TURKISH REPUBLIC OF NORTHERN CYPRUS
NEAR EAST UNIVERSITY
INSTITUTE OF HEATLH SCIENCES

EFFECTS OF ASPERULOSIDE MOLECULE ON THE VIABILITY OF MCF-7 AND MDA-MB-231 BREAST CANCER CELL LINES

HASAN AKYIL

MASTER THESIS IN MOLECULAR MEDICINE

THESIS ADVISORS:
Assoc. Prof. MAHMUT ÇERKEZ ERGÖREN, PhD
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2019, NICOSIA

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DECLARATION

I declare that I have no unethical behavior at all stages from the planning of the thesis to the writing, I have obtained all the information in this thesis within the academic and ethical rules, I cited all information and interpretations in the text and added these citations to the references part. I hereby, I did not violate patents and copyrights during the study and writing of this thesis.

HASAN AKYIL

PREFACE

This master thesis is the result of two year of hard work in a highly disiplined working conditions. It has been written to fulfill the graduation requirements of the Molecular Medicine masters course at Near East University, Faculty of Medicine. I was engaged in researching and writing this dissertation from March to November 2019.

I thank my supervisors, Assoc. Prof. Mahmut Çerkez Ergören and Prof. Gamze Mocan for their support as well as Assoc. Prof. Pınar Tulay providing critical and constructive feedback during my research. Additionally I appreciate the cooperative attitude of Hülya Şenol during the research.

I also wish to thank to my family members whose moral support was the most vital thing which made me reach to this point.

HASAN AKYIL

NOVEMBER 2019

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
ATM	Ataxia telangiectasia mutated
Bax	BCL2 Associated X
Bak	Bcl-2 homologous antagonist/killer
BCL-2	B-cell lymphoma 2
BRCA	Breast Cancer gene
BSA	Bovine serum albumin
CCK-8	Cell counting kit 8 (contains WST)
CD11b	Integrin Alpha M
CHEK1	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
CDK4/CDK6	Cyclin dependent kinase 4/6
CK	Cytokeratin
Co-Q	Ubiquinone
DCIS	Ductal Carcinoma In Situ
DNA	Deoxyribonucleic Acid
DMBA	7, 12-Dimethylbenz[a]anthracene
ECM	Extracellular Matrix
EMT	Epithelial-mesenchymal transition
EGFR	Epidermal growth factor receptor
ERa	Estrogen receptor alpha
ERK	Extracellular signal-regulated kinases
EDTA	Ethylenediaminetetraacetic acid

GSH	Glutathione
HDAC2	Histone deacetylase 2
HER2	Human Epidermal Growth Factor Receptor2
HR	Homologous recombination
IL-1	Interleukin 1
JNK	c-Jun N-terminal kinase
LCIS	Lobular carcinoma in situ
Mac-3	Macrophage 3 antigen
MUC4	Mucin 4
NK cell	Natural Killer cell
NF-κB	Nuclear Factor Kappa B
PALB2	Partner and Localizer of the BRCA2
PARP	Poly(ADP-ribose) polymerase
PRE	Progesterone Response Element
PTEN	Phosphotase and tensin homolog
PR-A	Progesterone Receptor-Alpha
PR	Progesterone receptor
PPAR-γ	Peroxisome proliferator-activated receptor gamma

PDE1A	Calcium/calmodulin-dependent3',5'-cyclic nucleotide phosphodiesterase 1A
Rb	Retinoblastoma
ROS	Reactive oxygen species
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
Snail	Zinc finger protein SNAI1
STK11	Serine Threonine Kinase 11
STAT3	Signal transducer and activator of transcription 3
TRPM8	Transient receptor potential cation channel subfamily M (melastatin) member 8
TP53	Tumor protein 53
TGF-B	Transforming Growth Factor Beta
Twist	Twist Family BHLH Transcription Factor
TNBC	Triple Negative Breast Cancer
UK-CGG	UK Cancer Genetics Group
UKGTN	UK Genetic Testing Network
VEGF	Vascular endothelial growth factor
Zeb	Zinc finger E-box-binding homeobox

µl	microliter
µM	micromolar
nM	nanomolar
mM	millimolar

ÖZET

ASPERULOSİD MOLEKÜLÜNÜN MCF-7 VE MDA-MB-231 MEME KANSERİ HÜCRE HATLARININ METABOLİK HIZI VE CANLILIĞI ÜZERİNE ETKİSİ

Hasan Akyıl

MOLEKÜLER TIP

Doç. Dr. Mahmut Çerkez Ergören, Prof. Dr. Gamze Mocan

AMAÇ:

Bu çalışmanın ana amacı saf Asperulosid molekülünün meme kanseri hücre hatları MCF-7 ve MDA-MB-231'nin canlılık ve metabolik hız parametrelerine etkisini araştırıp, meme kanseri farmakolojik tedavi araştırmaları literatürüne katkıda bulunmaktır.

YÖNTEM:

MCF-7 ve MDA-MB-231 meme kanser hücreleri 45ml DMEM, 5ml Fetal Bovin Serumu (%10), 125ml insülin (4g/ml), 0.5ml penisilin streptomisin (%1) içeren solüsyondan her bir flask için 15 ml alınarak T75 flask kültür ortamında çoğaltılmıştır. Hücreler 37˚C, 5% CO2 ortam içeren inkübatörde muhafaza edilmiştir. Medyumlar her hafta gün aşırı tazelenmiştir. Hücreler 80-100% confluent duruma geldiğinde pasajlanmıştır. Hazırlanan asperulosid stok çözeltisinden 50 µM, 25 µM, 10 µM, 1 µM ve 0.5 µM farklı konsantrasyonlar hazırlanmış ve hücre hatlarına eklenmiştir. 24, 48 ve 72 saat sonra asperulosidin hücre canlılığı ve metabolik hızına etkisi TEBU-BIO cell counting kit 8 (Code: 277CK04-11) = QTY: X1 ile ölçülmüştür. GraphPad® Prism software version 8 programı kullanılarak two-way ANOVA ve Tukey çoklu kıyaslama post-hoc analizi gerçekleştirilmiştir.

BULGULAR:

MCF-7 ve MDA-MB-231 meme kanseri hücreleri, farklı konsantrasyonlarda (50 µM, 25 µM, 10 µM, 1 µM, 0.5 µM) asperuloside maruz bırakılarak absorbans değerleri 24 saat, 48 saat ve 72 saatlik etkileşimden sonra ölçülmüştür.

Elde edilen veriler two-way ANOVA metodu ile analiz edilmiş ve hiçbir asperulosid maruziyeti yaşamamış MCF-7 ve MDA-MB-231 kontrol grubu hücrelerinin kuyulardaki canlılık ve metabolik hız parametreleri zaman geçtikçe düşerken, 24 saatlik 50 μM asperulosid tedavisi alan MCF-7 ve MDA-MB-231 ile 48 saatlik 50 μM asperulosid tedavisi alan MDA-MB-231 hücreleri hariç diğer bütün tedavi gruplarında bu parametrelerin aynı plateteki kontrollerine göre daha yüksek olduğu saptanmıştır.
24 saat maruziyette 50 μM tedavi grubu her iki hücre hattında canlılıkta gerilemeye sebep olurken bu etki MCF-7'de daha yüksek seviyede olduğu tespit edilmiştir. Tukey çoklu kıyaslama metoduna göre de 0,0024 P değeri ve -0,6318 ortalama fark ile MCF-7 ve MDA-MB-231 24 saat-50micromolar gruplar arasındaki kıyaslama statistiksel olarak anlamlı bulunmuştur.

SONUÇLAR:

Literatürde Asperuloside maddesinin MCF-7 ve MDA-MB 231 meme kanseri hücreleri üzerine antikanser ve sitotoksisite etkilerini inceleyen çok az sayıda çalışma bulunmaktadır. Yapılan bu çalışma, Putoria calabrica bitkisinden ekstrakte edilen asperulosid maddesinin MCF-7 ve MDA-MB 231 meme kanseri hücreleri üzerine metabolik hız ve canlılık etkilerini inceleyen ilk çalışmadır. Bu nedenle, yapılan çalışmanın literatüre katkı sağlayacağı düşünülmektedir.

Anahtar sözcükler:

MCF-7, MDA-MB231, Meme kanseri, Hücre canlılığı , Asperulosid

SUMMARY

EFFECT OF ASPERULOSIDE MOLECULE ON THE CELLULAR VIABILITY OF BREAST CANCER CELL LINES MCF-7 AND MDA-MB-231

Hasan Akyıl

MOLECULAR MEDICINE

ADVISORS: Assoc. Prof. Mahmut Çerkez Ergören, Prof. Gamze Mocan

AIM:

This study aimed to evaluate the effects of Asperuloside molecule on MCF-7 and MDAMB-231 cells line in vitro.

METHOD:

MCF-7 and MDA-MB-231 cells were grown in T75 flasks with the supplement of 15ml from the suspension of 45ml DMEM/F-12 (1:1) (1X) containing F-12 Nutrient Mixture (Ham) (+) L-Glutamate and (+) 15mM HEPES, 5ml Fetal Bovine Serum (%10), 125 ml insulin human (at 4mg/ml), 0.5ml penicillin streptomycin (%1) and incubated at 37 ˚C and in a 5% CO_2 containing humidified chamber. The medium was refreshed every consecutive day every week. Stock solution was prepared and used to prepare various concentrations of asperuloside as 50 μM, 25 μM, 10 μM, 1 μM and 0.5 μM. Cellular metabolic rate of asperuloside was evaluated by using TEBU-BIO cell counting kit 8 (Code: 277CK04-11) = QTY: X1. IC50 values were analyzed by using GraphPad® Prism software version 8.

RESULTS:

MCF-7 and MDA-MB-231 breast cancer cells were exposed to different concentrations of Asperuloside (50 μM, 25 μM, 10 μM, 1 μM, 0.5 μM) and absorbance values were obtained after 24,48 and 72 hours of molecule-cell interaction by first adding CCK-8 four hours prior to the taking absorbance readings.

Two-way ANOVA analysis was carried out and it has been observed that control cells' metabolic rate which were not exposed to the Asperuloside molecule declined as time passed.

Except 24-hour 50μM asperuloside treated MCF-7, MDA-MB-231 cell groups and 48 hour 50μM asperuloside treated MDA-MB-231 cell groups, all the other treatment groups were actually more viable and they were bearing a higher metabolic rate according to the their plates' control groups.

After 24-hours of exposure, 50 μM Asperuloside treated groups in both cell lines showed a significant decline at metabolic rate and viability with respect to the control group of that specific plate while this effect of 50μM asperuloside was more pronounced in MCF-7 cells.

Additionally, according to the Tukey's multiple comparison test post-hoc analysis after ANOVA, the comparison between MCF-7 and MDA-MB-231 24 hours results was found statistically significant with 0.0024 P value and -0.6318 mean difference value.

CONCLUSION:

To our knowledge, there is no study investigating anticancer and cytotoxicity effect of Asperuloside on the MCF-7 and MDA-B 231 breast cancer cells in the English literature. Thus, this is the first study which examined the metabolic effects of asperuloside extracted from Putoria Calabrica plant on MCF-7 and MDA-MB 231 breast cancer cell lines. This contributes to the significance of the study. Therefore, this study is thought to contribute to the literature.

Keywords:

MCF-7, MDA-MB231, Breast Cancer, Cellular viability, Asperuloside

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CHAPTER 1. INTRODUCTION AND AIM OF THE THESIS

1.1. INTRODUCTION

Cancer is the second leading cause of death worldwide due to the uncontrolled cell division leading to disruption of normal tissues and organ architecture. It occurs because of simply some cells within the organism gain ability to divide autonomously which means that they lose their social behaviour and they divide uncontrollably without consent of other cells within the original tissue. This autonomous behaviour is the consequence of genetic abnormality which cancer cells bear either sporadically or hereditary (Hanahan and Weinberg, 2011; Hanahan and Weinberg, 2000).

Due to the metazoan cells' endowment with autonomy and great versatility including the presence of same Deoxyribonucleic Acid (DNA) in every cell but different function and morphology of many different cell types, the evolution of extraordinary anatomical designs had been possible.

The ability of numerous cells in a mammal to grow and proliferate long after organism has reached to adulthood is vital for the replacement of dead cells and repair of wounds among many other processes.

The cells must gain access to a certain level of autonomy however, they must be incapable of reaching to the genomic information which is normally withheld from them due to cell and tissue type limitations. Pathologies violating this basic rule would create distortions in the normal tissue architecture during embryonic and cellular developmental stages as seen in teratomas (Peterson et al., 2012).

Moreover, the genomic information that is stored in the form of nucleotide sequence, histone and DNA marks are subject to change in response to environmental stimuli that can eventually form incompatible cells of which proliferative cellular pathways are overactive that makes them selfish in addition to being against to collaboration with other cells for the health of tissue and whole organism. This shows itself most as disruption of mechanical barriers (basement membrane in cancers of

epithelial origin) to invade more physical space and energy consumption imbalances within cell types in a tissue (Weinberg, 2014).

Cancers seem to occur progressively and they follow a sequence of deterioration and deviation from the normal cellular morphology and physiology. The order although not proven, starts with a normal healthy cell progressing to hyperplastic, dysplastic, neoplastic and finally to a metastatic cell (Schedin and Elias, 2004).

Breast cancer may develop from normal ductal breast epithelial cells that evolve through atypical hyperplasia (and eventually dysplasia), Ductal Carcinoma In Situ (DCIS) and invasive breast cancer. Multiple molecular alterations occur during this process, involving genetic and epigenetic alterations in precursor and neoplastic cells. Genetic predisposition can contribute to this process, but early molecular alterations (preceding DCIS) have not been well characterized(Yu and Lu, 2017).

1.2. HALLMARKS OF CANCER

1.2.1. Selective Growth and Proliferative Advantage

Tissue homeostasis is vital for the living organisms and it is maintained by the cellular interaction within the tissue that leads to tightly regulated cell cycle within individual cells. It is known that at least one part of this proliferative system is non-functional or in other words over-functional in cancer or pre-cancer cells. This can be due to an overabundance of growth signals (extracellular factor) or the presence of mutated receptors and cytosolic signalling molecules (intracellular factors) that enables the formation of the continuously active proliferative system. The overabundance of growth signals, unfortunately, is hard to be examined experimentally since in today's technology the spatiotemporal paracrine signalling cannot be worked on due to inability to mimic right extracellular matrix and tumour microenvironment experimentally *in vitro* (Antoni et al., 2015). It requires taking a more dynamic picture of the tumour physiology. However intracellular factors can, fortunately, be studied *in vitro* studies in connection with procedures scanning the whole genome and epigenome for so-called ''onco-mutations''. For instance, the *KRAS* gene encodes a

protein called K-Ras that acts as a signal transducer in signaling pathway known as the RAS/MAPK pathway. It works within the plasma membrane and nuclear membrane to relay cellular growth, proliferation, differentiation and maturation signals ("the *KRAS* gene - Genetics Home Reference - NIH"). The mutations in the DNA leading continuously active intracellular signalling proteins such as K-RAS in colon cancer is most of the time more prevalent in cancer cells. These mutations enable them to become self-sufficient which is more important in tumour growth (Hajdúch et al., 2010).

1.2.2. Loss of Negative Feedback Mechanisms (Brakes of Cellular Proliferation)

Eukaryotic cells have many auto-control mechanisms to restrict the sustained activation of cellular proliferation pathways thus these must be overcome by cells intended to transform into a cancerous cell. For instance, in the molecular level RAS oncoprotein has sustained activity partly due to activating mutations but these are not enough because even a molecule like RAS has its own auto-control and inhibitory domain (GTPase domain) which acts upon negative feedback (Weinberg, 2014).

1.2.3. Resistance to Development of Senescence in Case of Excessive Proliferative Signalling

The process of cellular senescence was first described in an influential study by Hayflick and Moorhead in 1961 based on their observation of normal human fibroblasts which had entered a state of irreversible growth arrest in the cell culture after a certain time of passaging (Hayflick and Moorhead, 1961). However, it was realized that cancer cells did not enter this growth arrest state and proliferated indefinitely.

There is a strong correlation between presence of excessive proliferative signaling and induction of senescence state for healthy mammalian cells(Zhu et al., 1998; Lin et al., 1998; Serrano et al., 1997).

However, the amplitude of proliferation signals is critically important to be below the threshold level in a cancer cell not to activate cellular protective mechanisms. But most of the time the senescence does occur in cancer cells and after that cancer cells sort out this problem by upregulating telomerase (Lowe et al., 2004; Degerman et al., 2010). Nevertheless, if these stress response mechanisms leading to senescence are not in a state of capability to detect excessive signaling somehow due to mutations affecting proteins involved in these mechanisms for instance due to a non fuctional p53 protein, cancer can evolve very rapidly even without a need for *telomerase* upregulation until their telomeres lead to an untolerable genomic instability. However, it had been demonstrated that telomerase null mice which also bears the Tumor protein 53 *(TP53)* gene mutation are highly prone to suffer from chromosomal non-reciprocal translocations and epithelial cancers so in the initial phases of cancer progression actually telomere shortening that leads to potential genomic instability might even be useful for further cancer progression to happen if it is no detected (Artandi et al., 2000).

Telomere shortening, the epigenetic derepression of the *INK4a/ARF* locus, and DNA damage have been shown to trigger irreversible growth arrest called senescence. Together these mechanisms may limit excessive or aberrant cellular proliferation protecting the cell against the development of cancer. These are also known as Hayflick factors which are all well appreciated now —telomere shortening, accumulation of DNA damage, and derepression of the INK4a/ARF locus—are summarized together with their main effectors, the tumor suppressors p53 and retinoblastoma (Rb) (Collado et al., 2007).

1.2.4. Avoiding the Growth Suppression Effects of Tumor Suppressors

The cellular environment consists of an internal clock which collects all the extracellular messages and signals, integrate them and give an output to lead the cell to continue into cell cycle or to stop the cell cycle processes until the conditions are right. This internal clock is named as protein RB. Unlike the auto-inhibitory domain of RAS, it is not regulated by negative feedback mechanisms. Its mechanism of action

is quite complex and directly dependent on external cues coming to the cell of interest and can be studied quantitatively (Zerjatke et al., 2017).

On the other hand, there is a more important protein molecule called Tumor protein 53 (p53) that imports cellular stress and abnormality signals from mediator molecules which actually detect mutations, oxidative stress, nucleotide depletion or suboptimal oxygenation and glucose levels and report to the p53 molecule for its effector functions such as further halting the cell cycle process and in case of continuous stress or irreparable genomic damage leading the cell to programmed cell death (Weinberg, 2014).

However, still, the absence of these two molecules cannot most of the time form cancer on its own as demonstrated by chimeric mouse experiments in which mouses contain a group of cells lacking these molecules do not develop cancer until late adulthood. This actually demonstrates the multi-stage nature of cancer development which takes years to accomplish all of the hallmarks (Williams et al., 1994).

1.2.5. Loss of Contact Inhibition

In the 1950s, Michael Abercrombie who was interested in the social behavior of cells had studied the contact inhibition of locomotion phenomena with the migrating chick heart embryonic fibroblasts and found out that their mean velocity had been inversely proportional to the density of their contacts with the other fibroblasts. Moreover, his observations had enabled him to discover the fact that not only velocity but directionality of the cells were also affected by the amount of contacts they made. In vitro cell culture studies had obviously demonstrated that reaching confluency in the cell culture leads the cells to enter senescence in response to contact inhibition phenomena. However, the same does not apply to cancerous cell lines indicating that violating adherence to normal cellular and tissue architecture and structure is one of the hallmarks of cancer development *in vivo* (Roycroft and Mayor, 2015).

There are multiple stages of contact inhibition of locomotion. A bipolar nature of migration is exhibited by freely moving cells. A) Ras-related C3 botulinum toxin

substrate 1 (Rac1) activity in one edge evokes a polarised form of protrusion. Microtubules orchestrate the stabilisation of cells during directional migration. Focal adhesions generate the mechanical forces while cells migrating along a substrate. B) Initially cells approach to each other and physical connection is established: the lamellae of the colliding cells overlap and cell–cell adhesions form between the two cells. The cytoskeletons of the colliding cells unite. C) Once the contact is achieved, the protrusive activity is abolished: Rac1 activity is replaced by Ras homolog gene family, member A(RhoA) activity at the contact site which reverses the approaching process and leads to moving away process. D) Rac1 activity in the free edge away from the contact leads repolarisation of cells and this supports the cells to protrude in a new direction. Focal adhesions again take part in the same role to stabilize new movement. E) The new migration events happen to relocalize after the cell–cell adhesions disassemble(Roycroft and Mayor, 2015).

Remodelling and Reorganizing the Transforming Growth Factor Beta (TGF-B) pathway

TGF-B pathway physiologically modulates the rate of proliferation but in cancer cells dysregulation for proliferative advantage is one of the usual steps seen after loss of contact inhibition to promote epithelial-mesenchymal transition (Hanahan and Weinberg, 2011; Hanahan and Weinberg, 2000).

1.2.6. Resisting to Induction of Programmed Cell Death

During the normal course of tumorigenesis, the cancer cells are exposed to many external and internal stresses for instance chemotherapy and activated intracellular cell death mechanisms due to genotoxicity they bear. Programmed cell death, apoptosis, is expected to act as a barrier to cancer progression and it has two different stages (extrinsic vs intrinsic) both culminating in cell death with the same mechanism (activating caspase8 and 9) (Mishra et al., 2018).

The extrinsic pathway is more prone to corruption in cancer cells thus the role of intrinsic pathway is more vital than the extrinsic pathway for the death of cancer cells.

In the intrinsic pathway, the balance of different functioning molecules is the determining factor whether the cell will undergo apoptosis or not eventually. The B-cell lymphoma 2 (BCL-2) family proteins are involved, some contributing as pro-apoptotic others anti-apoptotic factors when they are activated in the cellular environment. Briefly, the pro-apoptotic molecules such as BCL2 Associated X (Bax) and Bcl-2 homologous antagonist/killer (Bak), once get rid of the inhibition of anti-apoptotic molecules, disrupt the mitochondrial outer membrane which in turn releases the pro-apoptotic pathway upstream molecule, the cytochrome c (Redza-Dutordoir and Averill-Bates, 2016).

Some pathways such as PI3K-AKT-Mtor pathways which are stimulated by survival signals trigger apoptosis as well as autophagy when they do not get enough stimulation. Autophagy is a double-edged sword because it can both kill the cancer cells and promote their survival in different circumstances. It exceeds the basal level in nutrient deprivation conditions and intracellular organelles are broken down to provide nutrient to the cells. Cancer cells are almost always exposed to nutrient deprivation stress and this pathway can provide nutrients needed for cell growth and proliferation however in some situations old cells which have potential to become cancer cells die or the onco-molecules in cancer cells are recycled in the autophagy process and the overall rate of tumorigenesis is decreased (Apel et al., 2009; Isaka et al., 2017). There is another form of cell death called necrosis which can promote tumorigenesis more than other forms of cell death by promoting inflammation, unlike apoptosis and autophagy. There is ample evidence showing inflammation is the facilitating factor for tumour cells to fulfil all the hallmarks of cancer and in addition to the inflammation creating property of necrotic cells, they also release IL-1 which stimulates nearby cells to proliferate possibly due to wound healing reflex of the biological organisms although according to recent evidence necrosis sometimes can occur due to underlying genetic basis in individual cells (Weinberg, 2014).

1.2.7. Evading from Cellular Senescence and Replicative Crisis

Normal cells have two barriers that make them stay away from immortalization that is a characteristic of cancer cell lines. One is shortened telomere length which is associated with getting into cellular senescence after a certain number of cell divisions in both the parental cell and its progeny, the other factor is inactivated telomerase enzyme in the fully differentiated cells. However, in some cancer cells either telomerase is still active or HR (homologous recombination) mechanism is dysregulated to provide the cell with a continuous proliferation capability called immortalization. Telomerase lengthens the telomeres thus the hexanucleotide sequences continue to prevent genomic damage such as loss of chromosomal DNA or chromosome fusions.

Inappropriate homologous recombination is involved if the telomerase cannot be activated this is also a double-edged sword because according to recent evidence it may lead to genomic instability phenomena. While repairing damaged DNA when the telomeres are worn out it can cause more somatic mutations in the genome which can contribute clonal evolution of cancer. Additionally, it prevents programmed cell death to occur because it prevents dicentric chromosomal aberrations to be formed which are too far from being tolerable even for cancer cells.

1.2.8. Formation of New Vasculature for the Needs of Tumor

Sprouting is the main mechanism that contributes to tumour vasculature. It is briefly the formation of new blood vessels from existing quiescent vessels. Normally during embryogenesis, the blood vessel formation is very active, later all the formed vessels become quiescent unless the person is passing through female reproductive cycling or has wounds to be healed in normal physiological conditions.

All the tumour vasculature formation serves to the same rationale as the normal vasculature formation which is the disposal of metabolic wastes and getting adequate nutrient in addition to enough oxygen for the development of detectable tumour mass (Forster et al., 2017).

1.2.9. Metastasis Enabling Evolution

For cancer cells to be able to metastasize, it requires them to change their morphological appearance in addition to their way of interaction with the Extracellular Matrix (ECM) and nearby neighbouring cells. The strongest evidence that is related to these changes has come from molecular studies examining the presence or absence of E-cadherin molecule. Most of the time metastatic carcinoma cells are found to have lost their E-cadherin expression, a vital molecule in the cell to cell adhesion. N-Cadherin, another cadherin molecule however is found to be increased in cellular expression levels which is associated with increased cellular motility. This adhesion molecule normally encoded only in embryogenesis and inflammation to promote cellular migration, are most of the time found to be upregulated in metastatic carcinoma cells (Hazan et al., 2004; Onder et al., 2008).

However, the proportion of adhesion molecules to each other cannot explain the invasion-metastasis cascade on their own. The Epithelial-Mesenchymal Transition (EMT) program and pleiotropically acting transcription factors such as Zinc finger protein SNAI1 (Snail), Twist Family BHLH Transcription Factor (Twist) and Zinc finger E-box-binding homeobox (Zeb) are critical for the metastatic behaviour of especially epithelial cancer cells to appear. Their downstream effects include inducing morphological changes for instance from polygonal epithelial to spindly/fibroblastic morphology, matrix-degrading enzyme expression and secretion or resistance to apoptosis (Petrova et al., 2016).

Moreover, it has been demonstrated that only the cancer cells residing at the edge of the invasive margin of carcinoma but not the inner cancer cells undergo to EMT strongly suggesting the stimulative influence of tumour microenvironment (tumor-associated stromal cells) on cancer cells (Liu et al., 2016).

1.2.10. Evading Immune Destruction

The immune system is a natural barrier to the virus-induced cancers by eliminating virally infected cells from the body. However, in recent years especially the studies öwith genetically engineered mice have shown that at least some of the non-viral induced cancers are vulnerable to immune destruction and in case of immunocompromised organisms, they are prone to develop at a higher rate so incidence increases as the immune system surveillance decrease. CD8+ and CD4+ T cells along with Natural Killer (NK) cells were proved to be vital for tumour surveillance. Several transplantation experiments have shown that immunoediting causes selective growth of less immunogenic cancer cells in an immunocompetent organism and these less immunogenic tumour masses when transplanted to other organisms either immunocompetent or immunodeficient the secondary tumour masses almost always form however the tumour transplants derived from immunodeficient organisms cannot form secondary tumour masses in the immunocompetent organisms (Caligiuri, 2005; Kim, 2007; Teng et al., 2008; Smyth and Swann, 2007).

Moreover, in immunocompromised humans, transplantation of organs can induce the formation of tumour which had never appeared in the healthy immunocompetent donors(Hanahan and Weinberg, 2011; Hanahan and Weinberg, 2000).

1.3. BREAST CANCER

Breast cancer is the cancer of breast tissue cells. As there are several cell types forming breast tissue and many kinds of mutations they might bear, it is no surprise that there are many kinds of breast cancer classification schemes. According to breast tissue cell type from which cancer is originated (histological origin) and also according to its invasiveness potential (tumor stage), cellular differentiation status (grade) along with its clinical symptoms, there are 7 breast cancer types namely Angiosarcoma, DCIS, Inflammatory breast cancer, Invasive lobular carcinoma, Lobular carcinoma in situ (LCIS), Paget's disease of the breast and Phyllodes tumor. But, the cells which

cover the lining of milk ducts and lobules are the most susceptible ones and most of the time they are the origin of breast cancer ("Types of Breast Cancer | Different Breast Cancer Types", 2019 ; Calhoun et al., 2014; Dillon et al., 2014; Esteva and Gutiérrez, 2014; Nora, 2014; Overmeyer and Pierce, 2014).

Symptoms are specific to the kind of breast cancer. Change in the shape, size and skin of the whole breast or part of the breast is commonly encountered regardless of its subtype however, each subtype has also its specific symptoms such as inflammated orange colored and thickened skin seen in many cases of inflammatory breast cancer.

In developed countries, the 50's is the stage of life for women with the highest breast cancer frequency density. However, in contrast to developed countries, the women of developing country origin tend to suffer from this disease at earlier stage of their life with generally a worse prognosis (Anders et al., 2009; Hussein et al., 2013).

Hormones which are intercellular lipophilic signaling agents have significant effects on progression of this cancer along with some predisposing mutations such as the Breast Cancer Asssociate (*BRCA)* gene mutations that limits DNA repair capacity of the breast tissue. Oral contraceptives are proved to increase the risk significantly in women according to very recent studies even with modern low dose formulations (Busund et al., 2018; Morch et al., 2017). Moreover, it should also be noted that prevalence of breast cancer among pre-menouposal young women is much less than the one for post menouposal women most probably due to duration of hormonal exposure is less in the young pre-menoupousal group. While for the post-menouposal women the hormone replacement threapy has been associated with decrease in negative after-menopause side effects, there are some reports indicating an increased risk of breast cancer in this group (Rossouw et al., 2002). However, recent research indicates mixed results depending on the type of hormone replacement and interval between menopause and HRT and route of administration (Letendre and Lopes, 2012).

The main driver of progression is environmental genotoxic factors such as radiation or industrial and agricultural chemicals of which exposure has direct carcinogenic impact on the breast tissue (Hiatt and Brody, 2018).

1.3.1. Prevalence of Breast Cancer

Among women patients, breast cancer is one of the most prevalent types of cancer with a rate of 81.7 women out of 100,000 women in Cyprus in compliance with age-standardised rate classification system ("Breast cancer statistics", 2019). About one-quarter of all new cancer cases diagnosed in women worldwide is comprised of breast cancer cases and there were more than two million new cases worldwide in 2018. With regards to mortality rate, breast cancer is one of the major causes of cancer death in women (15.0%) ("Breast cancer statistics", 2019).

In the United States, while 1 in 8 women is expected to suffer from invasive breast cancer in the course of their lifetime, the incidence rate drops notably for men and 1 in 1000 men is expected to develop invasive breast cancer during their lifetime (2019).

In women, until the beginning of the menopausal period, each decade doubles the risk of breast cancer morbidity which reaches the plateau in this period in which there is an overall decrease in the female reproductive hormones. There is only a slight increase in morbidity risk after menopausal period. However, for women, post-menopausal period has still been the highest risk bearing period of their life in terms of breast cancer morbidity. Moreover, statistical data also indicates that percentage of deaths due to breast cancer peaks in this post-menopasusal period thus the prognosis for post-menopausal period breast cancer diagnosis is poor however still not poorer than the pre-menopausal period diagnosis if one takes the number of diagnosis per death ratio for both periods. Interestingly, the seemingly hormone-independent breast cancers are affected more by the menopausal period in terms of the incidence rates in women suggesting an indirect relationship with sex hormones and so-called hormone-independent breast cancer subtypes("Breast cancer statistics", 2019).

It has been proven that the universal biological reality of the breast cancer disease makes women more vulnerable to breast cancer diagnosis as their age increases until the menopausal period regardless of their genetic origin.

Studies of women who migrate from areas of low risk to areas of high risk indicate that after one or two generations their breast cancer morbidity rate is almost equalized

to the local women population showing that in addition to possible genetic aetiology of the cases, the epigenetic or environmental effects are also vital and contributes significantly to the incidence rates (Mccredie, 1998; Calle et al., 1997).

There are three types of risk factors indicated in the table below that can contribute to the development of all kinds of cancer. These are modifiable, unmodifiable and partially modifiable risk factors (Table 1.1) (Wu et al., 2018).

INTRINSIC RISK FACTORS	**NON-INTRINSIC RISK FACTORS**	
Random errors in DNA replication	Endogenous risk factors 1. Biologic aging 2. Genetic susceptibility 3. DNA repair machinery 4. Hormones 5. Growth factors 6. Inflammation etc…	Exogenous Risk Factors 1. Radiation 2. Chemical Carcinogens 3. Tumor causing viruses 4. Bad lifestyles such as smoking, lack of exercise, nutrient imbalance etc.
UNMODIFIABLE	PARTIALLY MODIFIABLE	MODIFIABLE

Table 1.1 demonstrates some of the potential biological and non-biological man-made contributors to the breast cancer morbidity risk. Biological factors are divided into unmodifiable and partially modifiable risk factors. Unmodifiable ones include purely randomly occuring genetic errors accumulating in lifetime while partially modifiable ones include factors occurring as a result of harmonization of genetic predisposition with epigenetic factors. Finally there are some possibly avoidable factors which acts purely in an exogenous manner (Adapted from Wu et al., 2018).

1.3.2. Breast Cancer Types

1.3.2.1. Somatic Cancer

Due to exposure to environmental exogenous carcinogens such as artificial hormones in food and chemicals in tobacco, the genome of somatic cells are vulnerable to undergoing mutations during the lifetime of the person. Some of these mutations do have the ability to cause somatic breast cancer and due to lack of those mutations in the germ cells, these patients have no responsibility for the next generations' breast cancer cases thus most of the time, somatic breast cancer cases are not accumulated in the families. However one should be cautious to say this because sometimes a multifactorial hereditary breast cancer case might seem like a pure somatic breast cancer case due to occurence of second hit in a critical gene such as *Tp53* or *BRCA* only in breast tissue cells and absence of other breast cancer cases in the family ("Breast cancer", 2019).

1.3.2.2. Hereditary Breast Cancer

In some families, the breast cancer diagnosis frequency density is higher than expected according to the other families in the population. This is most of the time due to the presence of critical gene mutations in the genome of the family. These intrinsic gene mutations pass from generation to generation and make the family members more susceptible to the environmental carcinogens thus they are prone to develop breast cancer along with some other related cancer types such as ovary and pancreatic cancer.

It is the era of genetic testing for hereditary breast cancer predisposition among women coming from families in which breast cancer incidence rate is higher relative to the population incidence rate. This helps in taking preventive measures. Moreover, early-onset cancer appearing at a younger age than expected and multiple cancers in the same person are also alarming factors which indicate the necessity of genetic testing for adjustment of appropriate treatment according to its molecular etiology for better prognosis. In patients with Ashkenazi Jewish origin regardless of

age and/or who have relative with male breast cancer are advised to undergo genetic screening and test according to guidelines (2019; Hampel et al., 2014; Robson et al., 2015). A gene panel is a test that checks for multiple genes at once for cancer-associated mutations. For instance, for BRCA genes mutations which have primary degree of breast cancer association, a gene panel reveals whether they are present in a person without the need for sequencing whole genome of that person (King et al., 2003; Rutgers et al., 2019).

Actually the criteria for gene selection for different gene panels might not match with individual person's needs. For instance, national health systems most of the time do not take care the needs of minorities living in the population who have different genomic background and daily habits. Even different laboratories have different gene panels with different measures to assess the risk factors. According to UK Cancer Genetics Group (UK-CGG) supported by the UK Genetic Testing Network (UKGTN), *ATM, BRCA1, BRCA2, CHEK2, PALB2, PTEN, STK11, TP53* genes are significant ones in breast cancer (Taylor et al., 2018).

However, there are many discrepancies with regards to use of these gene panels. Most of the genes used in gene panels are not in a strong association with the breast cancer incidence. Moreover epigenetical determinants such as environment, race and diet certainly have an impact whether any found risk factor is actually a risk factor for the specific person coming from a specific genetic background and culture. No one size fits all can be applied in these investigations since people coming from different genetic backgrounds may have different levels of vulnerability to the development of specific cancer even if they bear same mutation in their genome. Unfortunately, there is no standardized method to evaluate contribution of these epigenetical determinants while interpreting the gene panel results.

Moreover, it is true that germline mutations pass from one generation to another generation, however, it is not an obligation for a person to suffer from a cancer if he or she carries a critical gene mutation which can contribute to development of breast cancer such as *BRCA* genes mutation just because he or she inherited it from

his or her parents and bear it in all of the cells. One can certainly bear such a mutation in all cells and do not develop any cancer during his or her lifetime due to the absence of second hit for that gene. Thus there is no a strict line seperating the hereditary and somatic breast cancer.

1.3.3. Molecular Etiology of Breast Cancer

Many classification schemes have been proposed for the molecular etiologic subclassification of breast cancer. These are established according to gene expression profiles, proteomics, DNA copy number, alteration and chromosomal changes, mutation status, methylation and microRNAs of breast cancer cells and most of the time immunohistochemical methods have been used to identify to which subcategory of a specific breast cancer (Rakha and Green, 2017).

Most widely accepted molecular subtypes of breast cancer consist of nice categories namely Luminal A, B, Basal-like, Triple Negative Breast Cancer (TNBC), Human epidermal growth factor receptor (HER2) enriched, Normal-like, claudin low, molecular apocrine and interferon (Godone et al., 2018).

Both Luminal A and Luminal B type breast cancers express high levels of estrogen and progesterone intracellular hormone receptors. Luminal B can also express the HER2 receptor although this is not a strict requirement. A more important and distinctive feature of luminal B subtype is the highly elevated expression of ki-67 protein relative to luminal A. This microRNA is the key factor that predisposes luminal B to grow slightly faster than A.

TNBC has neither hormone receptor nor *HER2* expression and women with the Breast Cancer 1 *(BRCA1)* gene mutation are more susceptible to this type of breast cancer.

Basal-like is a subtype of triple negative breast cancer which has basal epithelial cell properties cytokeratins (CK5/6, CK14 and CK17), vimentin, and epidermal growth factor receptor (EGFR)

HER-2 enriched breast tumours exhibit tumour progression only via overexpression of Her2 so their growth fuel is supplied by human epidermal growth factor thus inhibition of this extracellular receptor by small molecule inhibitors most of the time proved to be efficacious (Meric-Bernstam et al., 2019). The *HER2* proto-oncogene encodes a transmembrane glycoprotein of 185 kDa (p185(HER2)) which exhibits tyrosine kinase activity (Akiyama et al., 1986). Amplification of the *HER2* gene and overexpression of its product induce cell transformation (King et al., 1985). It has been suggested that it amplifies the signal provided by other receptors of the HER family by heterodimerizing with them however it's specific ligand couldn't be identified yet (Burgess et al., 2003). HER1, HER3, and HER4 are activated by EGF or heregulin leading to heterodimerization with HER2 thus HER2 activation(Bazley and Gullick, 2005). HER2 overexpression makes the tumors vulnerable to doxorubicin, to cyclophosphamide, methotrexate, and fluorouracil (CMF), and to paclitaxel, whereas tamoxifen was proved to be ineffective in the best scenario with the possibility of having harming effects to patients (Cui et al., 2012).

Normal like breast tumours are almost exactly the same as luminal A subtype however their clinical prognosis is slightly worse than the luminal A type (Lal et al., 2017; Perou, 2011).

1.4. Therapeutic Targets in Breast Cancer

1.4.1. Nuclear Estrogen Receptor and its ligand 17B-Estradiol

As important hormone receptors in sexual maturation and gestation, once they are bound to their ligand 17B-Estradiol they form a complex which would be later classified as DNA-binding transcription factor after the complex has travelled to the nucleus affecting the transcription of several genes.

The receptor can be inhibited by small molecule inhibitors such as tamoxifen which is classified under selective-estrogen receptor modulators. However, because of estrogen hormone's tissue-specific actions resulting due to the differing ratio of estrogen receptor subtypes in different tissues, tamoxifen increases the risk of

uterine cancer slightly by its estrogen agonistic actions in the cells of the uterine. For instance, the expression of *ERa* in breast tumour cells is assumed to be a good indicator for endocrine therapy while its absence proved to be a factor in unresponsiveness to this therapy along with increased invasive potential in some studies (Hu et al., 2015; Osborne and Schiff, 2011).

1.4.2. Intracellular Progesterone Receptor

Although intracellular progesterone receptor-progesterone hormone interaction has more pronounced effects in breast cancer development, the role of the non-genomic rapid action of progesterone has also been proposed in breast cancer via extracellular plasma receptors such as growth factor receptors, neurotransmitter receptors and albeit plasma progesterone receptor (PR). The use of progesterone conjugated to bovine serum albumin (BSA) that cannot pass into the cell has proved its non-classical effects and some binding studies also confirm this non-genomic rapid actions in breast cancer development (Gellersen et al., 2009; Boonyaratanakornkit et al., 2018)

Progesterone is a steroid hormone and its production along with secretion take place mainly in ovaries, placenta, adrenal glands, and testis. On the other hand, it's *de novo* derivation from cholesterol or from circulating pregnenolone is carried out in the brain, spinal cord and peripheral nerves (Diotel et al., 2018).

Progesterone receptor is a protein molecule which responds to its ligand progesterone hormone that is involved in various processes from ovulation to sexual development (Diep et al., 2016).

Dimerization is not an obligation for PR transcription, and Progesterone Response Element (PRE) sequences are not the only sites where PRs can bind. In addition, PRE half-site elements have been shown to function similarly to complete PRE sequences indicating the possibility of PR binding to these sequences as a monomer (Jacobsen and Horwitz, 2012).

The ratio of PR isoform expression alters which genes will be expressed thus changes in built-in alternative splicing patterns affect the progression of breast cancer. *PR-A* overexpression in human breast cancers may be a reliable predictor of decreased tamoxifen responsiveness. Some experimental data suggest that antiprogestins combined with an ER-alpha receptor blockers may be the effective treatment strategy in these molecular subtype breast tumours (Rojas et al., 2017).

1.4.3. Human Epidermal Growth Factor Receptor

Her2 is a member of the human epidermal growth factor receptor family and it is a kind of receptor tyrosine kinase. It is known as proto-oncogene and the long arm of chromosome 17 is its chromosomal location. As with all receptor tyrosine kinases, after dimerisation autophosphorylation of its intracellular tyrosine residue takes place to activate intracellular downstream signalling pathways mainly pathways related to cellular differentiation, proliferation and growth.

Several antibody-based treatments were developed the most common and well known being the trastuzumab (Herceptin). In HER2 overexpressed breast cancer cases, the trastuzumab is highly effective until 12 months after which no additional benefits can be seen in clinical trials. Its mechanism of action briefly consists of the induced increase in p27 protein inside the cytoplasmic portion of the cell (Baselga et al., 2001).

It is also known that *HER2* expression can be modulated by estrogen receptor's transcriptional activity specifically the ratio between corepressors and coactivators of ER in the nucleus affects this transcriptional activity and in turn alters *HER2* expression. Thus, epigenetic mechanisms along with the interaction between ER and other signalling pathways have been attributed to contributing to endocrine therapy resistance (Aurilio et al., 2014).

Various molecular targets are being explored especially for TNBC including but not limited to, androgen receptor, EGFR, poly(ADP-ribose) polymerase (PARP), and vascular endothelial growth factor (VEGF). Receptors, protein tyrosine kinases, phosphatases, proteases, PI3K/Akt signalling pathway,

microRNAs (miRs) and long noncoding RNAs (lncRNAs) can potentially be targeted with molecular therapies (Munagala et al., 2011).

Poly (ADP-ribose) polymerase (PARP) family of nuclear enzymes are deemed with ability to recognize and repair DNA single-strand breaks. Approximately 10% of all breast cancer patients have a germ line mutation in *BRCA1* or *BRCA2* genes (Tung, N. et al., 2016). When this is taken into consideration, a treatment rationale appears at which breast cancer cells are introduced with PARP inhibitors that inactivate this DNA repair pathway which leads the pre-formed DNA single strand breaks progressing into being double strand breaks without being repaired. This has clinical implications especially in *BRCA* mutation bearing breast cancer cells because these kind of cancer cells are also problematic in terms of homologous recombination repair system which repair dna double strand breaks. Dual and simultaneous absence of two complementary DNA repair systems make these breast cancer cells very vulnerable to untolerable levels of genomic instability stress that eventually activates programmed cell death mechanisms inevitably thus hopefully results in tumor regression (Tutt et al., 2010).

1.4.4. Other therapeutic targets

In hormone positive breast cancers due to upregulated cyclin D1 levels which also lead to activated of cyclin-dependent kinases CDK4 and CDK6, sometimes CDK4/CDK6 inhibitors palbociclib, ribociclib and abemaciclib are also used in the treatment (Turner et al., 2015).

1.5. Herb Extract Approach to the Breast Cancer Treatment

According to the estimations among 877 small-molecule drugs which had come to the drug market worldwide between 1981 and 2002, only four out of 10 drugs are hundred percent sythetically developed by human effort and not originated from a similar plant compound. This means 60 percent of medications have plant-based roots and can be traced back to the original plant compound. Using plant based

molecules as starting point also reduces the cost of drug designing efforts from back to square one (Newman et al., 2003; Yuliana et al., 2011).

Not all of them but most of these plant originated drugs have low toxicity potential for the human biology which means they have wide therapeutic window unlike completely man-made chemicals which are most of the time become toxic in slightly overdosed cases like many cancer chemotherapy drugs (Grynkiewicz and Szeja, 2015).

Well-established chemotherapy based treatments are currently most used treatment modes for breast cancer along with radiotherapy and surgery ("American Cancer Society | Information and Resources about for Cancer: Breast, Colon, Lung, Prostate, Skin", 2019). However, they are non-specific despite their high costs, so they are literally not cost-effective for the patients. Thus, exploiting the power of herbal medicine has many advantages over traditional treatments.

Herbal treatments for breast cancer are mainly phytoestrogens and traditional Chinese medicines including alkaloids, coumarins, flavonoids and polyphenols, terpenoids, quinone, and artesunate (Yin et al., 2013).

Due to the presence of flavonoids and many other aromatic compounds with antioxidant and anti-inflammatory properties, the herbal approach may prove effective in ameliorating the high level of oxidative stress present in cancerous cells and inflammation in the tumour microenvironment without harm. An association between the consumption of fruits and vegetables with a lower risk of cancer is also widely accepted (Farvid et al., 2018).

However, sometimes the combination of different kinds of aromatic molecules in one plant or herb interact together to be able to possess some beneficial biological activity *in vivo* in contrast to *in vitro* chemosensitivity assays made with extracts of one specific molecule. For instance, there are many studies indicating beneficial effects of curcumin molecule for a bunch of diseases in vitro. Populational statistics data support this with much lower rates of some diseases encountered in Indian population however it is most of the time ignored that turmeric is the actual spice

consumed by the native Indian people and it contains hundreds of bioactive compounds apart from curcumin which might potentially work together in a living human biosystem to exert such effects (Zubair et al., 2017; Amalraj et al., 2017; Nardo et al., 2011).

Despite this disadvantage of chemosensitivity assays, many promising compounds are first explored in these assays (Blumenthal, 2005).

1.6. Chemical Properties of Iridoid Monoterpenoids

Iridoid monoterpenoids are in the general form of cyclopentanopyran. 8-oxogeranial is the source of iridoid monoterpenoids in living organisms in which iridoids are found. Monoterpenoids belong to the broad category of molecules called monoterpenes which are important in the modulation of inflammatory markers and thus inflammation (De Cássia Da Silveira E Sá et al., 2013). They can be acyclic linear molecules or cyclic in nature and monoterpenoids have generally modification in the carbon ten skeleton of the parental monoterpene molecule, this modification can be a missing methyl group or a functional group containing an oxygen atom. In plants, they occur as glycosides. These molecules mostly comprise the protection mechanism of plants in which they are found, from herbivores and microorganisms. Glycosides are molecules which contains glucose bound to their functional group via the glycosidic bond and upon cleavage of this glucose molecule by enzyme hydrolysis they are activated in most plant cells, so they are inactive molecules when they are bound to a glucose molecule. These secondary metabolites can be used as therapeutic agents according to some research. Two isoprene units (2-Methyl-1,3-butadiene 78-79-5) and molecular formula C10H16 are defining features of monoterpenes (Parvin et al., 2014).

1.6.1. Monoterpene Cancer Progression Suppressing Activity

Monoterpenes have various anti-tumour bioactivities detected so far; cell cycle arrest and induction of apoptosis, inhibition of the NF-κB (Nuclear Factor Kappa B) pathway, decrease of Mac-3 (Macrophage-3-antigen) and CD11b (Integrin alpha

M) markers of macrophages and granulocytes precursors, inhibition gene expression of topoisomerases I, II alpha, and II beta, TRPM8 (Transient receptor potential cation channel subfamily M (melastatin) member 8) channel activation by acting as TRPM8 agonist; cell cycle arrest, mitochondrial membrane depolarization via the TRPM8 channel, potentiation of selenocystine-induced apoptosis and activation of ROS (Reactive oxygen species)-mediated DNA damage, Inhibition of DNA synthesis, proteasome inhibition and induction of apoptosis, suppression of STAT3 (Signal transducer and activator of transcription 3) activation and induction of apoptosis, inactivation of the stress response pathway sensor CHEK1 (Checkpoint kinase 1) and induction of apoptosis, antioxidant activity, antiangiogenesis effect, induction of p53-independent apoptosis, involvement of reactive oxygen species and activation of ERK (extracellular signal-regulated kinases) and JNK(c-Jun N-terminal kinases) signaling, prooxidant cytotoxic mechanism, disruption in cell-cycle checkpoints, increase of ROS generation and decreased GSH (glutathione) levels, increase in the expression of the protein p53 and decrease in cyclin B1 protein, inhibition of telomerase, inhibition of PDE1A (phosphodiesterase 1A) expression, downregulated MUC4 (Mucin4) expression and induction of apoptosis, upregulation of PTEN expression, inhibition of Akt phosphorylation; induction of apoptosis; inhibition of HDAC2 (Histone deacetylase 2) proteins, modulation of the PPAR-γ (Peroxisome proliferator-activated receptor gamma) activation pathway (Sobral et. al., 2014).

Limonene can be given as an example to one of the most studied monoterpenes in mammary cancer and it prevents mammary cancer at the promotion/progression stage. The rats which were fed limonene after 2 weeks from the day of their 7, 12-Dimethylbenz[a]anthracene (DMBA) and N-methyl-N-nitrosourea ingestion didn't develop advanced cancer (Elegbede et al., 1984).

Mammary cancer in 43 women was also found to be regressed by limonene treatment applied 2-6 weeks before surgical excision of tumor mass and by investigating the tumor mass after operation (Miller et al., 2013).

Ductal epithelial cells which were induced constantly active ras gene using retroviral vectors were also responsive to limonene treatment (Miller et al., 2015).

Additionally, it has also been shown that monoterpenes inhibit the isoprenylation of certain proteins, the synthesis of ubiquinone (Co-Q), and the conversion of lathesterol to cholesterol all of which can bear physiological significance. It is especially known as ras and other G proteins get to either farnesylated or geranylgeranylated to localize to the cell membrane (Ren et al., 1998).

Linalool is one of the best monoterpenes to combine with the standard chemotherapy for breast cancer. It potentiates doxorubicin-induced cytotoxicity in MCF-7 and multidrug-resistant MCF-7 cell line (Ravizza et al., 2008).

1.6.2. Origin of Asperuloside Molecule

Asperuloside (rubichlorinic acid) is a monoterpenoid glycoside and it is isolated from Galium aparine, Hedyotis diffusa Willd (Rubiaceae) and Morinda citrifolia L. blossoms among many others while in this study it originated from Putoria Calabrica plant. It belongs to the iridoid glycoside class of monoterpenoids and it has a role as a metabolite (Grynkiewicz and Szeja, 2015; Mijatovic et al., 2007). The asperuloside molecule has been shown to have anti-tumour activity (Artanti et al., 2015).

1.6.3. Effects of Asperuloside Molecule in Mammals and Mammalian Cell lines

In many studies, the hedyotis diffusa plant which as a whole has been shown to act as anti-tumor agent on various cancer types(Niu and Meng, 2013)

In one study, cytotoxicity assay was carried out to assess fractions of Hedyotis corymbosa extract against breast cancer cell line T47D and in this study positive control was antimycin A3. Extract proved to be cytotoxic to the tumour cell lines studied (Andriyani et al., 2011). Hedyotis diffusa has also been shown to exhibit significant anti-proliferative effects via apoptotic responses on leukemia cells by modulating MAPK pathways (Wang, N. et al., 2013).

Another study again used T47D breast cancer cell line among many other cell lines to validate anti-tumour properties of asperuloside molecule with positive results. However, in this study, other molecules derived from hedyotis corymbosa extract were used as well (Andriyani et al., 2015).

In a study in which inflammation was induced via NF-kB and MAPK pathways by introducing Lipopolysaccharide (LPS) to the RAW 264.7 cells, asperuloside was shown to act as an anti-inflammatory agent. It was demonstrated that it reduced the production of nitric oxide (NO), prostaglandin E2 (PGE2), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) while inhibiting inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, and IL-6 mRNA expression in LPS-induced RAW 264.7 cells. It managed this effects by subtle intracellular phosphorylation of p38, ERK, IκB-α and JNK inhibitors to suppress their actions (He et al., 2018).

1.7. Work in this thesis

It is hypothesized that MCF-7 and MDA-MB-231 cell lines will react to the asperuloside molecule and their proliferation rate proportionately will regress as the time of their incubation with this molecule increases due to strong anti-tumor properties of this molecule. In addition, reduction in the ratio of viable versus death cell after different hours administration of the asperuloside molecule to the cells is also expected due to induction of cell death mechanisms. The main aim of this study is to make a contribution to very few but consisten studies demonstrating the cytostatic or cytotoxic effects of asperuloside molecule on MCF-7 and MDA-MB-231 breast cancer cell lines.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Suppliers

Sigma Aldrich (Poole, UK), Dojindo Europe (Munich, Germany), Thermo Fisher Scientific (Waltham, USA), American Type Culture Collection (ATCC) (Virginia, USA), Escoglobal (Friedberg, Germany), Merck(Taufkirchen, Germany), Molecular Devices (USA)

2.1.2. Cell Counting Kit-8

Tetrazolium salt WST8 (Dojindo EU Gmbh, Munich, Germany)

2.1.3 Chemicals used in cell culture

a) %10 (v/v) FBS (Fetal bovine serum) (Sigma Aldrich, Poole, UK)

It was used as growth factor and hormone source for cells. It also includes aprotinin which is useful for stopping trypsin action in subculturing procedure.

b) Tripsin-EDTA(w/v) 0.25X (Sigma Aldrich, Poole, UK)

It was used for detaching adherent epithelial breast cancer cells during subculturing and seeding cells to wells.

c) Dulbecco´s modified eagle medium F12(1:1) (1X) 500 ml (Gibco, Waltham, USA)

Biotin, Calcium Chloride (CaCl2) (anhyd.), Choline chloride, Cupric sulfate (CuSO4-5H2O), D-Calcium pantothenate, D-Glucose (Dextrose), Ferric Nitrate (Fe(NO3)3·9H2O), Ferric sulfate (FeSO4-7H2O), Folic Acid, Glycine, HEPES, Hypoxanthine Na, L-Alanine, L-Arginine hydrochloride, L-Asparagine-H2O, L-Aspartic acid, L-Cysteine hydrochloride-H2O, L-Cystine 2HCl, L-Glutamic Acid, L-Glutamine, L-Histidine hydrochloride-H2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, L-Valine, Linoleic Acid, Lipoic Acid, Magnesium Chloride (anhydrous), Magnesium Sulfate (MgSO4) (anhyd.),

Niacinamide, Phenol Red, Potassium Chloride (KCl), Putrescine 2HCl, Pyridoxine hydrochloride, Riboflavin, Sodium Bicarbonate (NaHCO3), Sodium Chloride (NaCl), Sodium Phosphate dibasic (Na2HPO4) anhydrous, Sodium Phosphate monobasic (NaH2PO4-H2O), Sodium Pyruvate, Thiamine hydrochloride, Thymidine, Vitamin B12, Zinc sulfate (ZnSO4-7H2O), i-Inositol

d) Aminoacids

Aminoacids was for cells to use them as macromolecule and protein building blocks. L-glutamine is the nitrogen source for the formation of other building blocks. It is also energy source for cells under hypoxic and low glucose conditions.

e) Inorganic salts

Inorganic ions regulate the osmotic balance of the cells and help maintain membrane potential by the providing sodium, potassium and calcium ions which are all components in the cell matrix used for cellular attachment. They also take role as enzyme cofactors.

f) Carbohydrates
Simple carbohydrates such as glucose are the major source of energy.

g) %1(w/v) Penicilline Streptomycin (Biochrom, Nicosia, Cyprus)

Multi-functional antibacterial agent. Prevents both gram-positive and negative bacteria to multiply and contaminate the cultures.

h) Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Poole, UK)
Cryoprotectant was used to prevent crystallized water bursting frozen cells.

i) %70 (v/v) Ethanol (Sigma Aldrich, Poole, UK)

It was used for disinfection purposes.

j) Phenol Red

It was used as visual pH indicator.

2.1.4. Cell Lines Used In Cell Culture

a) MCF-7 (ATTC, Virginia, USA)

MCF-7 cell line was established from primary tumour mass of invasive ductal breast carcinoma by pleural effusion method in 1973 at the Michigan Cancer Foundation-7 which would later be known as Barbara Ann Karmanos Cancer Institute. MCF-7 cell line is hormone positive breast cancer cell line and it shows proliferative response to estrogen, progesterone but it is marked by the absence of her2 gene amplification thus overexpression of her2 receptor is strictly not a feature of MCF-7 cell line.

b) MDA-MB-231(METU, Ankara, Turkey)

MDA-MB-231 cell line is from triple negative breast cancer cells. It has many characteristics such as absence of ER, PR expression on the nuclear membrane as well as the absence of her2 receptor overexpression on the cellular membrane and luminal androgen receptors (LAR) subtypes based on gene expression signatures.

Many different subgroups have been identified according to clinical, histopathological and molecular point of view towards the disease, tumour tissue and intracellular molecules of tumour cells respectively. A triple negative breast cancer (TNBC) can be basal like breast cancer but not all TNBC breast cancers necessarily have to be basal-like.

2.1.5. Plant Extract Asperuloside

In this work, asperuloside molecule was isolated from the Turkish plant *Putoria calabrica* (L.fil.) DC. and it was a kind gift from Near East University Faculty of Pharmacy. This molecule was first extracted in 2000 (Çalis, I. et al., 2001; Linden, A. et al., 2000).

The plant specimen was collected from Antakya, Turkey, in June 1999. The initial step was to dry the plant material from which the extract would later be obtained with MeOH at 323 K. Methanolic extract had many layers and water soluble layer was fractionated over polyamide resulting in several fractions. The

iridoid rich fractions were further subjected to column chromatography on silica gel using CH_2C_{l2}/MeOH/H_2O (90:10:1, 85:15:1.5) as eluants. This produced two additional fractions of which asperuloside was the major component. It was used in experiments in lyophilized form after crystallization.

2.2. Methods

2.2.1. Cell Culture Maintenance

2.2.1.1. Thawing process of frozen cell lines

Cell lines were purchased from American Type Culture Collection (ATCC Manassas, Virginia, USA) and were kept frozen for later use.

In the beginning of the cell proliferation experiment, frozen cells were thawed.

Cryovial which contains the frozen cells was removed from -80 degrees celcius storage and the vial was transferred into the laminar flow hood after outside of vial was cleaned with 70% (v/v) ethanol. After transferring the contents of the vial to the 15ml centrifuge tube, 14 ml (v/v) of pre-warmed complete growth medium was added dropwise into the centrifuge tube containing the thawed cells. Cell suspension was centrifuged at approximately 125 × g for ten minutes. Supernatant removed and remaining cell pellet resuspended in the 15 ml (v/v) complete growth media. From that 360-500.000 cells were seeded to a T75 flask and one third for the T25 flask.

2.2.1.2. Subculturing and collection of cells

One day were given to cells for overcoming stress induced on them in the thawing process and after their morphology was checked and found normal, media changed. Six to nine days later depending on the cells' adaptation rate, cell lines were subcultured once the 80% confluency was reached by the cells. Cell culture media was aspirated and disposed. The flask was first washed with 2 ml trypsin to neutralise any remaining serum. Cells were then detached using 6.5 ml %0.25 (w/v) trypsin containing Trypsin-EDTA solution for approximately 8-10 minutes. 2.5 ml %0.25 (w/v) trypsin was used for T25 flasks. To prevent over-trypsinization which leads attrition of cells in the culture, the cell culture was constantly checked with an inverted microscope until enough dissociation of cells was achieved. The remaining suspension was then neutralised with 2 ml of media. Later the suspension was transferred to a sterile falcon tube and the flask was rinsed with 2 ml media to collect any remaining sticked cells to the walls of the flask. That 2 ml solution was also carried to the same falcon tube. After that, cells collected by centrifugation for 8-10 minutes at 1000 rpm 128 g and 192 RCF. The supernatant was removed and cells were resuspended in the 1ml fresh complete growth medium. 100 µl was drawn and used for cell counting purposes.

Appropriate amount of cell number that would be added to wells was obtained (5000 cells) in both cell proliferation rate assesment and cell death experiment by drawing neeeded amount from the resuspended solution and if necessary by mixing it with additional media total volume not exceeding 100 µl.

2.2.1.3. Cell Counting and Seeding

Cell lines of a known genetic background were maintained as stated below in T-75 and T-25 cm^2 flasks. Prior to each experiment one 80-90 % confluent flask was trypsinised with % 0.25 (w/v) trypsin and cell suspension was centrifuged as described below, the supernatant was aspirated and the cell pellet resuspended in 1 ml of media and gently pipetted to prevent clumping of cells. 100 µl of cell solution was pipetted onto a Neubauer lam and cells counted by using inverted microscope. Approximately

5000 cells were seeded in each well of 96-well plate for both cell proliferation and cell death assay.

2.2.2. Cell Proliferation Rate Detection

CCK-8 kit exploits physiological electron transfer mechanisms mediated by intracellular reductors.

In case of lots of intracellular dehyrogenase activity which is direcly proportional with the viability of the cell, the NAD+ molecule is reduced into NADH molecule upon nucleophilic attack of the sulphur atom from the active site of the dehydrogenase enzyme to the carbonyl carbon of the aldehyde intermediates formed in the cellular metabolism cycles mainly in the mitochondrium compartment. However, in the presence of the electron mediator provided by the CCK-8 kit solution during this reduction, the mediator gets reduced finally in other words take hydrogen atom from the reduced NADH molecule turning it to again a NAD+. Later the reduced electron mediator transfers its hydrogen atom to the WST-8 molecule which in turn forms orange soluble formazan dye in the extacellular part of the plasma membrane and this is expected to increase absorbance value at 450 nm wavelength. As there are more viable cells the presence of formazan dye increase and so does absorbance value theoretically.

For each cell line, 5000 cells were seeded to the total three, 96 well plates in which cells would be incubated for 24, 48 and 72 hours for each cell line with five different concentrations of Asperuloside molecule (50, 25, 10, 1 and 0.5 μM).

The CCK-8 reagent was added after 24, 48 and 72 hours to the plates where cells were being held with the pure Asperuloside molecule before waiting for four hours and taking absorbance readings.

Interpretation of results was carried out with the raw absorbance data in Graphpad prism program's built in two-way ANOVA method. Both main column effect and main row effect has been evaluated by multiple comparison test at which columns were representing the absorbance values sorted according to cell line types and rows were representing the five different concentrations of the molecule under investigation.

CHAPTER 3. RESULTS

3.1. Mammalian Cell Lines Used in The Study

3.1.1. MCF-7 Human Breast Cancer Cell Line

Until today, there have been 25000 published reports about MCF-7 cell line which was obtained by pleural effusion method and represent hormone-positive human breast adenocarcinoma cells. These cells establish cellular aggregations which are formed from spherical cancer cells in the cell culture. It had been very difficult to establish human breast cancer cell lines bearing ER alpha in *in vitro* cell culture conditions, thus MCF-7 is nearly unique with its this aspect although not a new method had been applied while establishing this cell line in 1973 at Michigan Cancer Foundation labaratories (Lee et al., 2015).

MCF-7 thus allowed the scientific community to explore basics of ER action including but not limited to intracellular binding constants, transport mechanisms, and the mode of nuclear uptake. But most importantly it enabled researchers to get progress in the hormone responsive breast cancer research. Moreover, MCF-7 cells were used to purify the ER and obtain monoclonal antibodies againt it (Greene et al., 1980). These cells also express androgen, progesterone, and glucocorticoid receptors which means not only estrogen action in breast cancer but also its cooperative actions with other steroids can be studied with MCF-7 cell line (Horwitz et al., 1975).

3.1.2. MDA-MB-231 Human Breast Cancer Cell Line

The MDA-MB-231 cell line is an epithelial, human breast cancer cell line that was obtained from a 51-year-old caucasian female with a metastatic mammary adenocarcinoma. MDA-MB-231 represents triple-negative breast cancer (TNBC) cell type which is aggressive, invasive and poorly differentiated cell line. The absence of estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2 amplification is defining factors of this cell line.

MDA-MB-231 cell lines characteristically degrade the extracellular matrix by proteolytic mechanisms they bear which make their invasive potential extremely high (Liu et al.,2003). The cells belonging to this cell line down-regulate claudin-3 and claudinin-4 and have lower Ki-67 proliferation marker expression. Enrichment for markers associated with the epithelial-mesenchymal transition and the expression characteristics associated with mammary cancer stem cells (CSCs), such as the CD44+CD24-/low phenotype are within the very commonly encountered the signatures of this cell line (Holliday and Speirs, 2011).

3.2. Mammalian Cellular Energy and Metabolism Cycle

For the cellular metabolism to happen properly, a biochemical reaction network converting metabolites into end products must exist to enable the cell to fulfill biological functions. Some pathways catabolizes or in other words break down large molecules to provide energy and building blocks which in turn canalized into anabolic biosynthetic processes and to the mechanical work.

Adenosine Triphosphate (ATP) is the key molecule in all of the anabolic, catabolic and mechanical processes which happen in the cellular energy and metabolism cycle along with NADH and NADPH which are closely related in structure and function, namely NADPH is involved mainly in anabolic metabolism while NADH is involved in postranslational modifications and redox reactions of catabolism.

Thus, both normal and cancer cells rely heavily on the presence of enough ATP, NADH and NADPH molecules in the right place at the right time.

There are three classes of cellular physiological state, divided according to the amount of required energy by the cell at that specific moment.

3.2.1. Basal Metabolic State

Maintenance energy demand characterizes this metabolic state. According to the current theories and up to date knowledge the maintenance energy defines the energy cost expended to sustain the delicate ionic balance between intracellular and extracellular environment and it is independent of the cellular growth and division kinetics of that specific moment so it does not include the energy expense of growth and division cycle.

3.2.2. Functional Metabolic State

When cells do work in terms of movement, growing, dividing or fulfilling other cell type specific functions, they require extra energy.

Utilisation of both glycolysis and oxidative phosphorylation would be the next step for a normal healthy mammalian cell upon this extra energy need (Vazquez et al., 2010).

Glycolysis has a low yield of ATP per molecule of glucose transformation namely 2 molecule of ATP per molecule of glucose is derived via glycolysis although it is characterized by a high horsepower in terms of energy produced per volume of enzymes. Oxidative phosphorylation is much more efficient that as much as 32 molecules of ATP can be obtained per molecule of glucose conversion however it has a drawback of having a lower horsepower, thus too many enzymes are allocated in this process to obtain a relatively low ATP yield according to the number of enzymes work (Vazquez, 2017). Thus in order to reach to the highest rate of metabolic rate and biosythesis, mammalian cells have to select the aerobic glycolysis and uncoupling biosynthesis from NADH generated in the mitochondrial processes (Voet, D and Voet, J.G., 1995; Fernandez-de-Cossio-Diaz and Vazquez, 2017).

3.2.3. Maximal Metabolic Rate

Cells turn on maximal metabolic rate to respond extraordinary conditions or as result of a pathologic condition such as tumorigenesis. In the situations at which cells need to carry out their metabolical processes at the highest rate, only glycolysis with minimal OxPHOS (Oxidative Phosphorylation) usage can be a sustainable source of generating energy for both high mechanical work and biosyhthesis processes because as mentioned in the last section horsepower is low in the oxidative phosporylation. Moreover, the enzymes and reactions involved in OxPHOS have carbon depleting nature which makes the biosythetic anabolic reactions run out of carbon moieties normally used for duplicating macromolecules for supporting cellular growth and division (Figure 3.1) (Vinay et al., 2014).

3.3. Cancer Cell Energy and Metabolism Cycle

Cancer cells need more energy according to the normal cells due to their accelerated biological activities comprising of non-stop or nearly non-stop uncontrolled proliferation, migration during metastasis and adaptation to new tissue niches different from the primary invasion site. Constant energy supply is necessary for cancer cells in order to produce biomolecules that are required for these processes to happen. Moreover not only anabolic reactions and cellular mechanical movements require energy in the form of ATP but also sustained growth signalling as well as driver gene and mTOR activation does require ATP as a phosphate group supplier. Therefore, cancer cells need to have a huge supply of ATP.

Glycolysis is the key process in the cellular energy metabolism of a mammalian cancer cell. Warburg discovered that cancer cells benefited from high glycolytic rate and converted most of their glucose supply into lactate even in high oxygen conditions in contrast to normal cells which rely on glycolysis at a much lower rate or in other words which rely on oxidative phosphorylation at a much higher rate and obtain carbon dioxide and water after processing pyruvate in the mitochondria (Warburg, O. et.al., 1956). Moreover, the Warburg effect is followed by other sequential metabolic abnormal changes, the most significant and proven one being increased glutamine utilization and conversion of acetate into AcCoA in cancer cells (Sauer and Dauchy, 1983; Sauer et al., 1982; DeBerardinis et al., 2007).

Intrinsic mitochondrial dysfunction, deactivation of tumor suppressor proteins, the oxygen poor microenvironment, and dearrangements in built-in metabolic programme upon onco-protein activation all have potential to rewire cellular energy metabolism abnormally in a cancer cell by upregulating glycolytic genes (Kroemer and Pouyssegur, 2008). However, according to some recent studies, mitochondrial dysfunction is not an obligation in order to so-called “aerobic glycoysis” take place since it has been found that in most cancers mitochodrial function was intact (Scott et al., 2011).

In physiological conditions, ratio of glycolysis versus oxidative phosphorylation utilisation in a mammalian cell plays a role at the maintanance of delicate redox balance between the intra and extracellular microenvironment along with the cellular antioxidant enzyme capacity.

In this study an anti-inflammatory and anti-oxidant molecule had been chosen purposefully for exposing the cancer cells to it because pro-oxidative and pro-inflammatory signalling has detrimental effects on the redox balance of all mammalian cells and it has been shown that at cancer cells this balance is most of the time already distorted. Thus intervening to the inflammatory signals between cancer cells with an anti-inflammatory agent would theoretically disturb their increased proliferative capacity and reduce extracellular abnormally high oxidative state restoring intracellular reducing environment. This is expected to eventually lead to the death of cancer cells due to unfavorable conditions created against them.

CCK-8 kit was chosen purposefully to see whether if any change in metabolic rate occurs in MDA-MB-231 and MCF-7 cancer cell lines when exposed to Asperuloside molecule in a time-dependent manner.

Iridoid glycosides class of molecules to which Asperuloside belongs exhibit strong anti-inflammatory and anti-oxidant action at *in vitro* studies against various kinds of human cancer cell lines including but not limited to MCF-7 and MDA-MB-231 cell lines (Wang et al., 2017).

It has been shown that Asperuloside molecule reduces pro-inflammatory signalling molecules released by macrophages which were stimulated by LPS at in vitro studies (He et al., 2018). This may have some important implications for *in vivo* environment in terms of its anti-inflammatory effects since there is no immune cell *in vitro* environment chemosensitivity assays.

Preceding few and weak studies which used plant extracts also containing Asperuloside molecule had investigated the effect of these exctracts as a whole directly on the cancer cell lines and found that there is an anti-tumor and anti-cancer bioaction of these extracts.

However, in this study pure Asperuloside molecule was used instead of an extract of the plant from which it was derived.

3.4. Raw Absorbance Values Obtained From MCF-7 Cell Plates

Concentrations	MCF-7 (24 hours)	MCF-7 (24hours)	MEAN MCF-7
0 µM	0.5510	0.7310	0.6410
0.5 µM	0.8060	0.9130	0.8590
1 µM	0.6450	0.2740	0.4590
10 µM	0.9140	0.8730	0.8930
25 µM	0.1850	0.9340	0.5590
50 µM	0.0240	0.6830	0.3530
	MCF-7 (48 hours)	MCF-7 (48 hours)	MEAN MCF-7
0 µM	0.4570	0.3040	0.3805
0.5 µM	0.6570	0.7170	0.6870
1 µM	0.5700	0.7240	0.6470
10 µM	0.6130	0.7420	0.6775
25 µM	0.5690	0.6680	0.6185
50 µM	0.4710	0.6140	0.5425
	MCF-7 (72 hours)	MCF7 (72 hours)	MEAN MCF-7
0 µM	0.1960	0.3110	0.2535
0.5 µM	0.8410	0.0880	0.4645
1 µM	1.4240	0.5700	0.9970
10 µM	1.5060	0.5840	1.0450
25 µM	1.3520	0.5700	0.9610
50 µM	1.0910	0.7580	0.9245

Table 3.1 Absorbance values obtained from microplate reader in three consecutive days for MCF-7 cells as a function of Asperuloside concentration and duration of this exposure.

3.5. Raw Absorbance Values Obtained From MDA-MB-231 Cell Plates

Concentration	MDA-MB-231 (24 hours)	MDA-MB-231 (24 hours)	MEAN MDA-MB-231
0 µM	0.8290	1.3520	1.0905
0.5 µM	1.2920	1.4110	1.3515
1 µM	1.4520	1.5600	1.5060
10 µM	1.3430	1.5050	1.4240
25 µM	1.2080	1.4810	1.3445
50 µM	0.6180	1.0640	0.8410
	MDA-MB-231 (48 hours)	MDA-MB-231 (48 Hours)	MEAN MDA-MB-231
0 µM	0.3810	-0.0070	0.1870
0.5 µM	0.5050	-0.0050	0.2500
1 µM	0.4250	-0.0030	0.2110
10 µM	0.3530	-0.0030	0.1750
25 µM	0.3670	-0.0020	0.1830
50 µM	0.2550	-0.0100	0.1230
	MDA-MB-231 (72hours)	MDA-MB-231 (72 hours)	MEAN MDAMB231
0 µM	1.4050	1.5070	1.4560
0.5 µM	0.4870	0.7560	0.6215
1 µM	0.4470	0.8830	0.6650
10 µM	0.6780	0.5670	0.6225
25 µM	0.6190	0.4560	0.5375
50 µM	0.5430	0.4970	0.5200

Table 3.2 Absorbance values obtained from microplate reader in 3 consecutive days for MDA-MB-231 cancer cells as a function of Asperuloside exposure concentration and duration of this exposure.

3.6. Experimental Results Summary Graph

The bar chart indicate summary of raw absorbance values of Asperuloside treated samples with untreated cancer cell lines'(control group) absorbance values. 0 micromolar group is the control group for all exposure times.

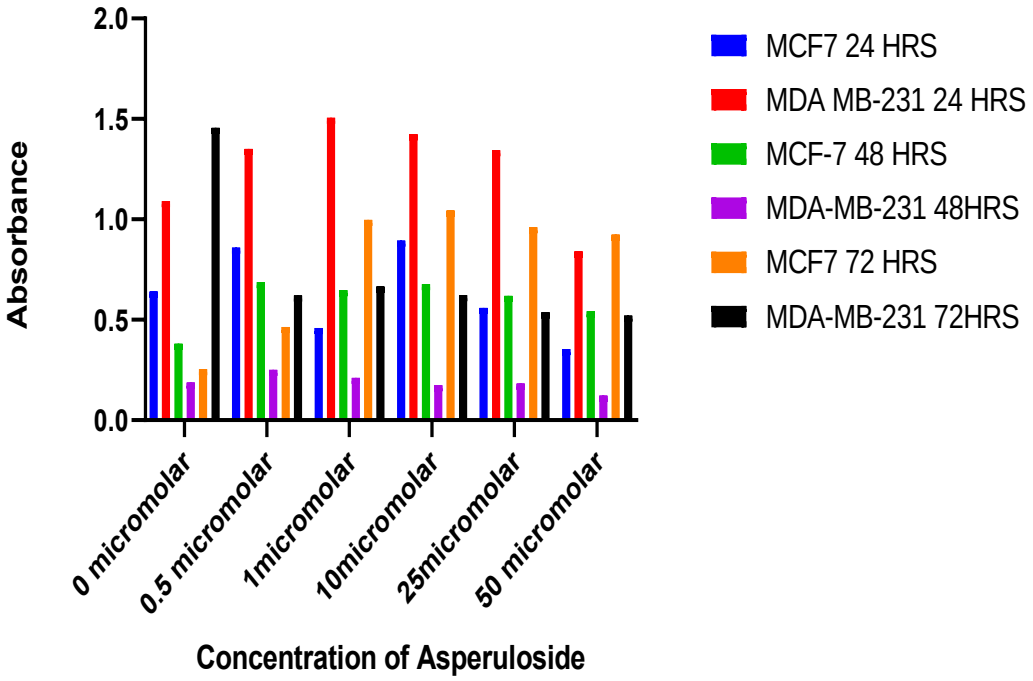

Figure 3.2: The bar chart representing the raw absorbance values of both cell lines at all exposure durations.

3.7. Effect of Asperuloside Molecule on the Proliferation and Metabolic Rate of Human Breast Cancer MCF-7 Cell Line

As time passed in the 3 consecutive days MCF-7 control cells' viability decreased. 50 micromolar concentration was the most effective dosage for MCF-7 cells at 24 hours with 1 micromolar being second most effective. At 48 hours and 72 hours, none of the concentrations was effective in reducing the proliferative rate of MCF-7 cancer cells.

3.8. Effect of Asperuloside Molecule on the Proliferation and Metabolic Rate of Human Breast Cancer MDA-MB-231 Cell Line

In 24 hours MCF-7 and MDA-MB-231 cells showed clear decrease in viability only in 50 micromolar treated wells with respect to their control groups' viability. Both of the cell lines' 50 micromolar treated cells were less viable according to the plate control cells. But, MCF-7 cells' viability was affected more in response to 50 micromolar Asperuloside treatment in 24 hours according to MDA-MB-231 cells since the difference between the MCF control group's viability and 50micromolar treated MCF group's viability was higher than the difference between MDA-MB-231 control group viability and treated MDA-MB-231 cells.

The proliferative rate of only 72 hours exposure group was decreased when compared to the MDA-MB-231 cells control group upon exposure to the Asperuloside although this was not a dose dependent specific response. The cells in the 48 hours plate were significantly less viable and metabolically active according to both 72 and 24 hours plate in all dosages however the 48 hour 50micromolar group's metabolic rate was nearly half of the control group's.

CHAPTER 4. DISCUSSION

4.1. Discussion

According to the Tukey's multiple comparison test-based post-hoc analysis, there were 7 significant comparisons between different responses from different plates. But, since it is not appropriate to compare the absorbance values from different exposure times with two-way ANOVA test, only 24 hours MCF-7 and MDA-MB-231 comparison is valid among these 7 significant comparisons.

According to this comparison the MDA-MB-231 cells were significantly more viable and metabolically active than the MCF-7 cells after 24 hours and this can possibly be explained by the intrinsic metastatic capability and agressive nature of MDA-MB-231 triple negative breast cancer cells but at first look, it might not necessarily mean that Asperuloside actually had affected the MCF-7 cells to a higher extent than MDA-MB-231 cells in 24 hours plates.

However, there was an actual exception to this in 24 hours plates which states that MCF-7 cells were affected more from Asperuloside treatment in 50 micromolar treatment group at which both MDA-MB-231 and MCF-7 cells were less viable according to the control group's metabolic rate but this difference between control group and 50micromolar group was more pronounced in MCF-7. So, this clearly shows that in 24 hours plates 50 micromolar of Asperuloside did worked to supress both cell lines' metabolic rate however it was more successful at MCF-7 cells at this work.

Unfortunately, all the other dosage group differences between two cell lines in 24 hours were probaby due to intrinsic feautures of the cell lines rather than the presence of any cytostatic or cytotoxic effect of the molecule itself on two cell lines. Additionally, between each cell line, comparisons among different dosage groups' responses except 24 hours 50 micromolar group were unmeaningful since their metabolic rate were higher than the control group.

The 48 hours MDA-MB-231 cells' response to 50micromolar Asperuloside was significant because viability of these cells were lower than the plate's control group's viability according to the raw absorbance values.

However overally it is not appropriate to make comparisons between treatment concentrations from different exposure duration groups. Such comparisons like 48 hours MCF-7 10 micromolar group versus 24 hours MDA-MB-231 25 micromolar group cannot be examined via two-way ANOVA test. Moreover, some experimental imprecision such as unequal distribution of cells to the wells might have had an impact which restricts our ability to make robust comparisons.

4.1.1. Possible Explanations To The Higher Metabolic Rate of Treatment Groups with Respect to Control Cells

4.1.1.1. Experimental Imprecision

Although interpretation of experimental results by statistical analysis reveals that Asperuloside does not have any effect on the viability of cancer cell lines except 24 hours 50micromolar group, this doesnt necessarily have to be the universal reality. It can be the reality of this experiment or in other words its effect on the cancer cell lines might be hidden due to experimental imprecision.

Even the control wells had a wide variability in their absorbance values for the same cell line in the same plate. The replicates for the five concentrations of Asperuloside has also shown a wide variability for the same cell line. This might be related to the non-equal distribution of cells to the wells due to the absence of automatic cell counter facility in the lab where the work was carried out.

To prevent the effect of a this kind of imprecision, the cell number per well could be held higher (sample size) and different pipette tip for each well could be used to prevent accumulation of sticked cells in the same pipette tip after many seeding processes to fill the 36 wells with cells in the three microplates.

Application of this measure would not prevent the unequal distribution of cells to the wells however, the usage of same distribution method (mainly pipetting) and also with different pipette tips to disribute a much higher cell number to the each well would make this imprecion's background effect on absorbance less important with regards to the overall absorbance of the total cell population in the wells which reflects their overall viability with respect to other wells and also cellular accumulation in the pipette tip could be prevented for wells to which cellular seeding applied lastly by simply

replacing the pipette tip for every single well while cell seeding. Thus more homogenous cellular density between wells could be achieved.

4.1.1.2. Apoptosis Induced Proliferation in Low Asperuloside Dosages and High Exposure Durations

On the other hand, there is a relatively recently acknowledged phenomena which is called apoptosis induced proliferation. According to this phenomena, the dying cells release some diffusable mitogenic signals to induce proliferation of neigbouring cells to replace the dead cell fraction of the tissue (Ryoo and Bergmann, 2012; Fan and Bergmann, 2008). If this also takes place in vitro environment, it could explain the higher metabolic rate of some concentrations of Asperuloside treated samples than the control wells. To speculate, one could for instance claim that apoptosis was higher in treated samples but not enough to kill all the cells so they grew even harder in response to this treatment by the power of proliferative cytokines released from small amount of dying cells in the wells.

4.2. Conclusion

Overall ineffectiveness of different concetrations of Asperuloside molecule in different exposure durations and only 50 micromolar's partial action of slowing down the proliferation rate of cancer cells in 24 hours plate was revealed not only by means of intuitive reviewing of absorbance values but also via various statistical tests such as two-way ANOVA and post-hoc analysis comprising of Tukey's multiple comparison test.

According to these results, Asperuloside would neither be a good option as a direct treatment in breast cancer threapy in low doses nor as a reference starting molecule in drug discovery search. Morever, it can potentially be harmful in these low doses when looked at the increased proliferation rate of cancer cells with respect to control groups except 50 micromolar group in only 24 hours group.

4.3. Future Aspects

In the future, the same cell lines can be exposed to 50micromolar or higher concentrations of Asperuloside in order to investigate the apoptotic effects of Asperuloside along with presence or absence of apoptosis-induced proliferation phenomena *in vitro* environment. Normal breast tissue cells must be used as well in a potential apotosis assay to evaluate the specificity of the molecule in terms of its cytotoxic potential.

Moreover, animal studies can be conducted with higher dosages to see *in vivo* effects since anti-inflammatory and anti-oxidant action of Asperuloside might be present only in the environment in which there is extracellular matrix and immune cells because as mentioned before in the text it has been shown that Asperuloside molecule reduces pro-inflammatory signalling molecules released by macrophages which were stimulated by LPS at *in vitro* studies.

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