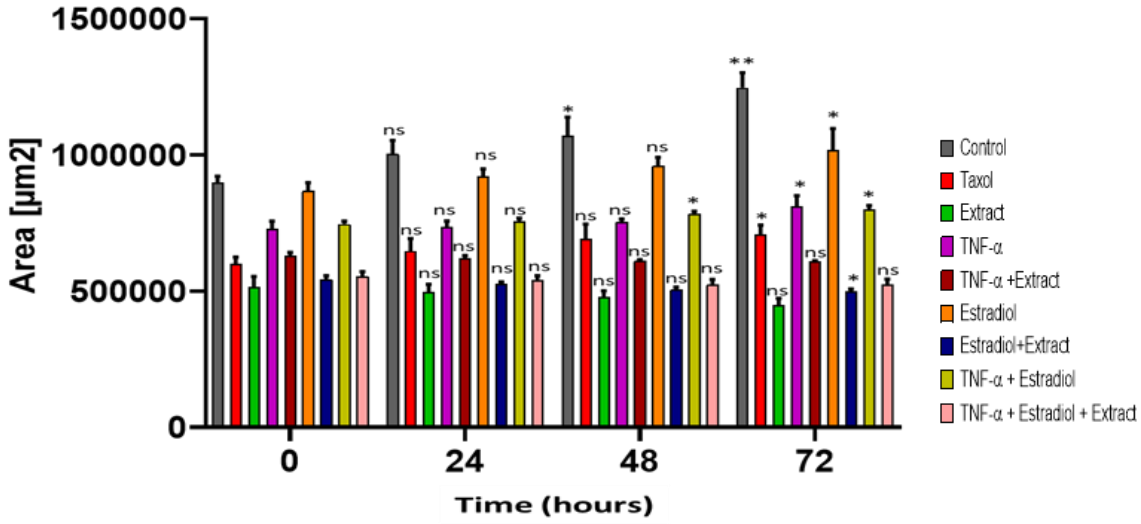


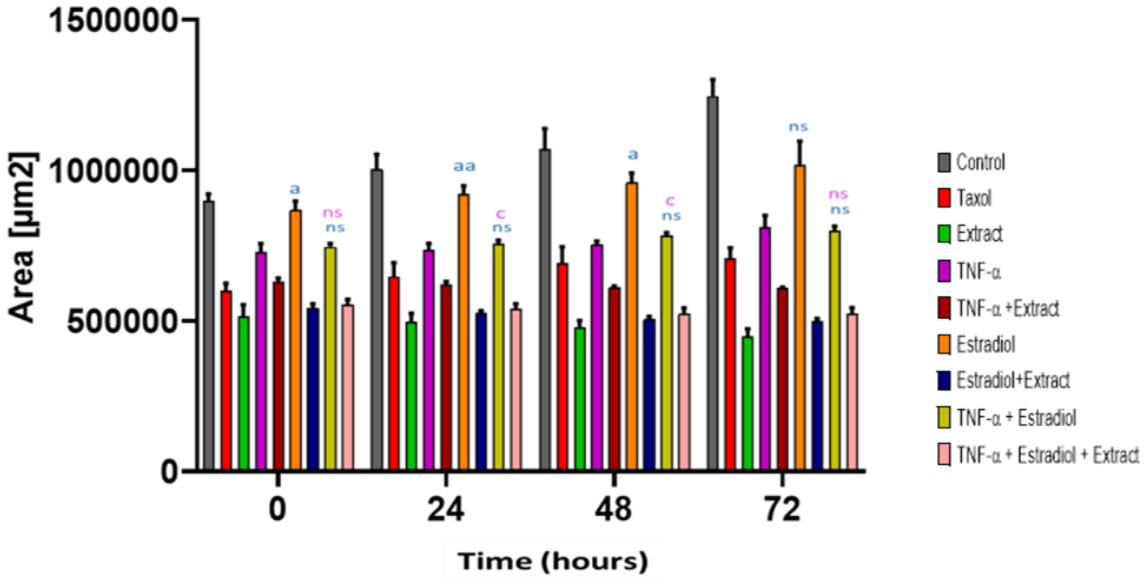
Figure 5.10: The effects of aqueous extract of *Bauhinia variegata* L. leaves on cell proliferation in MCF-7 spheroids. a) presence of aqueous extract and Taxol. b) presence of TNF-α and aqueous extract in MCF-7 spheroids. c) presence of estradiol and aqueous extract in MCF-7 spheroids. d) presence of TNF-α + estradiol and aqueous extract in MCF-7 spheroids.

Difference in the cell area was measured in the all the spheroids groups and graph was plotted.

a)



b)



c)

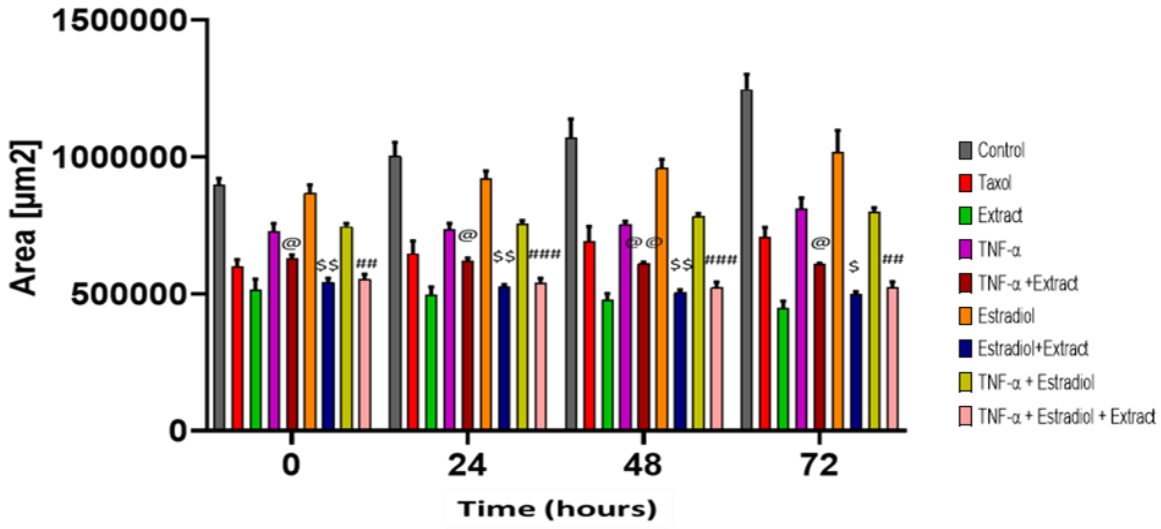


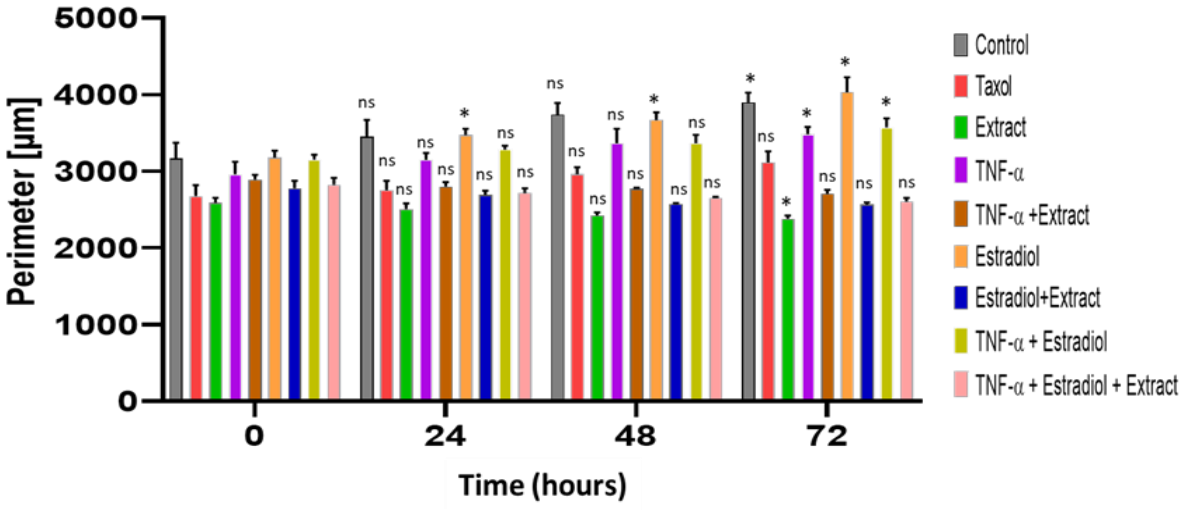
Figure 5.11: Effect of aqueous extract of *Bauhinia variegata* leaves on cell area in absence and presence of TNF-α and estradiol in MCF-7 spheroids. a) shows the difference in the area of the cells at different time interval (24th to 72nd) with respective group at 0th hour. b) shows the difference in area for TNF-α, estradiol and their combination spheroids at 0th, 24th, 48th and 72nd hour. c) shows the difference in the cell invasion area in the spheroids of extract treated the spheroid group with respect to their TNF-α, estradiol and their combination treated spheroids at 0th, 24th, 48th and 72nd hour. In graph a, * and ns indicates comparison with corresponding bar for all the hour groups with respect to 0 hours. In graph b, a stand for comparison between TNF-α and estradiol at same time point, b stand for comparison between TNF-α and TNF-α + estradiol at same time point ns stand for non-significance between the group at the same time point. c and ns stand for comparison between estradiol and TNF-α + estradiol at same time point. In graph c, @ stand for comparison between TNF-α and TNF-α + extract at same time point (0th, 24th, 48th and 72nd hour), $ stand for comparison between estradiol and estradiol + extract at same time point and # stand for comparison between TNF-α + estradiol and TNF-α + estradiol + extract at same time point.

In figure 5.11, the graph (a) shows that there was significant increase in the area of untreated and TNF-α + estradiol spheroids from 48th hour whereas significant increase was observed in presence of Taxol, TNF-α and estradiol spheroids at 72nd hour compared to 0th hour. There was no difference in the area of extract treated, TNF-α + extract and TNF-α + estradiol + extract spheroids from 0th to 72nd hour. No difference in the area of spheroid was observed from 0 to 48th hour estradiol + extract treated MCF-7 cells but a decrease was observed at 72nd hour. This indicates the efficiency of the aqueous extract in restricting the invasiveness in the different treated groups in presence of the extract. In graph b, comparison was made between TNF-α, estradiol

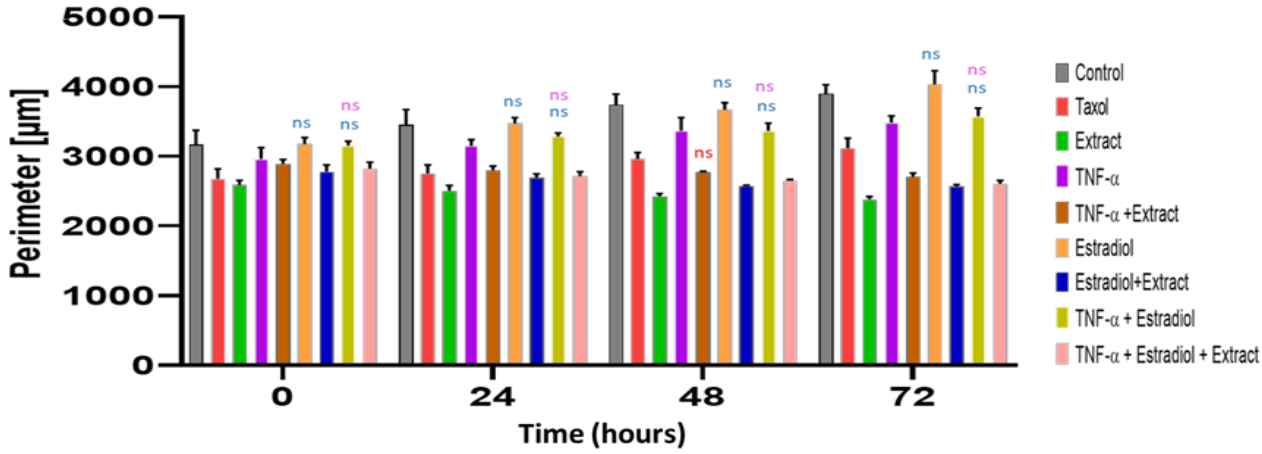
and TNF-α + estradiol spheroids area, it showed significant increase in the area of estradiol spheroids with respect to TNF-α spheroids. There was no difference in the TNF-α and TNF-α + estradiol spheroids. In graph c, at all the time point, significant decrease in the spheroid area was observed in the TNF-α + extract, estradiol + extract and TNF-α + estradiol+ extract treated spheroids with respect to TNF-α, estradiol and TNF-α + estradiol spheroids indicating the inhibitory ability of the aqueous extract of *Bauhinia variegata* leaves.

Difference in the cell perimeter was measured in the all the spheroids groups and graph was plotted.

a



b



c)

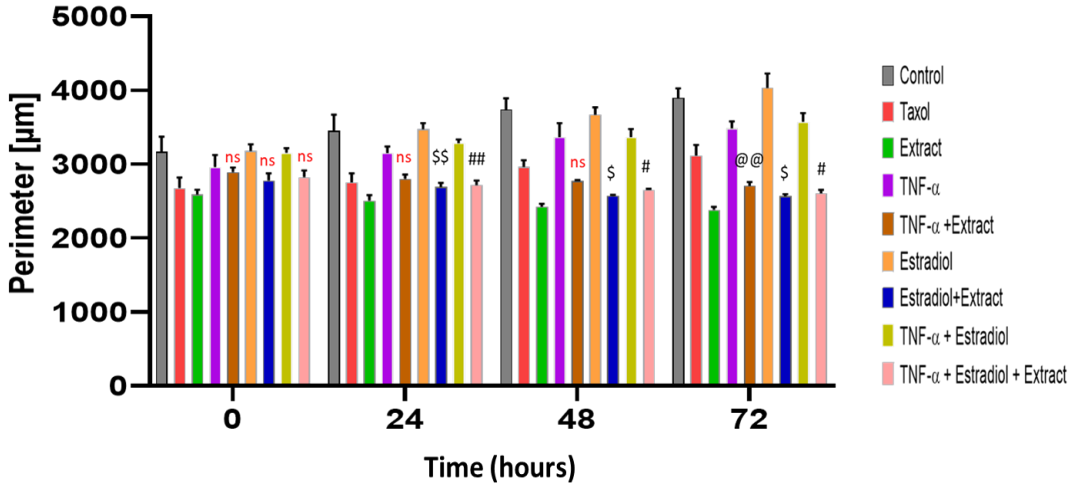


Figure 5.12: The effect of aqueous extract of *Bauhinia variegata* leaves on cell perimeter in absence and presence of TNF-α and estradiol in MCF-7 spheroids. a) shows the difference in the cell perimeter in the treatment groups spheroids at different time interval (24th to 72nd) with respective group at 0th hour. b) shows the difference in the cell perimeter in the spheroids of the TNF-α, estradiol and their combination spheroids at 0th, 24th, 48th and 72nd hour. c) shows the difference in the cell perimeter in the spheroids of extract treated the spheroid group with respect to their TNF-α, estradiol and their combination treated spheroids at 0th, 24th, 48th and 72nd hour. In graph a, * and ns indicates comparison with corresponding bar for all the hour groups with respect to 0 hours. In graph b, a stand for comparison between TNF-α and estradiol at same time point, b stand for comparison between TNF-α and TNF-α + estradiol at same time point ns stand for non-significance between the group at the same time point. c and ns stand for comparison between estradiol and TNF-α + estradiol at same time point. In graph c, @ stand for comparison between TNF-α and TNF-α + extract at same time point (0th, 24th, 48th and 72nd hour), $ stand for comparison between estradiol and estradiol + extract at same time point and # stand for comparison between TNF-α + estradiol and TNF-α + estradiol + extract at same time point.

In figure 5.12, (a) shows no significant increase in the perimeter of untreated spheroids until 72 hours. A significant increase was observed in the perimeter of the estradiol treated group from 0^{th} to 72^{nd} hour. A significant decrease was observed in extract treated spheroids at 72^{nd} hour compared to 0, 24^{th} and 48^{th} hour. There was no significant difference in the perimeter of the all the other groups with respect to 0^{th} hour. In graph b, no significant difference was seen in the perimeter of the TNF-α and estradiol, TNF-α and TNF-α + estradiol, and estradiol and TNF-α + estradiol spheroids at all the time points. In graph c, no significant difference was observed in the TNF-α, estradiol and TNF-α + estradiol group in compared to spheroids exposed to extract in respective groups. A significant decrease was seen in the TNF-α + extract spheroids with respect to TNF-α spheroids at 72^{nd} hour. Significant decrease in the perimeter of the estradiol + extract and TNF-α + estradiol+ extract was observed in compared to estradiol and TNF-α + estradiol spheroids at 24^{th}, 48^{th}, and 72^{nd} hour.

The aqueous extract of *Bauhinia variegata* leaves restricts the cell invasion in MCF-7 spheroids even in the presence of TNF-α and Estradiol. This is the first time that effect of phytocomponents on spheroids of MCF 7 is reported. Henceforth, it can be concluded that the phytocomponents present in the aqueous extract of *Bauhinia variegata* leaves might be having TNF-α modulating ability and anti-invasive properties which should be further explored from the cancer therapeutic point of view.

5.9 Discussion

The study of cell migration and invasion in cancer research is of prime importance as it is the main cause of cancer associated mortality. Matrix metalloproteinases (MMPs) degrade the extracellular matrix which facilitates tumor cell migration and hence contributing towards metastasis. A study on *Bauhinia variegata* candida (Bvc) stem reported that the F3 fraction from ethyl acetate partition inhibited matrix metalloproteinases MMP-2 and MMP-9 activity and decreased HeLa cell migration and invasion. This suggests that FR3 inhibits cell migration of HeLa cells which may be due to presence of phytocomponents with potential anti-migratory and anti-invasive action (K. M. Santos et al., 2018). Another study on *Bauhinia ungulate*, also displayed inhibition of MMP-2 and MMP-9 activity in both hydroalcoholic partition and ethyl acetate partition. They were also found to be rich in phytochemicals like alkaloids and flavonoids which have important pharmacological properties (K. Santos, Nunes, Faria,

Da Silva, & Ribeiro, 2015). One more report from the same group revealed that the ID7 fraction obtained from ethyl acetate partition of *Bauhinia variegata* stem was effective against three cell lines with different hormonal responsiveness BT-20, MCF-7 and MDA-MB-231 breast cancer cells and inhibited metastatic progression *in vitro* and in vivo (Monteiro et al., 2019). The results of present study on migration and invasion assays, showed that the aqueous extract of *Bauhinia variegata* leaves was able to inhibit the migration and invasion in both the breast cancer cell lines MCF-7 and MDA-MB-231. This study also found the presence of alkaloids and flavonoids in aqueous extract of *Bauhinia variegata* leaves (showed in chapter 4). Gunalan et al., 2013 also showed presence of alkaloids and flavonoids. It is suggested that alkaloids and flavonoids are correlated with such anti-cancer activities. Berbamine and despapaverine belonging to alkaloid group were found to be present in aqueous extract of *Bauhinia variegata* leaves. Literature also supports anti-cancer activity of Berbamine and Papaverine (mentioned in chapter 4). Clonogenic ability of both the ER/PR +ve (MCF-7) & ER/PR -ve (MDA-MB-231) cell-lines was decreased when exposed to aqueous extract of *Bauhinia variegata* leaves. Hence the aqueous extract of *Bauhinia variegata* leaves has anti-tumorigenic effect also. Thus, a promising phytocomponent with tumor inhibitory characteristics may be present in the aqueous extract of *Bauhinia variegata* leaves.

Tumor microenvironment of the solid tumor including breast cancer also had high level of TNF-α which promote the tumorigenesis. TNF-α also promotes the migration of both MCF-7 and MDA-MB-231 breast cancer cells. TNF-α can enhance the invasive ability of MCF-7 cells, partly by regulating a series of metastasis related genes (Xiaofeng Chen et al., 2008). There is evidence that indicates that TNF-α is involved in the transformation, proliferation, angiogenesis, invasion and metastasis of many cancers (Y. Wu & Zhou, 2010). TNF-α promoted the migration of both MCF-7 and MDA-MB-231 breast cancer cells is shown by a scratch assay and transwell assay (Wolczyk et al., 2016). Earlier there were reports of TNF-α was down-regulated in MCF-7 cells when treated by E2 for different time periods (Frasor et al., 2003) and also of TNF-α regulating the growth of MCF-7 breast cancer cells through the down-regulation of ERα expression (S. H. Lee & Nam, 2008). It was also found that the TNF- α enhances estrogen-induced cell proliferation of estrogen-dependent breast tumor cells through a complex containing nuclear factor-kappa B (M. F. Rubio et al., 2006). Treatment of

MCF-7, T47D and ZR-75 breast cancer cells with 17-β-estradiol increased TNF-α mRNA and protein expression and secretion(To et al., 2014). This study finds that TNF-α, expressed in ER+ tumour epithelial cells is a tumor-derived factor, and is regulated by 17-β-estradiol. Resistance of the estrogen receptor alpha (ER)-positive, chemosensitive MCF-7 breast cancer cell line to tumor necrosis factor (TNF-α) was associated with loss of ER expression and a multi-drug resistant phenotype. (Antoon et al., 2012). Another study shows that TNF-α together with Estrogen and epidermal growth factor had significant effect on breast cancer cells leading to EMT transition and cell migration (Weitzenfeld et al., 2013). The results of current study showed that the aqueous extract modulates TNF-α induced cell proliferation in both the hormone responsive and triple negative cell-lines and also affects the migration in MCF-7 spheroids.

Spheroid generation by Hanging drop is a simple method which generates tissue-like cellular aggregates. It is used for the cancer research as it shows cell-cell interaction and cell-ECM interactions mimicking physiological conditions similar to in vivo tumor (Teng, 2015). Spheroids or 3D microtissues formed using scaffold-free agarose hydrogels in MCF-7 human breast cancer cells, cells self-organize to form microtissues that contain a luminal space, suggestive of the in vivo structure of the mammary gland and these MCF-7 spheroids are responsive to exposure to 17β-estradiol and showed increased expression of luminal epithelial markers keratin 8 and 19 (Vantangoli, Madnick, Huse, Weston, & Boekelheide, 2015). Similar type of morphology changes like formation of cortical buds, elongated tubular structure and acinar spheroids over the period of time from 5^{th} day of spheroid formation until 30^{th} day was observed (do Amaral, Rezende-Teixeira, Freitas, & Machado-Santelli, 2011).

In present study, cells were migrating from the periphery of the spheroid in the presence of TNF-α and Estradiol when treated independently. Cells when treated in combination of TNF-α and Estradiol, substantial decrease in migration was observed compared to the independent exposure of the TNF-α and Estradiol. But when TNF-α and Estradiol were given along with the extract, there no migration observed. So, the extract is capable of inhibiting the migratory property of the cells. The aqueous extract of *Bauhinia variegata* leaves is efficient in inhibiting the cell migration and invasion of breast cancer cell lines, indicating presence of anti-migratory phytocomponents in the

extract which possess TNF- α modulating properties. To our knowledge till date this is the first study showing inhibitory effect aqueous extract of *Bauhinia variegata* leaves on cell proliferation and cell migration in presence of TNF- α and estradiol on breast cancer cell lines and spheroids.