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CYTOTOXIC EFFECT AND APOPTOSIS INDUCTION OF VERBASCOSIDE IN MCF-7 AND MDA-MB-231

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MASTER THESIS

MOLECULAR MEDICINE

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NICOSIA, 2019

DECLERATION

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.

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PREFACE

This master thesis is the result of two year of hard work. It has been written as thesis for the master of Molecular Medicine at Nearest University, Faculty of Medicine.

I thank my supervisors, Assoc. Prof. Pınar Tulay and Prof. Gamze Mocan for their support, providing critical and constructive feedback during my research. I also thank Prof. İhsan Çalış at Pharmacy Department of Nearest University for extracting and supplying verbascoside from *Phlomis nissolii* L. plant and I thank Middle East Technical University for supplying MDA-MB 231 breast cancer cell line for my research.

Hülya Şenol
September, 2019

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LIST OF ABBREVIATIONS AND SYMBOLS

5-FU : Fluorouracil

μl.: Microliter

μM: Micromolar

nM: Nanomolar

Aβ(25–35): Amyloid β-Peptide 25–35

Aβ42: Amyloid β-Peptide 42

AChE : Acetylcholinesterase

AhR: Aryl hydrocarbon receptor transcription factor

AP-1 : Activator protein 1

BALB/c Nude mice: concatenation of Bagg and Albino

Bax gene : Member of the Bcl-2 gene family

Bcl-2 gene : B-cell lymphoma 2

BRCA-1 gene : Breast cancer type 1 gene

BRCA-2 gene : Breast cancer type 2 gene

caspase-3: Protein product of CASP3 gene., member of cysteine-aspartic acid protease (caspase) family.

CCK-8 assay: Cell counting kit 8 (contains WST)

CD44: Cluster of Differentiation 44

CDK2: Cyclin-dependent kinase 2

CDK6: Cell division protein kinase 6

CO_2: Carbondioxide

COX-2: Cyclooxygenase 2

CRC HCT-116: human colon cancer cell line

CYP1A1 : Cytochrome P450, family 1, subfamily A, polypeptide 1

CYP1B1: Cytochrome P450 Family 1, Subfamily B, Member 1

Cyclin D1,D2,D3: G1-phase regulators

DMSO-Dethyl Sulfoxide

E-cadherin: Cell adhesion molecule E

E2 : Estrogen

EGFR gene: Epidermal growth factor receptor

ERα : Estrogen receptor alpha

Erβ: Estrogen receptor beta

ER: Estrogen receptor

EDTA : Ethylenediaminetetraacetic acid

FBS: Fetal Bovine Serum

GSH: Reduced glutathione

HCT-116 colorectal cell lines

HeLa : Cervical cancer cell line

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER-2/Neu: Human epidermal growth factor receptor 2/ proto-oncogene Neu

HIPK2: Homeodomain Interacting Protein Kinase 2

HL-60 cells: Human promyelocytic leukaemia cells

HN4 , HN6: Head and neck squamous cell carcinoma cell lines

HT-29: Human colon cancer cell line

IAP : Inhibitors of apoptosis proteins

IFN-γ: Interferon gamma

IgE : Immunoglobulin E

IGF-1 : Insulin-like growth factor 1

IL-8 : Interleukin 8

IL-6:Interleukin 6

IL-12p70: Interleukin-12

(IKK)/NF-κB: IκB kinase complex

iNOS: Nitric oxide synthase

K562 : Myelogenous leukemia cells

LoVo: Colorectal cell lines

LPS/IFN-γ: Lipopolysaccharide/ Gamma interferon

MPP: 1-methyl-4-phenylpyridinium ion

MCP-1 : Monocyte chemoattractant protein 1

MAPK : Mitogen-activated protein kinases

MCF-7 : Breast cancer cell line isolated by Michigan Cancer Foundation

MDA-MB-231: Breast cancer cell line isolated by M. D. Anderson

MMP-2: Metalloproteinase 2

mL : Milliliter

Mg: Magnesium

MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

n-6 PUFA: n-6 polyunsaturated fatty acid

NF-kappaB p65: Nuclear factor kappa-light-chain-enhancer of activated B cells

NFκB : Nuclear factor-Kb

NRP1: Neuropilin-1

Nrf2: Nuclear factor erythroid 2-related factor 2

OSCC : Human oral squamous cell carcinoma

p53 : TP53 : Type of tumor suppressor gene

P450 : Cytochrome P450 gene

PC12 : Cell line from a pheochromocytoma of the rat adrenal medull

PR: Progesterone receptor

POP: Prolyl oligopeptidase

Prostaglandin E2: Medication that terminates pregnancy

RB gene : The retinoblastoma gene

RAC3 : Ras-Related C3 Botulinum Toxin Substrate 3

RCF : Revolutions per minute in a centrifuge

ROS: Reactive oxygen species

SHP-1 : Protein-Tyrosine Phosphatase

STAT3: Signal transducer and activator of transcription 3

SCC : Human oral squamous cell carcinoma

SH-SY5Y : Neuroblastoma cell line

SOD: Superoxide dismutase

SW620 : Colorectal cell line

Th 1, Th 2 : Helper cells

TNFα: Tumor necrosis factor alpha

TRNC: Turkish Republic of Northern Cyprus

TAK-1: Mitogen-activated protein kinase 7 (MAP3K7)

TNBC: Triple-negative breast cancer

U937 : Human myeloid leukaemia cell line

UV : Ultraviolet

WHO: World Health Organization

WST-8: 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

VEGF : Vascular endothelial growth factor

VB: Verbascoside

VEGFR1: Vascular endothelial growth factor receptor 1

VEGFR2: Vascular endothelial growth factor receptor 2

VEGF-A: Vascular endothelial growth factor A

VEGF-C: Vascular endothelial growth factor C

VEGF-D: Vascular endothelial growth factor D

ÖZET

VERBASCOSİDİN MCF-7 VE MDA-MB-231 MEME KANSERİ HÜCRE HATLARI ÜZERİNE SİTOTOKSİK VE APOPTOTİK İNDÜKLEME ETKİSİ

Hülya Şenol

Moleküler Tıp Ana Bilim Dalı

Tez Danışmanları: Assoc. Prof. Pınar Tulay, Prof. Gamze Mocan

Amaç:

Bu çalışmanın amacı verbascoside maddesinin *in vitro* MCF-7 and MDA-MB-231 meme kanseri hücre hatları üzerine genotoksik etkisini araştırmaktır.

Gereç:

Kimyasal maddeler :

Penisilin Streptomisin (+10000 units/mL penicillin and +10000 μg/mL streptomycin); Insülin, İnsan (4mg/ml); Fetal Bovine Serumu, DMEM /F-12(1:1) (1x) (Dulbecco's Modified Eagle medium F-12, +L-glutamat, +15mM HEPES, Nutrient Mixture (ham); Tripsin/EDTA çözeltisi (0.25% (depolama sıcaklığı -20^{0}C)

Kitler:

Hücre canlılığı / sitotoksisite: TEBU-BIO cell counting kit 8 (depolama sıcaklığı 0-5^{0}C).

Araç-gereçler:

ESCO Class II Type A2 biyolojik güvenlik kabini, inkübatör (37 °C, 5% CO2), Sentrifuj, Invert mikroskop,Versa max tunable microplate okuyucu, SOFTmaxÆ PRO software (absorbance 450 nm), GraphPad® Prism software version 8, Buzluk, Su Banyosu, Falcon tupleri, Cryotüpler, Pipet Kontrolör, Pipetler, Mikropipetler, Hemocytometer,T-75 flasklar, 96-çukurlu plateler.

Bitkisel madde:

Çalışmada kullanılan verbascoside maddesi *Phlomis nissolii* L. bitkisinden

Yakındoğu Eczacılık Fakültesi öğretim üyesi Prof. Dr.İhsan Çalış tarafından ekstre edilmiştir.

Meme Kanseri Hücre Hatları :

MCF-7 ve MDA-MB-231

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 T75 flasklar içindeki kültür ortamında çoğaltılmıştır. Hücreler 37˚C, 5% CO_2 ortam içeren inkübatörde muhafaza edilmiştir. Besi yeri her hafta gün aşırı tazelenmiştir. Hücreler 80-100% confluent duruma geldiğinde pasajlanmıştır. Hazırlanan verbascoside stok çözeltisinden 100, 48, 25, 10, 1, 0.5 ve 0.1 μM farklı konsantrasyonlar hazırlanmış ve hücre hatlarına eklenmiştir. 24, 48 ve 72 saat sonra verbascosidin hücre canlılığı /sitotoksisite etkisi TEBU-BIO cell counting kit 8 ile ölçülmüştür. IC_{50} değerleri GraphPad® Prism software version 8 programı kullanılarak non-linear regresyon curve fit analizi ile saptanmıştır. Control grup ve verbascosidin farklı konsantrasyonlarının ortalamaları arasında önemli bir farkın olup olmadığını tespit etmek için Two-way Anova multiple comparisons test uygulanmıştır.

Bulgular:

MCF-7 meme kanseri hücreleri, farklı konsantrasyonlarda (100 μM,48 μM,25 μM,10 μM,1 μM,0.5 μM ve 0.1 μM) verbascoside ile muamele edilerek absorbans yüzdeleri 24 saat, 48 saat ve 72 saatlik maruziyetten sonra ölçülmüştür. Elde edilen veriler Nonlinear Regresyon Metodu ile analiz edilmiştir. IC50 değerleri sırasıyla 0.127, 0.2174 and 0.2828 μM dır. Hesaplanan R^2 değerleri ise sırasıyla 0,9630, 0,8789 and 0,8752 dir. Verbascosidin 48, 25,10,1, 0.5 and 0.1 μM konsantrasyonları 24, 48 ve 72 saatlik maruziyet süresince MCF-7 meme kanseri hücreleri üzerine sitotoksik etki göstermemiştir. 100 μM verbascosidin ise sadece 72 saatlik maruziyeti MCF-7 meme kanser hücreleri üzerinde en yüksek sitotoksik etkiyi göstermiştir.

MDA-MB 231 meme kanseri hücreleri farklı konsantrasyonlarda (100 μM, 48 μM, 25 μM, 10 μM, 1μM, 0.5 μM ve 0.1 μM) verbascoside ile muamele edilerek

absorbans yüzdeleri 24 saat, 48 saat ve 72 saatlik maruziyetten sonra ölçülmüştür. Elde edilen veriler Nonlinear Regresyon Metodu ile analiz edilmiştir. IC50 değerleri ise sırasıyla 0.1597, 0.2584 and 0.2563 μM dır. Hesaplanan R^2 değerleri ise sırasıyla 0.8438, 0.5107 and 0.9203 tür. Verbascosidin 48, 25,10,1, 0.5 and 0.1 μM konsantrasyonları 24, 48 ve 72 saatlik maruziyet süresince MDA-MB 231 hücreleri üzerine sitotoksik etki göstermemiştir 100 μM verbascosidin ise 24, 48 ve 72 saatlik maruziyetleri MDA-MB 231 meme kanser hücreleri üzerinde en yüksek sitotoksik etkiyi göstermiştir

Sonuçlar:

Literatürde Verbascoside maddesinin MCF-7 ve MDA-MB 231 meme kanseri hücreleri üzerine antikanser ve sitotoksisite etkilerini inceleyen çok az sayıda çalışma bulunmaktadır. Yapılan bu çalışma, *Phlomis nissolii L.* bitkisinden ekstrakte edilen verbascoside maddesinin MCF-7 ve MDA-MB 231 meme kanseri hücreleri üzerine genotoksisite etkilerini inceleyen ilk çalışmadır. Bu nedenle, yapılan çalışmanın literatüre katkı sağlayacağı düşünülmektedir.

Anahtar sözcükler: MCF-7, MDA-MB231, Meme kanseri, Genotoksisite, Verbascoside

ABSTRACT

CYTOTOXIC EFFECT AND APOPTOSIS INDUCTION OF VERBASCOSIDE IN MCF-7 AND MDA-MB-231

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Molecular Medicine

Advisors: Assoc.Prof.Pınar Tulay, Prof. Gamze Mocan

Aim:

This study aimed to evaluate the genotoxic effects of Verbascoside on MCF-7 and MDA-MB-231 cells line *in vitro.*

Materials:

Chemicals:

Penicillin Streptomycin (+10000 units/mL penicillin and +10000 µg/mL streptomycin); Insulin, Human (at 4mg/ml); Fetal Bovine Serum and DMEM /F-12(1:1)(1x) (Dulbecco's Modified Eagle medium F-12, +L-glutamate, +15mM HEPES, Nutrient Mixture (ham), 500mL; Trypsin/EDTA Solution, 0.25% (Stored at (-20^{0}C).

Kits:

Cell viability/cytotoxicity: TEBU-BIO cell counting kit 8 (Stored at 0-5^{0}C).

Instrumentation:

ESCO Class II Type A2 Biological Safety Cabinet, Humidified incubator (at 37 °C, 5% CO2), Centrifuge, Inverted microscope,Versa max tunable microplate reader connected to an external computer, SOFTmaxÆ PRO software (absorbance at 450 nm), GraphPad® Prism software version 8 (for calculation of IC_{50} values), Freezer,Water bath, Falcon tubes, Cryotubes, Pipette controller, pipettes, micropipettes, Hemocytometer (counting chamber), Cell culture T-75 flasks, 96-well plates.

Plant Extract:

Verbascoside was extracted from *Phlomis nissolii* L. plant by Prof. Dr. İhsan Çalış at Pharmacy Department of Nearest University.

Cell Lines:

Breast cancer luminal A molecular subtype MCF-7 and Triple negative MDA-MB-231 cell lines

Method:

MCF-7 and MDA-MB-231 cells were grown in T75 flasks with the supplement of 45ml DMEM/F-12 (1:1) (1X)containing F-12 Nutrient Mixture (Ham) (+) L-Glutamate and (+) 15mM HEPES, 5ml Fetal Bovine Serum (%10), 125ml 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 other day. Verbascoside stock solution was prepared and diluted to working concentrations of 100, 48, 25, 10, 1, 0.5 and 0.1 μM. Cell viability/Cytotoxicity activity of verbascoside was evaluated by using TEBU-BIO cell counting kit 8. IC_{50} values were analyzed by using GraphPad® Prism software version 8. Two-way Anova multiple comparisons test was performed for MCF-7 and MDA-MB 231 breast cancer cell lines to determine the significance of mean difference between control and other concentrations of verbascoside for 24h, 48h and 72h of exposure respectively.

Results:

MCF-7 cells were treated with different concentrations of verbascoside (100, 48, 25, 10, 1 ,0.5 and 0.1 μM) and absorbancy percentages were measured after 24h,48h and 72 h of exposure. IC50 values for MCF-7 cell line after 24 hour, 48 hour and 72 hour exposure to different concentrations of verbascoside were found as 0.127, 0.2174 and 0.2828 μM respectively. R^2 values for 24h, 48h and 72h exposure to verbascoside were calculated as 0,9630, 0,8789 and 0,8752 respectively. Two way Anova multiple

comparisons test was performed for MCF-7 cell line to determine the significance of mean difference between control and other concentrations of verbascoside for 24h, 48h and 72h respectively. 48, 25,10,1, 0.5 and 0.1 μM concentrations of verbascoside are not effective on MCF-7 breast cancer cells after 24h, 48h and 72h exposure. 100 μM verbascoside has the highest cytotoxic effect on MCF-7 breast cancer cells after 72h exposure.

MDA-MB 231 cells were treated with different concentrations of verbascoside (50, 25, 10, 1, 0.5 and 0.1 μM) and absorbancy percentages were measured after 24h, 48h and 72 h of exposure. IC50 values for MDA-MB 231 cell line after 24 hour, 48 hour and 72 hour of exposure to different concentrations of verbascoside were found as 0.1597, 0.2584 and 0.2563 μM respectively. R^2 values for 24h,48h and 72h exposure to verbascoside were calculated as 0.8438, 0.5107 and 0.9203 respectively. Two way Anova multiple comparisons test was performed for MDA-MB 231 cell line to determine the significance of mean difference between control and other concentrations of verbascoside for 24h, 48h and 72h respectively. 48, 25,10,1, 0.5 and 0.1 μM concentrations of verbascoside are not effective on MDA-MB 231 breast cancer cells after 24h and 48h and 72h exposure. 100 μM verbascoside has the highest cytotoxic effect on MDA-MB 231 breast cancer cells after 24h , 48h and 72 h exposure.

Conclusion:

There are few studies investigating anticancer and cytotoxicity effect of Verbascoside on the MCF-7 and MDA-B 231 breast cancer cells in the literature. This is the first study which examined the genotoxicity effects of verbascoside extracted from *Phlomis nissolii* L plant on MCF-7 and MDA-MB 231 breast cancer cell lines. This adds importance to the study. Therefore, this study is thought to contribute to the literature

Key words: Apoptosis, Breast Cancer, Genotoxicity, MCF-7, MDA-MB231, Verbascoside

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CHAPTER I

INTRODUCTION

1.1.General Information

1.1.1. What is cancer? What are the Hallmarks of Cancer?

Cancer refers to a large group of diseases and it is characterized by the abnormal growth of the cells that would invade and spread from an origin site to the other parts of the body (National Cancer Institute, 2007). Cancer caused 9.6 million death in 2018 globally. While the most common cancer types are prostate, lung, colorectal, liver and stomach among the men; breast, lung, thyroid, colorectal and cervix cancers are the most common types among the women (Bray et al., 2018).

There are over 100 types of cancer and tissue origin of each gives distinguishing characteristics. It is known that transformation of only one of the 10^{14} cells in the body can create a tumor. A benign tumor does not spread throughout the body but some of the benign tumors can be life threatening due to their location (for example: it is very difficult to remove a benign brain tumor). But malignant tumors are not encapsulated and they can invade, and metastasize (Pecorino,2012). Cancers that arise from epitelial cells are called carcinoma, cancers that origin from mesoderm cells (for example bone and muscle) are called sarcoma and the cancers that origin from glandular tissue (for example breast) are called adenocarcinomas. Lymphoma arises from hematopoietic cells, leave the bone marrow and mature in lymph nodes. Leukemia arises from hematopoietic cell that leave the bone marrow then mature in the blood.germ cell tumors are derived from pluripotent cells and blastoma derives from immmature cells (precursor cells) or embryonic tissue. Cancers have distinctive properties depending on their origins and also each type has a different major cause. In vitro culture conditions, cancer cells show different features from normal cells. Normal cells produce monolayer because contact of the normal cells with neighbouring cells inhibits growth and this is called contact inhibition but cancer cells have a round morphology, they are not flat or extended and do not show contact inhibition so that they grow as piles of cells (foci) and also can grow in low serum (Pecorino,2012).

Hanahan and Weinberg (2000) defined six hallmarks of cancers. They proposed that evasion of growth inhibitory signals, unlimited replicative potential, evasion of cell death, growth signal autonomy, invasion-metastasis and angiogenesis are essential for carcinogenesis. In 2011, they reported two enabling characteristics because these characteristics are very crucial to acquire six hallmarks of cancer. These are tumor-promoting inflammation and genome instability. Also, avoiding immune destruction and reprogramming energy metabolism are emerging hallmarks. (Hanahan and Weinberg, 2011).

Faulty DNA repair pathways is one of the factors which contribute to genomic instability. This causes acquiring core hallmarks of cancer. Proto-oncogenes are mutated and they are activated then become oncogenes. Oncogene is a type of mutated gene and its protein product may be produced in high quantities or its activity may be increased. This initiates tumor formation in dominant way (Hanahan and Weinberg, 2011). This means that one mutation in only one allele shows an effect such as point mutations/deletions in coding sequences which may lead to structural and functional changes of proto-oncogene products. Deletions and point mutations in regulatory sequences may cause the over-expression. Fusion proteins and translocations of chromosomes alter expression (for example:translocation in immunoglobulin regulatory and c-myc sequences). Increase in gene dose and protein production is called gene amplification. For example, erbB2 is activated in breast cancer (Hanahan and Weinberg, 2011). Tumor suppressor genes code proteins which inhibit growth and also formation of a tumor. If there is a mutation causing a loss of function of tumor suppressor genes, growth can not be inhibited anymore. Mutations in tumor suppresor genes are recessive. Knudson's two-hit hypothesis states that both alleles should be mutated to initiate carcinogenesis. A patient can inherit one mutated tumor suppressor allele and also it is possible for this patient to get a second somatic mutation in time. Recent evidence suggests that haploinsufficiency (one allele mutated only) leads to having cancer phenotype (Fodde and Smits, 2002). In this case, only one of the normal alleles can produce half (haplo) of the amount of the protein that is produced by the normal cells. This amount cannot suppress formation of a tumor. Haploinsufficiency leads to genetic instability because it occurs in genes which regulate DNA repair and DNA-

damage response. Spectrum of a tumor may be affected by gene dosage (Fodde and Smits, 2002). Mutations in the p53 pathway lead to cancer. 75 % of these mutations are missense mutations that lead to single amino acid substitutions. 90 percent of them are in the DNA-binding domain which encode for amino acids 102–292 and 30% in six codons and they are referred to as "hotspots". Many mutated p53 molecules accumulate in the cells and they show "gain of function". Overexpression of the MDM2 protein alters the regulation of p53. Inactivation of Bax and FASR (downstream effectors) perturbs the apoptotic response. Retinoblastoma (Rb) is another tumor suppressor gene which has role in regulation of the cell cycle . This gene is mutated in many of the cancer types. Protein product of Rb gene inhibit cell proliferation normally. Activity of retinoblastoma gene is regulated the cyclin-dependent kinases (4/6) and cyclin D via phosphorylation(Pecorino,2012). The family of protein kinases are also included in cancer biology. Specific role of protein kinases is adding a phosphate group to the hydroxyl group of particular amino acids in proteins. Tyrosine residues are phosphorylated by tyrosine kinases and serine, threonine residues are phosphorylated by serine-threonine kinases and this causes a conformational change. Kinases are in the nucleus, at the surface of the cell as transmembrane receptors or in the cells as intracellular transducers. Kinases have major roles in transcription, in progression of the cell cycle and signal transduction thats why they are molecular targets for designing drugs to treat cancer. Ras acts to binding of a growth factor to its receptor so that it takes part in transmitting a signal from the receptor through the cell. G proteins are on the intracellular side of the plasma membrane. They are activated when GDP is exchanged for GTP in the cells. In over 50% of certain cancers, Ras family is found mutated (genomic instability) (Hanahan and Weinberg, 2011).

All tumors contain inflammatory immune cells. Acquisition of core hallmarks of cancer is facilitated by inflammation. Because these cells promote angiogenesis and invasion by supplying enzymes and growth factors. Also inflammatory immune cells cause mutations by releasing oxygen species (tumor-promoting inflammation) (Hanahan and Weinberg, 2011). Normal cells need growth factors to divide as external signals but cancer cells don't because short-circuit growth factor pathways and also acquired mutations cause unregulated growth

(Growth signal autonomy) (Hanahan and Weinberg, 2011). Cancer cells do not respond to the inhibitory signals for the maintenance of homeostasis. Because gene silencing and acquired mutations interfere inhibitory pathways (Evasion of growth inhibitory signal) (Hanahan and Weinberg, 2011). Successful cancer cells do not initiate an immune response to avoid destruction by the immune system therefore avoide immune destruction. Normal cells can divide 50 times (Hay Flick Limit) then become senescent because chromosomal ends (telomeres) shorten after every round of the DNA replication but telomeres in cancer cells do not shorten and this results in unlimited replicative potential (Unlimited replicative potential) (Hanahan and Weinberg, 2011).

Cancer cells move from their origin to other parts of the body but normal cells do not migrate. This can be because of alterations of the genome in cancer cells . This effects levels and activities of the enzymes that are involved in invasion or molecules that are involved in cellular–extracellular or cell–cell adhesion (Invasion and metastasis). Tumor cells invade, intravasate, transport, extravasate, and do metastatic colonization. Methylation of promoter part of *E-cadherin* gene, mutations in the extracellular domain and also alterations in expression of integrin receptor in the tumor cells help mobility and the invasion of the metastasizing cells because of modification of the membrane. E-cadherin (epithelial marker) like the others is downregulated, N-cadherin as other members of mesenchymal proteins is upregulated(Hanahan and Weinberg, 2011). Cancer cells and migrating tumor cells secrete MMPs (matrix metalloproteinases) and serine proteases. On the membrane of the tumor cells, EMMPRIN (extracellular matrix metalloprotease inducer) is upregulated so that MMP production is induced in adjacent stromal cells. Tumor cells can enter into a lymphatic vessel or blood (intravasation) then attach to the stromal face of the vessel. Tumor cells use MMPs and degrade the basement membrane. Serine proteases then pass (transendothelial migration) into the bloodstream through the endothelial cells. New blood vessels become tortuous and leaky when stimulated by the tumor cells (Hanahan and Weinberg, 2011).

Tumor cells also produce colony-stimulating factor 1(CSF1) which lead to chemotaxis-mediated co-migration. Tumor cells move in blood singly or as clumps combined with platelets that is known as emboli. Tumor cells are protected against

sheer forces in the bloodstream by emboli. Lungs and liver are first-pass organs. Tumor cells exit from lymphatic vessel or blood vessel by extravasation(Hanahan and Weinberg, 2011). Cancer cells stick to the endothelium via binding to adhesion molecule (E-selection) and this helps cancer cells to migrate transendothelialy. Metastatic colonization is progressive growth of a tumor at a distant part of the body with new blood vessels produced form the existing blood vessels by angiogenesis. When mature vessels are destabilized, the endothelial cells grow, migrate so that new blood vessels form called sprouting. Pro-angiogenic factors are *VEGF, FGF, HGF* and *EGF*. Tumor cells grow and produce hypoxic conditions by the help of hypoxia-inducible factor-1α (HIF-1α) and this induces angiogenesis. Promoter region of *VEGF gene* has hypoxia response element so that HIF-1 α targets *VEGF gene*. Loss of tumor suppressors and oncogenic proteins help the modification of the angiogenic switch. Growth factors are produced aberrantly so that endothelial cells grow. *VEGF* is upregulated by the oncogenic proteins such as intracellular transducers (such as Ras), receptor tyrosine kinases (for example, EGFR), intracellular tyrosine kinases (such as Src) and transcription factors (for example, Fos, Jun). Some of the tumor suppressor proteins upregulate/increase angiogenic inhibitors normally. *P53* binds and activates the promoter region of *thrombospondin-1 gene*. But if the *p53* gene is mutated, angiogenic inhibitors are decreased so that this will lead to angiogenesis. Tumor cells also can form vascular-like structures like endothelial cells . This is called vasculogenic mimicry (Hanahan and Weinberg, 2011).

Normal cells have inactive procaspases. These inactive procaspases are stimulated by apoptotic signals. Tumor cells have defective apoptotic pathway because of acquired mutations. Cancer cells have activated caspases but upregulated IAPs inhibit these activated caspases. Apoptotic signals stop IAP inhibition of activated caspases. In this way, cancer cells avoid apoptosis and more mutations accumulate (Evasion of cell death) (Hanahan and Weinberg, 2011). Glycolysis takes place in cancer cells even in the presence of oxygen. Unlike normal cells, intermediates of the glycolysis are used in biosynthetic pathways (Reprogramming energy metabolism) (Hanahan and Weinberg, 2011).

DNA repair is a type of defense against mutations. DNA repair systems are nucleotide excision repair (NER), one-step repair, base excision repair (BER), recombinational repair and mismatch repair. DNA lesions are repaired before cell replication. If not, this contributes to formation of carcinogenesis (Pecorino,2012).

Epigenetic modifications are heritable alterations but not because of the changes in the sequence of nucleotides in DNA so that they are not mutations. Epigenetic modifications, also called "tags," alter DNA accessibility and chromatin structure. DNA methylation and histone modification are examples of epigenetic alterations. Epigenetics affect conformation of chromatin and regulation of the transcription (Handy et.al.,2012). In several cancer types, mutation of DNA methyltransferase has been demonstrated. Hypermethylation occurs in the unmethylated CpG islands of gene promoters. Gene silencing by methylation can be a cause of carcinogenesis. For example, inactivation of *BRCA1* is due to hypermethylation in non-inherited breast cancer. C→T transitions may cause increased mutation rate that can be observed in methylated CpG islands. Methylation affects many genes such as *DAPK, Rb, APC, p16 INK4a* and the *estrogen receptor gene*(Pecorino,2012). There may be hypermethylation in specific genes and also hypomethylation may occur in repetitive DNA sequences or in the coding regions at the same time in cancer cells. This affects transcriptional activity because of activation of genes which are not expressed normally in cells. Mutations may disable some enzymes which are very important for epigenetic regulations. Epigenetic alterations can cause more epigenetic alterations and this leads to genomic instability and genome-wide alterations (Pecorino, 2012).

1.1.2. Breast Cancer Incidence in the World and in TRNC

Breast cancer has the highest rate in Belgium among women. Luxembourg and Netherlands follow Belgium as having second and third highest rate of breast cancer . This rate is the lowest in Canada (Bray et al. 2018). Breast cancer rates are higher among women living in more developed countries than undeveloped countries and still these rates are increasing globally (World Health Organization, 2012). Hincal et.al. (2008) evaluated cancer incidence in North Cyprus in comparison with North and South Europian countries covering the period of 1990-2004.

According to data obtained, there were 110 and 120 per 100,000 cases of cancer in males and females. The order of prevalence of cancer incidence among males were lung, skin, colorectal, prostate, brain, bladder, liver and stomach. The order of prevalence of cancer incidence for females is breast, gynaecological, skin, colorectal, lung, liver, brain, stomach and bladder. Lung cancer (male) and skin cancer (both sexes) were more common than in South and North Europe but breast (female), stomach (female), prostate, bladder (both sexes), corpus and cervix were less frequent. Total incidence rate of all cancers in North Cyprus was 201 per 100,000 in 2011 with prevalence rate of 460 per 100,000 (breast cancer (19.8%), prostate cancer (12.6%), colorectal cancer (9.7%), thyroid cancer (6.4%), and lung cancer (5.6%)). Güzelyurt Lefke has the highest rate of cancer than other districts (Gökyiğit and Demirdamar, 2016). 1854 men and 1809 women were diagnosed with cancer between the years 2012-2016 in North Cyprus according to statistical data obtained by TRNC Ministry of Health. It is seen that the majority of cancer cases in both sexes are diagnosed between the ages of 50-69. The incidence of cancer increases from age of 30 in women and from age of 50 in men (TRNC Ministry of Health, 2019) (Table 1).

AGE GROUP	MALE	FEMALE
0-14	1.2 %	1.1%
15-29	2.7%	2.9%
30-49	10.5%	28.0%
50-69	48.5%	43.3%
70+	37.1%	24.8%

Table 1 Percentage Distribution of Number of Cancer Cases According to Age Groups in TRNC (2012-2016): adapted from data of TRNC Ministry of Health

Breast cancer has the highest incidence among the women and prostate cancer has the highest incidence among the men(Table 2).

TYPE OF CANCER	MALE	FEMALE
Breast	6	547
Thyroid	83	260
Colon,rectum,anus	195	104
Lung,trachea,bronchus	236	48
Prostate	256	-
Bladder	206	45
Lymphoma	88	80
Stomach	64	39
Corpus and uterus NOS	-	81
Leukaemia	42	31

Table 2. Number of Cancer Cases in TRNC between years 2012-2016 , five-year average rates of new cancer cases per 100,000 population per year (TRNC Ministry of Health, published on 5 April 2019)

Breast cancer incidence among the women in North Cyprus is lower than incidence in Europe but higher than incidence in the world (Table 3)

Region	Incidence
South Cyprus	81.7
Europe	74.4
TRNC	62.2
World	46.3
Turkey	45.6

Table 3. Incidence of breast cancer in TRNC between years 2012-2016 (TRNC Ministry of Health, published on 5 April 2019)

1.1.3. Breast Cancer Classification

Patients having breast cancer express estrogen receptor, progesterone receptor and have HER-2/Neu amplification. These help clinicans to classify type of breast tumor.

There are 4 major subtypes of breast cancer: Luminal A, Luminal B, basal-like and HER2 amplified. Breast cancers can be classified according to their

molecular and histologic characteristics and can be sorted into one of at least four subtypes (Brenton et.al.,2005) as shown in the table below:

SUBTYPE	ER	PR	HER2
Luminal A	+	+/-	_
Luminal B	+	+/-	+
Basal (triple negative)	_	_	_
HER2 amplified	_	_	+

Table 4: Classification of Breast Cancer (Ayoup et al., 2017)

Luminal A type expresses proliferation marker Ki67 in low amount and it is responsive to hormone therapy and chemotherapy. Luminal B type expresses proliferation marker Ki67 in high amount and it is responsive to hormone therapy, chemotherapy and to HER2 antibody therapies. Basal (triple negative) type expresses proliferation marker Ki67 in very high amount, expresses EGFR+ and basal cell marker cytokeratin 5/6. It is often responsive to chemotherapy but not responsive to hormone therapy. HER2 amplified type expresses proliferation marker Ki67 in high amount and it is often responsive to HER2 therapies and chemotherapies (Ayoup et al., 2017).

1.1.4. Risk Factors For Breast Cancer

Factors that may cause a risk for breast cancer are never being pregnant, having only one pregnancy rather than many, no breast feeding, aging, having breast cancer history in family, mutations on *BRCA1*, *BRCA2*, *RB* and *p53* genes, postmenopausal estrogen substitution, postmenopausal hormone substitution as treatments, intake of contraceptives orally, less intake of vegetables and fiber, high intake of alcohol and fat and tobacco smoking (Wu et al., 2002). 70% of the female patients having breast cancer is about age of 50 or over and risk is 4x more (Somunoğlu 2007).

People having breast cancer history in their family tend to have breast cancer at early ages and this is very clear for whose mothers have breast cancer (Telo 2006). Although risk is around %7.8 in 80-year life duration, this risk increases to %13.3 if

there is one primary relative with breast cancer and increases to 21.1% when there are two primary relatives with breast cancer according to epidemiologic studies (Campbell, 2002; Bryant, 2004; Rogers et al., 2002; Williams et al., 2002; Smith et al., 2003). Hereditary breast cancers are only 5-10% of all breast cancers. In hereditary breast cancer, mutations on tumor suppressor genes *BRCA1*, *BRCA2*, *RB* and *p53* genes are effective. *BRCA1* gene is on chromosome 17, shows autosomal dominant transmission. *BRCA2* gene is on chromosome 13 and has role on early generation of breast cancer and bilateral diseases (Clamp et al., 2003). Catching breast cancer risk of women having *BRCA1* gene defect is 85% and catching breast cancer risk of women having *BRCA2* gene defect is 87% (Bryant 2004). Recessive *Retinoblastoma* gene is on chromosome 13 and loss of heterogeneity leads to breast cancer. *p53* gene is on chromosome 17 and there is a relation between loss of this gene and breast cancer. Also *erb-B2* oncogene gives valuable information to determine prognosis of breast cancer (Clamp et al., 2003). Probability of a woman to develop breast cancer is 3-4 times higher if the woman had breast cancer and was treated before than women who had no breast cancer history (Manjer et al., 2000, Campbell 2002)

Giving birth after age of 30 and not having pregnancy during life increase risk of having breast cancer (Clavel-Chapelon & Gerber, 2002; Campbell, 2002; Cuzick, 2003). This may be due to having lower levels of prolactin due to labour which has a protective affect against breast cancer (Telo 2006). Being pregnant and breast feeding reduce risk of having breast cancer about 4-7 times (Yoo et al., 2002). This risk reduces by 11% with breast feeding about 4-12 months and reduces by 25% with breast feeding about 2 years or more (Telo 2006). Women are under effect of estrogen longer if they have early menstruation and late menapouse. This increases risk of having breast cancer (Kruk and Aboul-Enein 2003). Estrogen treatment more than 10 years increases risk of having breast cancer (Driedger and Eyles, 2001;Clavel- Chapelon and Gerber, 2002, Cuzick, 2003). Contraceptive pills contain estrogen and supresses ovulation. Risk of having breast cancer increases by 36% if a woman takes contraceptives orally during 10 years (Telo 2006).

Putting on weight and being obese increase postmenapousal breast cancer risk by 30-50% but this is reverse for premenapousal period (McTiernan, 2003) because

longer menstrual cycles reduce effect of estrogen on breast cells (Ziegler et al., 1996). Diet poor in fat but rich in fruits, vegetables, fiber and complex carbohydrates reduces breast cancer risk. Diet which is rich in fiber reduces breast cancer risk due to inhibiting the absorption of estrogen from intestines (Scmizu et al., 1990). Medium or high intake of alcohol increases risk of premenapousal and postmenapousal breast cancer development (McTiernan 2003) . Because alcohol intake reduces amount of folate, vitamin C and beta-karoten in body and causes DNA damage that may lead to cancer (Singletary and Gapstur, 2001). Breast cancer has higher incidence in developed countries than underdeveloped countries due to not being pregnant, not having breast feeding, overconsumption of fat and alcohol (Özmen et al., 2009)

In addition to causes mentioned above, environmental factors which may lead to cancer are pollutants in soil, air, water; arsenic contamination of drinking water; pollution from heating and cooking; exposure to radon and asbestos; diesel engine emissions; second-hand tobacco smoke (Boffetta,2013). Having radiation therapy between puberty and age of 30 increases risk of having breast cancer. Because breast tissue is more active and more sensitive to carcinogenic effects of radiation (Clemons et al., 2000). Levels of heavy metals in environment higher than recomended levels by US Environmental Protection Agency are potential carcinogens. Humans are exposed to heavy metals by drinking water, consuming vegetables and fruits grown in contaminated soil. Heavy metals in water and soil in North Cyprus were analyzed by CRF/KAV in partnership with Frederick Institute of Technology. 225 composite soil samples from North Cyprus and 256 samples from South Cyprus collected from different areas of agriculture were analyzed. According to data obtained in North Cyprus, average levels of selenium was between 0.00-0.26 in rainy season and upper limit was 0.41 in dry season. 0.2 ppm optimum level for driving health benefits recommended by US Environmental Protection Agency. In 2005, same team collected 140 composite samples from different regions of North Cyprus as Güzelyurt, Yuvacık, Lefkoşa, Alevkayası, Mesarya, Bostancı, Karpaz and Kırnı. Level of lead was between 5.7-224.9 ppm which is lower than maxium allowed limit (400 ppm). Cadmium level was between 0.2-1.89 ppm which is higher than recommended value (1ppm) by EPA. Arsenic concentrations were between 0.2-

18.5ppm which are higher than safe limit (10ppm). Copper mining was started by Romans and Phoenicians 2000 years ago and deposits were reactivated from 1920 till 1974 in Güzelyurt-Lefke and there are 10 million tonnes of tailings containing heavy metals (Djamgoz et.al., 2017). Güzelyurt-Lefke has high incidence of cancer and further research needs to be done to measure the effects of mining and agrochemical usage in Lefke (Gökyiğit and Demirdamar, 2016).

1.1.5. Treatment of Breast Cancer

Early diagnosis is very important in that case appropriate type of treatment should be started after identification of the stage of the disease (Spears and Bartlett, 2009). Tumors having amplified HER-2/Neu can be treated with chemicals/ agents which inhibit HER-2/Neu. Triple-negative breast cancer can be treated with chemotherapy (Brenton et.al.,2005). Triple-negative breast cancer is an aggressive form of breast cancer with limited treatment options. Strategies used to treat breast cancer are surgery, radiation therapy, hormone therapy and radiotherapy plus adjuvant chemotherapy, (Arı 2010).

Basis of breast cancer treatment is chemotherapy. Usually combination of drugs used in chemotherapy is more effective to treat breast cancer (Sayek 2004). Metotreksat (M) 5-fluorourasil (F), siklofosfamid (C), epirubisin (E), adriamisin (A) are main anti-cancer drugs that are used in combinations like CMF, FAC, FEC, AC. Vinerelbin, dosetaksel and paklitaksel are also used on secondary stage. Mitomisin C and prednizon are the anti-cancer agents that are used in late stage of this disease (Mavroudis et al., 2009). However, these therapies have many side effects on body. For example, radiation therapy may cause rib fracture, second non-breast infield malignancies, tissue necrosis, brachial plexopathy. Cancer stem cells are very resistant to theraphies. Chemotherapy reduces white and red blood cells, increases risk of having infection and anemia, causes diarrhea, fatique, hair loss, sore throat, ulcers, nausea, constipation, loss of apetite and change in color of the skin. Some of the examples of complications associated with adjuvant treatment are skin changes, cardiac toxicity, neuropathy, reproductive dysfunction, arm lymph edema and pneumonitis (Agrawal, 2014). Due to these side effects, there has been a growing interest in alternative

treatment modalities. Finding alternative therapies with less or no side effects are essential. Many plant extracts have been used as alternative to treat cancer for many years. Verbascoside which may be extracted from many different species of plants has attracted great attention due to its pharmacological features (Schonbichler et al., 2013) such as its anti-inflammatory, anti-oxidative, anti-microbial, neuroprotective effects.

1.1.6. Chemical Properties of Verbascoside

Verbascoside known as acteoside is one of the disaccharide caffeoyl esters (Alipieva et al., 2014) and one of the phenylethanoid glycosides which is water soluble, naturally occuring in many plants. This chemical is characterised by hydroxyphenylethyl (C_6–C_2) moieties and cinnamic acid (C_6–C_3) which are linked to a β-glucopyranose by glycosidic bond (Dembitsky,2005). Verbascoside is characterized by having caffeic acid linked by a β-(D)-glucopyranoside to 4,5-hydroxyphenylethanol (hydroxytyrosol) bound through ester and glycosidic links, with a rhamnose in sequence (1–3) to glucose molecule (Dell'Aquila et al. 2014).

Scarpati and Monache (1963) first extracted phenylethanoid glycoside from *Verbascum sinuatum* L.; Scrophulariaceae and they called this substance as verbascoside. Birkofer et al. (1968) isolated the same substance from *Syringa vulgaris* flowers, Oleaceae and identified its structure as 2-(3, 4-dihydroxyphenyl)ethyl-1-O-α-$_L$- rhamnopyranosyl-(1→3)-(4-O-E-caffeoyl)-β-$_D$-glucopyranoside then called this substance as acteoside. Also, Andary et al. (1982) achieved to isolate verbascoside from another plant called broomrape (*Orobanche rapum-genistae*, Orobanchaceae). One year later, in 1983, Sakurai and Kato (1983) isolated this substance from Lamiaceae, *Clerodendron trichotomum* Thunb and identified this substance as kusaginin. Alipieva et al.(2014) reported that Verbascoside has been found in primary and secondary roots, also in stems, leaves and flowers of Verbascum species. It is found in many plant species (more than 200) at varying levels such as *Buddleja brasiliensis*, *Striga asiatica*, *Olea europea*, *Paulownia tomentosa* var. *tomentosa*, *Lippia javanica*, *Lantana camara*, and *Lippia citriodora*. Additionally, in olive mill waste water, verbascoside is abundant (De Marco et al., 2007; Dell'Aquila et al. 2014). Properties of Verbascoside are shown in table 5. Research in bioavability and metabolism of verbascoside is scarce. Sinico et al.

(2008) suggested that transdermal delivery of this chemical can be increased if it is combined by lipogels or liposomes. Quirantes-Pine et al. (2013) fed the rats with Lippia citriodora extracts and found plasma levels of verbascoside and isoverbascoside as approximately 80 and 60 ng/mL, respectively. It was confirmed that verbascoside is absorbed quickly by reaching highest plasma concentration after 15 minutes and is eliminated from the body of the rats (Li et al., 2014).

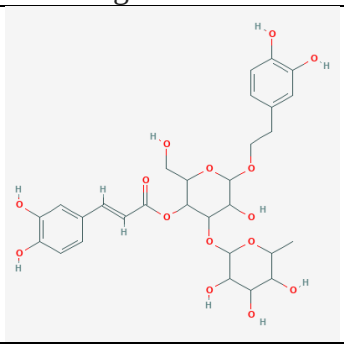
PubChem CID of verbascoside:	5459010
Chemical Names of verbascoside:	Verbascoside; Kusaginin; NSC603831; AC1NUR14; SCHEMBL657971; SCHEMBL13978365 More...
Molecular Formula of verbascoside:	**$C_{29}H_{36}O_{15}$**
Molecular Weight of verbascoside:	624.592 g/mol
Chemical diagram of verbascoside	
Hydrogen bond donor number	9
Hydrogen bond acceptor number	15
Rotatable bond number	11
Complexity of substance	936
Surface area (Topological polar)	245 A^2
Mass (monoisotopic)	624.205 g/mol
Mass	624.205 g/mol
Verbascoside is canonicalized	true
Formal charge	0
Heavy atom number	44
Defined atom, stereocenter number	0
Undefined atom, stereocenter number	10
Defined bond, stereocenter number	1
Undefined bond, stereocenter number	0
Isotopic atom number	0
Covalently-Bonded Unit number	1

Table 5: Features of verbascoside
(https://pubchem.ncbi.nlm.nih.gov/compound/5459010#section=Computed-Properties&fullscreen=true)

1.1.7. Literature About Verbascoside

Anti-Inflammatory Effects of Verbascoside

Many studies suggest that verbascoside has anti-inflammatory effects and may be used to treat Atopic dermatitis (Li et al., 2008). The researchers evaluated the effects of verbascoside on AD and found out that this substance relieved symptoms of AD like scratching and severity of skin lesion and significantly reduced Th2 cytokines and IgE induced by DNCB in peripheral blood, IL-6, IL-4 mRNA, TNF-α in human monocytes, THP-1 model at the site of skin lesion in a dose-dependent manner. De Moura et al. (2018) found similar results about the effects of verbascoside on inflammation in LPS induced microglial murine cells (N9). Authors displayed that verbascoside significantly reduced INFγ, IL-6,TNFα, MCP-1 and IL-12p70.

In a study, Mazzon et al.(2009) examined the effects of verbascoside in rats having 2,4 dinitrobenzene sulfonic acid (25 mg/rat) induced colitis. Verbascoside significantly reduced pro-MMP-9 activity, MMP-2 activity and the degree of NF-kappaB p65. This study suggested that verbascoside can treat inflammatory bowel disease because of being radical scavenger in cells. In another research, verbascoside extracted from *Plantago lanceolata* L. down- regulated secretion of IFN-γ that causes bowl disease induced by dextran sulphate in humans (Lenoir et al., 2011). Isoverbascoside and verbascoside isolated from *Castilleja tenuiflora* Benth provided protection against acute gastric ulcer in oedema model in mouse in vivo when compared with dexamethasone (Sanchez et al.,2013). Verbascoside prevented protein nitrosylation and intestinal damage in swine when it is supplied in diet (Di Giancamillo et al.,2013). When verbascoside is topically administered, it heals excision and scarification wounds in animal models (Korkina et al., 2007). Verbascoside extracted from Lemon verbena with combination of omega-3 fatty acids significantly reduces stiffness and pain and also increases physical functioning of subjects having joint discomfort (Caturla et al., 2011). Verbascoside has regulatory role in vascular inflammation caused by bacterial lipopolysaccharides and oxidised low density lipoproteins (Kostyuk et al.,2011). Lee et al.(2006) isolated acteoside from *Clerodendron trichotomum Thunberg* and investigated its effects on release of arachidonic acid and histamine in RBL 2H3 mast cells. This study showed

that in a dose-dependent manner, acteoside inhibited release of histamine caused by arachidonic acid, melittin, and thapsigargin in absence or the presence of extracellular Ca^{+2} and also inhibited release of arachidonic acid and production of prostaglandin E2 significantly induced by the substance melittin. Anti-inflammatory effect depends on down regulation of MAPK signalling (Ca^{2+}-dependent) in basophilic cells (Motojima et al., 2013); NO, TNF-α inhibition and production of IL-12 (Rao et al., 2009); inhibition of IL-8 secretion (Czerwińska et al.,2018); inducible nitric oxide synthase (iNOS) inhibition and release of NO from the macrophages if stimulated by the lipopolysaccharides of bacteria (Picerno et al., 2005); induction of heme oxygenase 1, high mobility group box 1 suppression (Seo et al., 2013). According to Speranza et al. (2010) verbascoside inhibits the iNOS, inhibits production of superoxide anion (intracellular) and suppresses glutathione, peroxidase catalase and SOD. Anti-INOS mechanism of verbascoside is related to the down-regulation of AP-1 and NFκB transcription factors which are important modulators of inflammatory processes (Lee et al.,2005). Also, verbascoside inhibits growth factors and downstream pro-inflammatory cytokines because, this chemical binds with aryl hydrocarbon receptor transcription factor and contributes to the transcription of detoxification genes that code glutathione S-transferase (GST), Nrf2, the cyto-chrome P450 CYP1 subfamily and antioxidant enzymes (Korkina et al., 2011; Potapovich et al., 2011). Verbascoside may be used to treat inflammatory diseases because it down-regulates TAK-1/JNK/AP-1 signallings which are pro-inflammatory signal transduction pathways. Verbascoside increases tyrosine phosphatase SHP-1 activity in U937 cell line (Pesce et al., 2015).

Anti-Oxidant Effects of Verbascoside

Verbascoside exhibited antioxidant activities in many experimental studies. Beyond well-investigated anti-inflammatory activities, it was demonstrated that verbascoside has also direct anti-oxidative, free radical scavenging effects (Sgarbossa et al.,2012).

Verbascoside is water soluble and penetrates into cell layers of the porcine skin slowly (Abdelouahab and Heard,2008) so that verbascoside extracted from *Buddleia davidii* has higher anti-oxidant capacity (Vertuani et al.,2011).

Lemon verbena extract containing high amount of verbascoside combined with omega-3 fatty acids showed very strong anti-oxidant properties in subjects having joint discomforts. It was suggested that verbascoside protected the blood components against oxidative stress associated with exercise (Catura et al. 2011) because this chemical modulates activity of GSH-reductase (Carrera-Quintanar et al., 2012). Also Lemon verbana extract protect neutrophils against oxidative damage produced during chronic exercise (Funes et al.,2011). Verbascoside enhanced glutathione-dependent enzymes and superoxide dismutase in the blood cells of female swimmers but also decreased sex hormone levels (Mestre-Alfaro et al., 2011). Liao et al. (1999) investigated effect of verbascoside by measuring contractility of Bufo gastrocnemius muscle which was stimulated electrically *in vitro*. Verbascoside resisted muscle fatique at 20.0 microM after 30 minutes of pretreatment because of its antioxidative properties. Instead of isolating verbascoside from plant parts, Cardinali et al.(2012) extracted phenolics from olive mill wastewater and identified components and assayed anti-oxidant properties of LH-20 fraction. Their results showed that LH-20 fraction of olive mill waste water contains isoverbascoside, verbascoside, β-hydroxyisoverbascoside and β-hydroxyverbascoside and verbascoside was the most active scavenger of ROS by protecting low-density lipoproteins against oxidative damage among the other chemicals in fraction. Di Giancamillo et al. (2015) evaluated the dietary verbascoside's effect on the oxidative stress produced by the intake of n-6 PUFA (n-6 polyunsaturated fatty acid) in blood and liver of weaned female Hypor piglets and pointed out that verbascoside partially restored the antioxidant role of the piglet liver but didn't affect the systemic responses. However, despite of the large data on anti-inflammatory effects of verbascoside, further clinical studies are needed to reveal mechanisms underlying these effects of verbascoside (Alipieva et al.,2014).

Protective Effects of Verbascoside From UV Irradiation

Excessive exposure to UV radiation induces photochemical reactions in skin surface lipids, modifies components of keratinocytes, accelerates proliferation of keratinocytes and modifies lipids in stratum corneum, causes irreversible damage to

proteins in skin and DNA when UV radiation reaches to epidermis and components of dermis in long term. Due to overproduction of reactive oxygen species and the absence of anti-oxidants, UV radiation leads to ageing and skin cancer. In addition, UVB induces CYP1A1, CYP1B1, cytochrome P450 subfamilies (Korkina et al., 2009; Pastore et al., 2012; Kostyuk et al., 2013).

Verbascoside absorbs UV light in 300–400 nm the wavelength range. Kostyuk et al. (2013) showed that verbascoside provides photo protection and prevents non-melanoma skin cancers induced by UV radiation. It suggested that verbascoside inhibited second phase of inflammation and responses of metabolism in keratinocytes to solar UV radiation (Potapovich et al., 2013). Also, *in vitro*, verbascoside inhibited melanin production and down regulated tyrosinase enyzme and inhibited α-melanocyte-stimulating hormone in melanoma cells (Munoz et al., 2013).

Neuroprotective Effects of Verbascoside

Sheng et al.(2002) examined effects of verbascoside on pheochromocytoma neuronal cells and determined the neuroprotective effects by using MTT assay, flow cytometry and measured levels of caspase-2 activity and extracellular hydrogen peroxide. The authors concluded that verbascoside decreased apoptotic death due to MPP+(1-methyl-4-phenylpyridinium ion) and also increased levels of extracellular hydrogen peroxide.

Pu et al.(2003) also showed that verbascoside inhibited apoptosis and neurotoxicity induced by 1-methyl -4-phenylpyridinium ion neurotoxin in cerebellar granule neurons in rats. Researchers suggested that verbascoside deactivated caspase-3 and proteolytic poly polymerase expression.

Deng et al.(2008) made a similar measurement about effects of verbascoside on SH-SY5Y neuronal cells against 1-methyl-4-phenylpyridinium ion-induced injury and suggested that verbascoside could be used to treat Parkinson's disease because this chemical decreased apoptosis. In another study, verbascoside showed protective effects on SH-SY5Y cell injury caused by Aβ(25–35) because this substance modulated apoptosis via cytochrome c release, Bcl-2 and caspase-3 cleavage (Wang et al.,2009). Esposito et al.(2010) proved

that neuroprotective effects of verbascoside on central nervous inflammation induced by LPS/IFN-γ by inhibiting expression of the neuronal nitric oxide synthase and averting activation of COX-2 in glioma cells.

Additionally, it was demonstrated that verbascoside has anti-amyloid effects because of its catechol moiety so that this chemical may be used to treat Alzheimer's disease because verbascoside activates HO-1 and Nrf2 nuclear translocation (Wang et al.,2012) in PC12 neuronal cells, inhibits aggregation of Aβ42 in a dose-dependent manner (Kurisu et al., 2013); acetyl-cholinesterase and butyrylcholinesterase (Georgiev et al., 2011); prolyl oligopeptidase (POP) (Filho et al., 2012); cholinesterase enzyme family (Kahraman et al., 2010). Lin et al. (2012) proved memory enhancing effects of verbascoside by using mice having memory deficit induced by scopolamine. Verbascoside improved the function of central cholinergic system in mice. In addition to neuroprotective effects, it was suggested that isoverbascoside has analgesic and anti-nociceptive effects as ibuprofen (Backhouse et al., 2008).

Anti-Microbial and Anti-Parasitic Features of Verbascoside

Recent studies suggest that verbascoside isolated from *Lippia* species has very high anti-*Cryptococcus neoformans* effects (Funari et al.,2012). Azimi et al. (2012) reported that pure verbascoside isolated from *Camellia sinensis* and *Commiphora mukul* may also be used to treat acne vulgaris because of its anti-inflammatory, anti-bacterial and also anti-androgen properties. In a study, Maquiaveli et al. (2017) suggested that verbascoside was very active against L. amazonensis amastigotes and suggested that this effect may be associated with the reduction in the protective oxidative mechanism of parasite because verbascoside inhibited parasite arginase.

Anti-Cancer Effects of Verbascoside

According to the recent publications, verbascoside has anti-metastatic properties and anti-estrogenic functions (Korkina, 2007) so that it may be used for chemoprevention in skin cancer, UV-associated cutaneous and non-melanoma malignancies (Kostyuk et al., 2013), myelo and other leukaemia types (Wartenberg

et al., 2003), human gastric carcinoma (Zhang et al., 2002); colorectal cancer (Zhou et al., 2014), oral squamous cell carcinoma (Zhang et al., 2018); Glioblastoma (Jia et al., 2018) because of inhibiting tumour cell proliferation, apoptosis induction (Wartenberg et al., 2003); repair of DNA damage due to oxidative stress (Li et al., 2000). In a study, Lee et al. (2007) showed that verbascoside inhibited human promyelocytic leukaemia HL-60 cell proliferation by 50% in concentration of 30 μM. It was suggested that verbascoside induces cell cycle arrest because this chemical blocks D2, D3 and E cyclins and CDK2, CDK6 cyclin-dependent proteins. Zhou et al. (2014) implanted CRC HCT-116 cells in the BALB/c nude mice and determined the effects of verbascoside on colorectal cancer *in vivo* and *in vitro* experiments. They administered control, 20, 40, or 80 mg/mL VB, or 1 mg/mL of fluorouracil (5-FU) to mice. The researchers determined expression of *p53, Bcl-2, HIPK2* and *Bax* by immunohistochemistry, effects of verbascoside on cell proliferation and apoptosis by using flowcytometry and CCK-8 assay and western blot was used to measure *Bax, Bcl-2, p53, HIPK2* and *p-p53*. It was seen that *in vitro,* verbascoside inhibited the proliferation of HCT-116, LoVo, SW620 and HT-29 cells in a dose and time dependent manner and also enhanced expression of *Bax, p53, HIPK2, p-p53,* decreased expression of anti-apoptotic *Bcl-2* in colorectal cells and increased cell apoptosis. In one of the newest study, Zhang et. al. (2018) tried to find out mechanism and function of verbascoside in human oral squamous cell carcinoma cells and showed that verbascoside promoted apoptosis, decreased metastasis and viability of HN4 and HN6 tumor cells, inhibited lung metastasis by suppressing activation of IκB kinase complex (IKK)/NF-κB-related signaling when it is injected intraperitoneally. In the same year, Jia et al.(2018) explored molecular mechanism of verbascoside in SHP-1 and STAT3 in glioblastoma xenograft mouse model. Results of this study demonstrated that verbascoside inhibited proliferation, migration, invasion of cells and promoted apoptosis by activating SHP-1 and by inhibiting phosphorylation of STAT3. In recent years, drug-loaded nanoparticles have attracted attention because of their therapeutic effects and become a hot topic (Auffinger et al., 2013). Chen et al. (2013) extracted verbascoside from Chinese Tsoong herb (Banchunmaxianhao, BCM) and coated with Ni nanoparticles and proved that VB-Ni induced apoptosis more than VB or Ni alone and resisted the

growth of K562 myelogenous leukemia cells that are doxorubicin-resistant *in vitro* and *in vivo*. Nga et al.(2017) isolated 5β,6β-dihydroxyantirrhide from Pseuderanthemum carruthersii (Seem.) Guill. var. atropurpureum (Bull.) Fosb. (Acanthaceae) leaves and thirteen different compounds including verbascoside and assayed cytotic activities and acetylcholinesterase inhibition against MCF-7 breast cancer cells and cervical cancer cells at a concentration of 100μg/mL. Isoverbascoside and verbascoside showed fairly weak AChE inhibitory activity but strong cytotoxic activity against MCF-7 breast cancer cells.

1.2. AIM OF THE STUDY

This study aimed to evaluate the genotoxic effects of Verbascoside on MCF-7 and MDA-MB-231 cells line *in vitro.*

1.3.IMPORTANCE OF THE STUDY

Breast cancer is causing many deaths among women in the world (Chen et al., 2013). Treatment strategies for breast cancer are radiotherapy with an adjuvant chemotherapy, radiation therapy, hormone therapy and surgery but each of these treatments have many side effects on human body. That's why finding alternative therapies with less or no side effects is essential. Extracts of many plants have been used as an alternative for treatment of cancer many years. Verbascoside which may be extracted from many different species of plants has attracted attention because of its pharmacological features (Schonbichler et al., 2013) such as anti-inflammatory, anti-oxidative, neuroprotective and anti-microbial effects. This chemical protects the skin against UV radiation. The data about the anti-metastatic and chemopreventive effect of verbascoside is limited *in vitro* and in animal studies but it is seen as a good candidate drug for cancer treatment such as UV associated cutaneous and non-melanoma malignancies, human gastric carcinoma, myelo and other leukaemia types.

Studies about the cytotoxic effects, underlining mechanism and function of verbascoside on breast cancer are scarce so that intensive studies are needed to confirm the effects of verbascoside on breast cancer. This increases the importance of this study.

1.4.LIMITATIONS

This study is limited with exploring the cytotoxic effect of verbascoside only on MCF-7 and MDA-MB-231 cell line *in vitro*.

CHAPTER II

2.1. MATERIALS

2.1.1. Chemicals

Penicillin-Streptomycin (+10000 μg/mL streptomycin, +10000 units/mL penicillin); Insulin, Human (at 4mg/ml); Fetal Bovine Serum (and DMEM /F-12(1:1)(1x) (Dulbecco's Modified Eagle medium F-12 suplemented with +L-glutamate, +15mM HEPES, Nutrient Mixture (ham); Trypsin/EDTA Solution, 0.25% (Stored at -20^{0}C) were purchased from Gibco, USA. DMSO-Dethyl Sulfoxide was obtained from Che Cruz, USA.

2.1.2. Kits

Cell viability/cytotoxicity: TEBU-BIO cell counting kit 8 (CCK8) was purchased from Tebu, France. Nonradioactive CCK-8 kit is used for sensitive colorimetric assays to calculate number of viable cells in the cytotoxicity assays and cell proliferation. This one-bottle solution does not require premixing of components. It uses tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). WST-8 is reduced by dehydrogenases to give water-soluble formazan dye (orange colored) in cells. The amount of formazan is directly proportional to the number of viable cells.

2.1.3. Instrumentation

Class II Type A2 Biological Safety Cabinet (ESCO, Singapore)

Humidified incubator (at 37 °C, 5% CO2), ((Binder,USA)

Centrifuge (Sigma 3-18K, UK)

Inverted microscope (Olympus IX53, UK)

Tunable microplate reader (Versa max,USA)

External computer (Bross,USA)

SOFTmaxÆ PRO software,USA

GraphPad® Prism software version 8, San Diego

Freezer (Panasonic, Tweenguard,Japan)

Water bath (Combatible control, CC1, Huber, USA)

Falcon tubes (Eschau, Germany)

Cryotubes (Eschau, Germany)

Pipette controller,pipettes,micropipettes(Eppendorf research,Hamburg,Germany)

Hemocytometer (counting chamber, Neubauer,Germany)

Cell culture T-75 flasks (NEST, Jiangsu, China)

96-well plates (NEST, Jiangsu, China)

2.1.4. Plant Extract

The genus Phlomis L. has 34 species in Turkey and *Phlomis nissolii L.* (Lamiaceae) which is endemic to Turkey was collected from Mersin, Gülnar, South Anatolia, Turkey and verbascoside was extracted from *Phlomis nissolii* L. plant by Prof. Dr. İhsan Çalış (Kırmızıbekmez et.al., 2004).

2.1.5. Cell Lines

Breast cancer luminal A molecular subtype MCF-7 cell line was obtained from the American Type Culture Collection (ATCC), Rockvilles, Maryland, USA. MDA-MB 231 cell line was supplied by Middle East Technical University,Turkey.

2.1.5.1 MCF-7 Breast Cancer Cell Line

Name of the MCF breast cancer cell line derived from Michigan Cancer Foundation by Dr. Soule and his colleagues in 1973. MCF -7 cells were first isolated from metastatic breast mammary gland of a Caucasian woman at age of 69 having metastatic disease, Adenocarcinoma by the pleural effusion (Soule et al., 1973). It was shown that tamoxifen is an anti-estrogen agent and can inhibit MCF-7 cell growth but also it was demonstrated that this effect could be reversed by estrogen (Levenson and Jordan,1997).

Characterization of MCF-7 breast cancer cells

MCF -7 cells produce 3D multicellular aggregates that produce lumen-containing spheroids (Do Amaral et.al., 2010). MCF-7pl cells form compact polygonal shaped colonies and these colonies are in contact with each other. Some of

the cells having fibroblastic shape at the border of colonies and move away from the colony. Cell–cell adhesion is due to E-cadherin protein in spheroids (Dittmer et.al., 2009) . But MCF-7A3 colonies are not compact and move away from the parental colony (Pérez-Yépez et.al., 2012). MCF-7 cells are estrogen receptor positive breast cancer cells. Many sub-clones of MCF-7 cells represent different classes of ER-positive tumors having different levels of nuclear receptor expression (Sweeney et al., 2013). These cells are PR (progesterone receptor)-positive (Shirazi, 2011), belong to luminal A molecular subtype (Done, 2011) and they are non-invasive, poorly-aggressive cell lines (Gest et al., 2013). MCF-7 breast cancer cells have low metastatic potential (Shirazi, 2011) and present extensive aneuploidy (chromosome numbers ranging from 60 to 140). They present cytogenetic differences such as having specific marker chromosomes or not and have an elevated level of genetic instability. This cell line contains stem cells which are able to generate clonal variability and different variants undergo divergence at RNA expression levels and genomic levels (Nugoli et al., 2003). MCF-7 breast cancer cells suits for anti-hormone therapy resistance research very well because of retaining ER expression during culturing (Sweeney et al., 2013).

Molecular profile of MCF-7 breast cancer cells

MCF-7 breast cancer cells depend on E2 to proliferate (Perrot-Applanat and Di Benedetto, 2012). They express *ERα* in very high levels and *ERβ* in very low levels *(*Buteau-Iozano et al., 2002) and contain 17β-estradiol receptor (Brooks et al., 1973). They strongly express progesterone in the parental line but absent or weak in tamoxifen resistant sublines (Baguley and Leung, 2011). Reduced rate of proliferation lasts for about a month after estrogen is removed (Sweeney et al., 2013). Responsiveness of MCF-7 breast cancer cells to E2 is because of dependence on an autocrine factor that activates IGF-IR (Hamelers et.al., 2003). IGF-1 signaling has role in the regulation of miRNAs (Martin et.al., 2012). ER, human epidermal growth factor receptor-2, PR and EGFR activated by EGF control MCF-7 cell growth (Baguley and Leung, 2011). Triple negative sub-lines have the origin in the ER-positive MCF-7 cell line (Leung et.al., 2011. MCF-7 cells show differentiated mammary epithelium features. They

are positive for various epithelial markers including β-catenin, cytokeratin 18 and E-cadherin (D'Anselmi et.al., 2013) . MCF-7 cells are negative for the mesenchymal markers such as smooth muscle actin (SMA) and vimentin (D' Anselmi et.al., 2013). MCF-7 parental cells express claudins (as specific molecular marker of epithelial cells) (Pérez-Yépez et.al., 2012). They express zona occuldens protein 1 (ZO-1) forming intercellular junctions (Pérez-Yépez et.al., 2012). MCF-7 cells are deficient of CD44 (Dittmer et.al., 2009) . MCF-7 cells do not express GHRHR (Barabutis et.al., 2007). The MCF-7 cells abundantly express VEGF receptor 1 and neuropilin-1 but express VEGFR2 poorly (Guo et.al., 2003; Lee et al., 2007) and secrete low amount of VEGF-A, VEGF-D and VEGF-C (Timoshenko et.al.,2006). MCF-7 breast cancer cells have poor angiogenic potential (Aonuma et al., 1999). Interestingly, serum deprivation causes dramatic reduction in proliferation but does not cause apoptosis (Barabutis et.al., 2007).

2.1.5.2. MDA-MB-231 Breast Cancer Cell Line

The MDA-MB-231 breast cancer cell line is epithelial and was first isolated from a 51-year-old Caucasian female by pleural effusion. This woman had metastatic mammary adenocarcinoma and invasive ductal carcinoma (Cailleau et.al.,1978). MDA-MD-231 cells show endothelial-like morphology (Harrell et.al.,2014) and have stellate projections that bridge multiple cell colonies (Kenny et.al.,2007). MDA-MD-231 cells can metastasize to the lymph nodes and its subclones can metastasize to the brain, lungs and bones (Chavez et.al.,2010). MDA-MB-231 adenocarcinoma cells are triple negative. Their receptor status is ER-, PR-, HER2-, E-cadherin-, EGFR+ , Caspase 2 and 3 +. These cells express mutated *p53*. They are very agressive, invasive, poorly differentiated and also resistant to several anti-cancer agents (Islam and Resat,2017; Chavez et.al.,2010; Gest et al.,2013).

2.2. METHODOLOGY

2.2.1 Freezing and Thawing Cell Lines

Cell lines were stored in cryotubes containing DMSO (Dimethyl sulfoxide (%5) in a freezer at (-) 80^0C. This prevents formation of ice crystals.

Frozen cells were thawn in a waterbath at 37 ^{0}C and then transferred into a centrifuge tube containing 15ml culture medium. Contents in tube were centrifuged (speed 1000, RCF 192, time 8min., temperature 22^{0}C). Then supernatant was discarded from the tube.

2.2.2. Cell Culture

MCF-7 and MDA-MB-231 cell lines were grown in T75 flasks with the supplement of 45ml DMEM/F-12 (1:1) (1X) containing F-12 Nutrient Mixture (Ham), L-Glutamate, 15mM HEPES, 5ml Fetal Bovine Serum (%10), 125ml insulin human (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 other day.

2.2.3. Subculturing

T-75 flasks were taken out from the incubator and examined under inverted microscope at low power. Cells were subcultured if there were no floating cells and they were 80-100% confluent. Culture medium was removed from the flasks. Then cell layer was rinsed briefly with 2ml, 0.25% (w/v) Trypsin/EDTA to remove traces of serum having Trypsin/EDTA inhibitor. 7ml trypsin was added to flask and placed in incubator and left for 10 minutes at 37°C. Then cells were examined under inverted microscope to be sure that all the cells are detached and floating. 6.0 to 8.0 mL of growth medium was added into flask and cells were aspirated by pipetting gently. After transfer of cell suspension into the centrifuge tube containing medium and cells, this tube was centrifuged for 5 minutes (RCF:192, temperature: 24°C, speed: 1000). Then supernatant was discarded. 1000 microliter of medium was added into the centrifuge tube containing only cells. 100 microliter of suspension is placed on hemocytometer (counting chamber) and number of cells were counted. 2.1 x 10^6 cells were seeded into a new sterile flask containing 15ml medium. Then flask was placed in incubator and incubated at 37°C, 5% CO_2 .

2.2.4. Preparation of stock solution and various concentrations of verbascoside

2.5 mg of verbascoside was dissolved in 1000 µl of distilled water. Then stock solution containing 0.0625 mg verbascoside and 975 µl of medium was prepared. This stock solution was used to prepare various concentrations of verbascoside as 100 µM, 48 µM, 25 µM, 10 µM, 1 µM, 0.5 µM and 0.1 µM.

2.2.5. Measuring cell viability/Cytotoxicity

Cytotoxic activity of verbascoside was evaluated by using TEBU-BIO cell counting kit 8 on breast cancer cell lines MCF-7 and MDA-MB231. Following procedure recomended by the company was applied. 100 µl of cell suspension was dispensed (5000 cells/ well) in each of 3 x 96-well plate. Plates were pre-incubated for 24 hours in a humidified incubator (at 37°C, 5% CO_2). Plates were observed under inverted microscope to be sure that they were attached. Then medium in each well was removed. 10 µl of various concentrations of verbascoside (100 µM, 48 µM, 25 µM, 10 µM,1 µM, 0.5 µM and 0,1 µM) were added to be tested to the plates. Each of the three plates was labeled as duration of exposure, name of cells and date. First plate was incubated for 24hours, second plate was incubated for 48 hours and third plate was incubated for 72 hours in the incubator. 10 µl of CCK-8 was added into each well of the plates with attention at the end of the incubation period. The plates were incubated for 4 hours in the incubator. The absorbance was measured at 450nm by using Versa max tunable microplate reader connected to an external computer.

2.2.6. Statistical Analysis

GraphPad® Prism software version 8 was used to calculate IC_{50} values by the application of non-linear regression curve fit analysis. Further statistical analysis was performed using Two-way Anova Multiple Comparisons Test to determine the significance of mean difference between control and other concentrations of verbascoside on MCF-7 and MDA-MB 231 cell lines for 24h, 48h and 72h of exposure respectively.

CHAPTER III

RESULTS

3.1. CYTOTOXICITY RESULTS FOR MCF-7 BREAST CANCER CELL LINE

Different concentrations of verbascoside 100, 48, 25,10,1, 0.5 and 0.1 μM were exposed to MCF-7 breast cancer cells and the absorbance values were measured at 450nm using Versa max tunable microplate reader. Concentrations of verbascoside used in the study were converted to molar to be able to enter data into graph pad prism 8 programme. All absorbancy values were normalized to control (max.100-min.0). Normalized values were analyzed by using nonlinear regression analysis and IC50 (%95 CI) values were calculated. IC50 values calculated in μM and R^2 values for each exposure time to verbascoside are shown in table 6. IC50 value and R^2 value for MCF-7 breast cancer cells are 0.127 μM and 0.9630 respectively after 24h of exposure to different concentrations of verbascoside. IC50 value and R^2 value for MCF-7 breast cancer cells are 0.2174 μM and 0.8789 respectively after 48h of exposure to different concentrations of verbascoside. IC50 value and R^2 value for MCF-7 breast cancer cells are 0.2828 μM and 0.8752 respectively after 72h of exposure to different concentrations of verbascoside.

Exposure Time to verbascoside	**IC50 (μM)**	**R^2**
24 hour	0.127	0,9630
48 hour	0.2174	0,8789
72 hour	0.2828	0,8752

Table 6: IC50 values in μM and R^2 values for MCF-7 cell line

3.2. ANOVA TEST RESULTS FOR MCF-7 CELL LINE

Two-way Anova Multiple Comparisons Test was performed to determine the significance of mean difference between control and other concentrations of verbascoside for MCF-7 cell line after 24h, 48h and 72h exposure respectively.

Two-way Anova multiple comparisons test results for MCF-7 cell line after 24h exposure to different concentrations of verbascoside are shown in figure 1. Mean difference is not significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 48, 25 and 10 µM verbascoside concentrations. Mean difference is significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 100, 1, 0.5 and 0.1 µM verbascoside concentrations. When concentration of verbascoside is decreased from 100 to 10 µM, absorbancy increases so that number of alive cells increases. When concentration of verbascoside is further decreased from 10 to 0.1 µM , absorbancy decreases so that number of alive cells decreases but number of dead cells increases. But all absorbacy values are higher than control so that 100, 48, 25,10,1, 0.5 and 0.1 µM concentrations of verbascoside are not effective on MCF-7 breast cancer cells after 24h exposure.

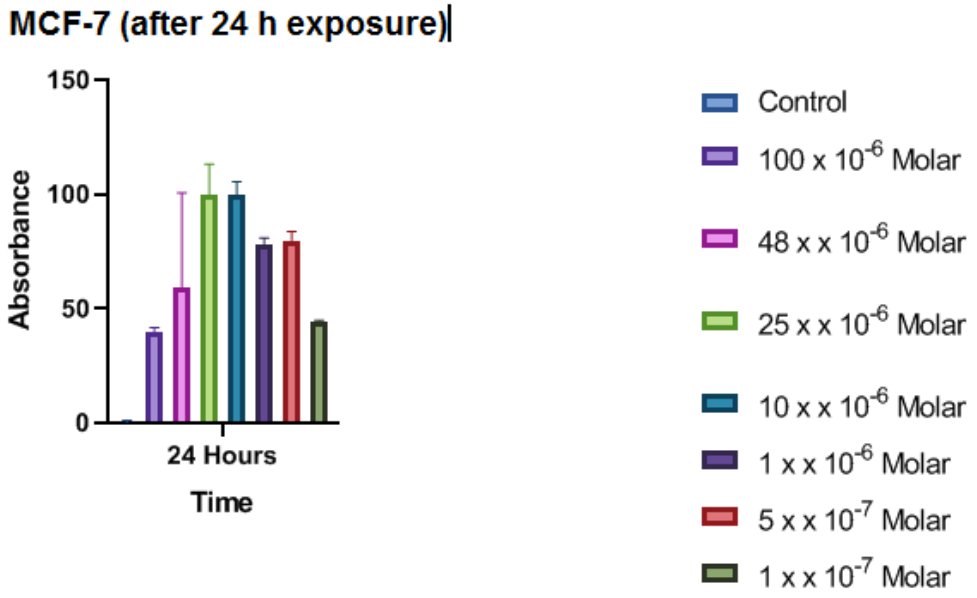


Figure 1 Two-way Anova multiple comparisons test results for MCF-7 cell line after 24h exposure to different concentrations of verbascoside

Two-way Anova multiple comparisons test results for MCF-7 breast cancer cell line after 48h exposure to different concentrations of verbascoside are shown in figure 2. Mean difference is not significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 100, 48, 25, 10, 1, 0.5 and 0.1 µM verbascoside concentrations. When concentration of verbascoside is decreased from 100 to 10 µM, absorbacy increases so that number of alive cells increases. When concentration of verbascoside is further decreased from 10 to 0.1 µM , absorbancy decreases so that number of alive cells decreases but number of dead cells increases. But all absorbacy values are higher than control so that 100, 48, 25, 10, 1, 0.5 and 0.1 µM concentrations of verbascoside are not effective on MCF-7 breast cancer cells after 48h exposure.

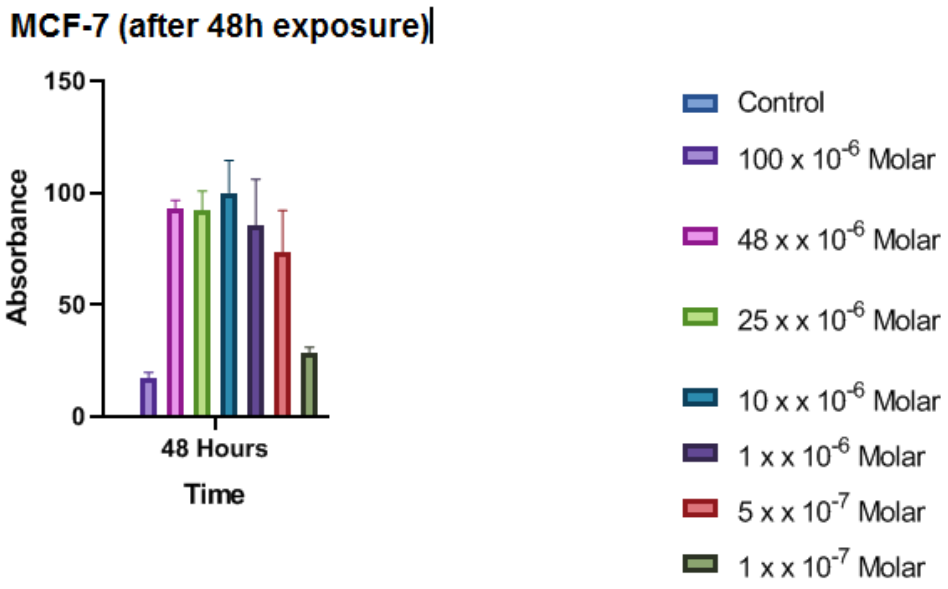


Figure 2 Two-way Anova multiple comparisons test results for MCF-7 cell line after 48h exposure to different concentrations of verbascoside

Two-way Anova multiple comparisons test results for MCF-7 breast cancer cell line after 72h exposure to different concentrations of verbascoside are shown in figure 3. Mean difference is not significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 100, 48, 25, 10, 1, 0.5 and 0.1 µM verbascoside concentrations. When concentration of verbascoside is

decreased from 100 to 25 µM, absorbancy increases so that number of alive cells increases. When concentration of verbascoside is further decreased from 25 to 0.1 µM, absorbancy decreases so that number of alive cells decreases but number of dead cells increases. Absorbancy value at 100 µM verbascoside is the lowest among the other absorbancy values so that lowest number of alive cells but highest number of dead cells are at this concentration. 100 µM verbascoside has the highest cytotoxic effect on MCF-7 breast cancer cells after 72h exposure.

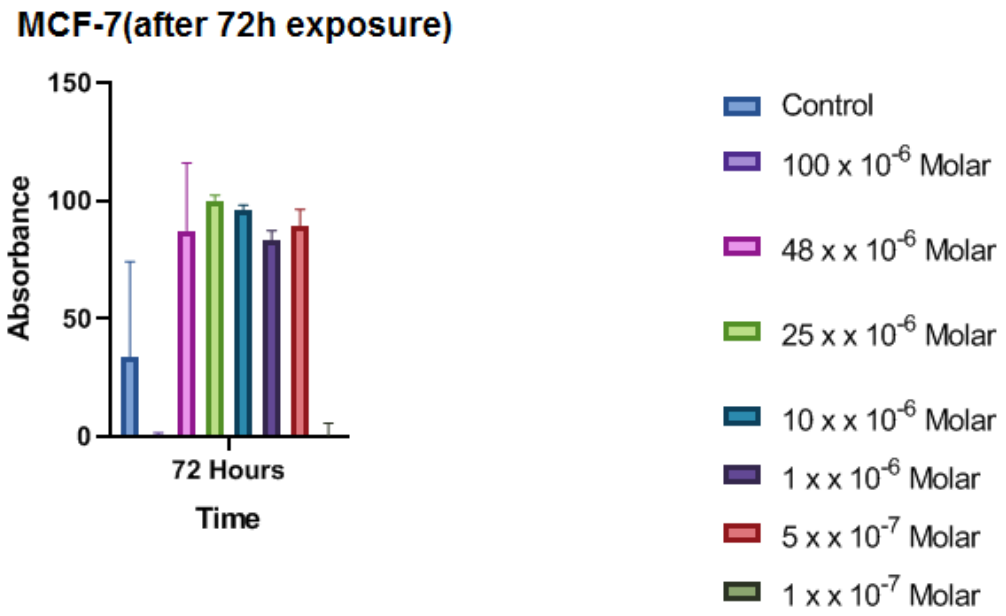


Figure 3 Two-way Anova multiple comparisons test results for MCF-7 cell line after 72h exposure to different concentrations of verbascoside

3.3. CYTOTOXICITY RESULTS FOR MDA-MB 231 BREAST CANCER CELL LINE

Different concentrations of verbascoside 100, 48, 25,10,1, 0.5 and 0.1 µM were exposed to MDA-MB 231 breast cancer cells and the absorbance values were measured at 450nm using Versa max tunable microplate reader. Concentrations of verbascoside used in the study were converted to molar to be able to enter data into graph pad prism 8 programme. All absorbancy values were normalized to control

(max.100-min.0). Normalized values were analyzed by using nonlinear regression analysis and IC50 (%95 CI) values were calculated. IC50 values calculated in μM and R^2 values for each exposure time to verbascoside are shown in table 7. IC50 value and R^2 value for MDA-MB 231 breast cancer cells are 0.1597 μM and 0,8438 respectively after 24h of exposure to different concentrations of verbascoside. IC50 value and R^2 value for MDA-MB 231 breast cancer cells are 0.2584 μM and 0,5107 respectively after 48h of exposure to different concentrations of verbascoside. IC50 value and R^2 value for MDA-MB 231 breast cancer cells are 0.2563 μM and 0. 9203 respectively after 72h of exposure to different concentrations of verbascoside.

Exposure Time to verbascoside	**IC50 (μM)**	**R^2**
24 hour	0.1597	0,8438
48 hour	0.2584	0,5107
72 hour	0.2563	0,9203

Table 7: IC50 values in μM and R^2 values for MDA-MB 231 breast cancer cell line

3.4. ANOVA TEST RESULTS FOR MDA-MB 231 BREAST CANCER CELL LINE

Two-way Anova multiple comparisons test results for MDA-MB 231 breast cancer cell line after 24h exposure to different concentrations of verbascoside are shown in figure 4. Mean difference is not significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 100, 48, 25, 10, 1, 0.5 and 0.1 μM verbascoside concentrations. When concentration of verbascoside is decreased from 100 to 0.5 μM, absorbancy increases so that number of alive cells increases. When concentration of verbascoside is further decreased from 0.5 to 0.1 μM, absorbancy decreases so that number of alive cells decreases but number of dead cells increases. Absorbancy value at 100 μM verbascoside is the lowest among the other absorbancy values so that lowest number of alive cells but

the highest number of dead cells are at this concentration. 100 µM verbascoside has the highest cytotoxic effect on MDA-MB 231 breast cancer cells after 24h exposure.

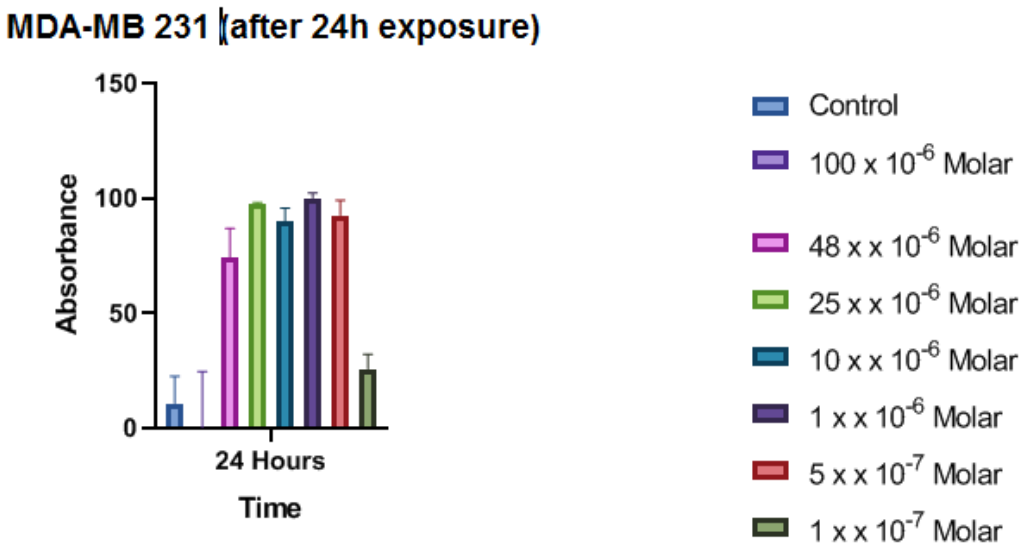


Figure 4 Two way Anova multiple comparisons test results for MDA-MB231 cell line for 24h exposure of verbascoside

Two-way Anova multiple comparisons test results for MDA-MB 231 breast cancer cell line after 48h exposure to different concentrations of verbascoside are shown in figure 5. Mean difference is not significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 100, 48, 25, 10,0.5, 1 and 0.1 µM verbascoside concentrations. When concentration of verbascoside is decreased from 100 to 25 µM, absorbancy increases so that number of alive cells increases. When concentration of verbascoside is further decreased from 25 to 0.1 µM , absorbancy decreases so that number of alive cells decreases but number of dead cells increases. Absorbancy value at 100 µM verbascoside is the lowest among the other absorbancy values so that lowest number of alive cells but the highest number of dead cells are at this concentration. 100 µM verbascoside has the highest cytotoxic effect on MDA-MB 231 breast cancer cells after 48h exposure.

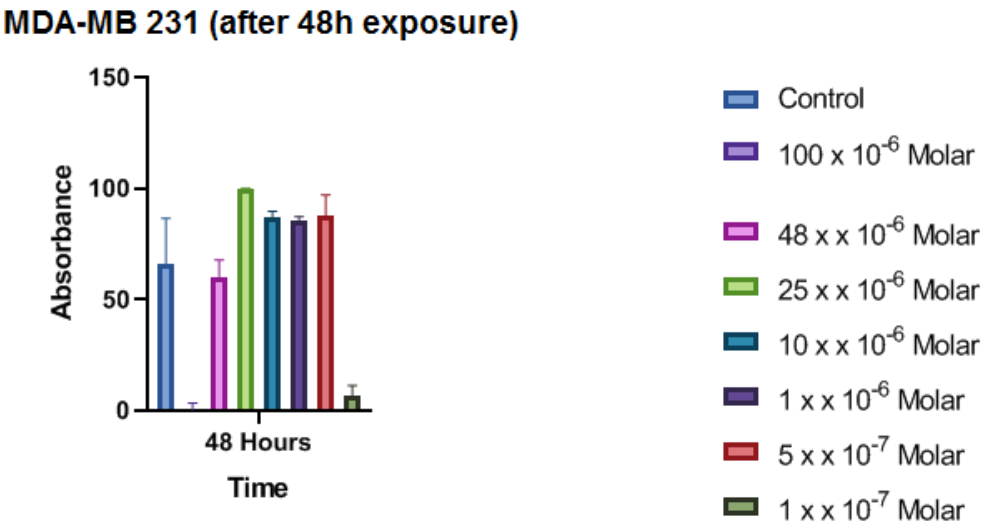


Figure 5 Two- way Anova multiple comparisons test results for MDA-MB231 cell line for 48h exposure of verbascoside

Two-way Anova multiple comparisons test results for MDA-MB 231 breast cancer cell line after 72h exposure to different concentrations of verbascoside are shown in figure 6. Mean difference is not significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 48, 25, 10 and 1 µM verbascoside concentrations. But mean difference is significant at 95 % confidence level between control absorbancy value and absorbancy values obtained at 100, 0.5 and 0.1 µM verbascoside concentrations.When concentration of verbascoside is decreased from 100 to 0.5 µM, absorbacy increases so that number of alive cells increases. When concentration of verbascoside is further decreased from 0.5 to 0.1 µM , absorbancy decreases so that number of alive cells decreases but number of dead cells increases. Absorbancy value at 100 µM verbascoside is the lowest among the other absorbancy values so that lowest number of alive cells but the highest number of dead cells are at this concentration. 100 µM verbascoside has the highest cytotoxic effect on MDA-MB 231 breast cancer cells after 72h exposure.

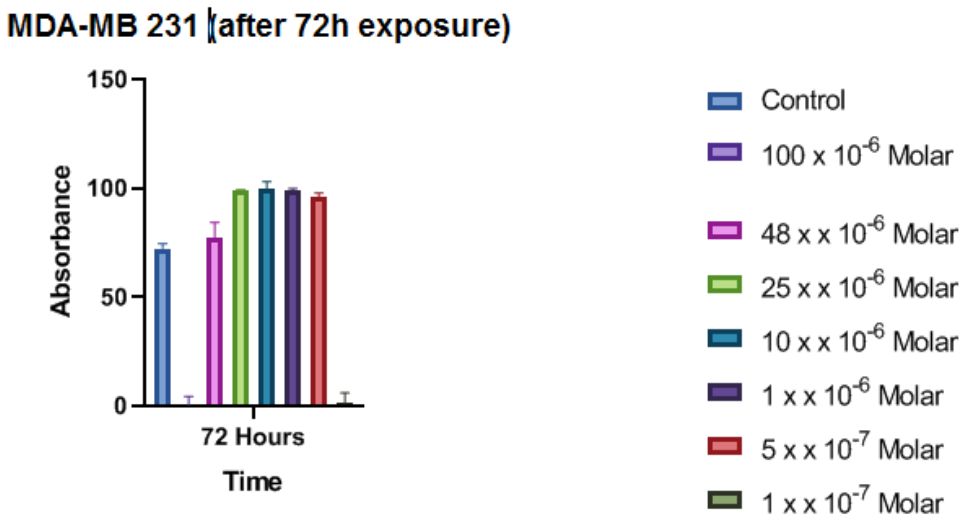


Figure 6 Two way Anova multiple comparisons test results for MDA-MB231 cell line for 72h exposure of verbascoside

CHAPTER IV

4.1 DISCUSSION AND CONCLUSION

Breast cancer affects 1.2 million people every year, especially women in the world (World Health Organization). The prevalence and increasing frequency of breast cancer, being diagnosed and treatable in the early stages increases its importance (Parkin 2002). Despite advances in treatment in the early stage of breast cancer, many women experience recurrence and metastasis. Although the treatment strategies are limited, the main focus is on medical treatment. The importance of classical treatment methods in cancer treatment is indisputable (Aggarwal and Shishir, 2006). Treatment strategies are surgery, radiation therapy, hormone therapy and radiotherapy with adjuvant chemotherapy but these therapies have many side effects on human body. However, increase in the number of cancer cases and resistance to the drugs increase the need for new diagnostic and treatment methods. Since the success of traditional treatments is limited, most cancer patients try complementary and alternative medical therapies.. There has been a growing interest in alternative treatment modalities. Finding alternative therapies with less or no side effects are essential.

Verbascoside is one of the phenylethanoid glycosides and can be extracted from different species of plants such as *Verbascum sinuatum* L.; Scrophulariaceae, *Syringa vulgaris*. Verbascoside which may be extracted from many different species of plants has gained great attention because of its pharmacological features (Schonbichler et al., 2013) anti-inflammatory (Lee et al., 2006 ; Li et al., 2008; Mazzon et al., 2009; Rao et al., 2009; Lenoir et al., 2011; Kostyuk et al., 2011; Pesce et al., 2015), anti-oxidative (Vertuani et al., 2011; Catura et al. 2011; Mestre-Alfaro et al., 2011; Carrera-Quintanar et al., 2012; Cardinali et al. 2012; Sgarbossa et al., 2012; Alipieva et al., 2014; Di Giancamillo et al., 2015), neuroprotective (Sheng et al., 2002; Pu et al. , 2003; Backhouse et al., 2008; Deng et al., 2008; Wang et al., 2009; Esposito et al., 2010; Kahraman et al., 2010; Filho et al., 2012; Lin et al., 2012; Wang et al., 2012; Kurisu et al., 2013) and anti-microbial effects (Azimi et al., 2012; Funari et al., 2012; Maquiaveli et al.; 2017).

This chemical also protects the skin against UV radiation (Korkina et al., 2009; Pastore et al., 2012; Kostyuk et al., 2013; Munoz et al., 2013; Potapovich et al., 2013).

Verbascoside has also anti-metastatic properties, anti-estrogenic functions (Korkina, 2007). Verbascoside can be used for chemoprevention in skin cancer and to treat UV-associated cutaneous and non-melanoma malignancies (Kostyuk et al., 2013), myelo and leukaemia types (Wartenberg et al., 2003; Zhang et al., 2002; Lee et al., 2007; Chen et al., 2013) , human gastric carcinoma (Zhang et al., 2002); colorectal cancer (Zhou et al.,2014), human oral squamous cell carcinoma (OSCC) (Zhang et al., 2018); Glioblastoma (Jia et al., 2018). It has gained significance due to its properties of inhibition of tumour cell proliferation, induction of apoptosis (Wartenberg et al., 2003) and repair mechanism of DNA damage caused by oxidative stress (Li et al., 2000).

This study evaluated the cytotoxic effect of Verbascoside extracted from *Phlomis nissolii L.* plant (Lamiaceae) in MCF-7 and MDA-MB 231 breast cancer cell lines *in vitro*.

IC50 values for MCF-7 breast cancer cell line after 24 hour, 48 hour and 72 hour exposure to different concentrations of verbascoside were found as 0.127, 0.2174 and 0.2828 μM respectively. R^2 values for 24h, 48h and 72h exposure to verbascoside were calculated as 0,9630, 0,8789 and 0,8752 respectively. Two-way Anova multiple comparisons test was performed for MCF-7 cell line to determine the significance of mean difference between control and other concentrations of verbascoside for 24h, 48h and 72h respectively. 48, 25,10,1, 0.5 and 0.1 μM concentrations of verbascoside are not effective on MCF-7 breast cancer cells after 24h, 48h and 72h exposure. 100 μM verbascoside has the highest cytotoxic effect on MCF-7 breast cancer cells only after 72h exposure. Delazar et. al. (2019) extracted verbascoside from *Scrophularia subaphylla L.* and examined the effect of 1 to 1000 μg/mL verbascoside on MCF-7 cells and found IC50 value as 0.39 (+/- 0.015) μg/mL after 48 hours of exposure. Nga et al. (2017) isolated 5β,6β-dihydroxyantirrhide from *Pseuderanthemum carruthersii (Seem.) Guill. var. atropurpureum (Bull.) Fosb.* (Acanthaceae) leaves and thirteen different compounds including verbascoside and assayed cytotic activities and acetylcholinesterase inhibition against MCF-7 and HeLa cells at a concentration of 100μg/mL.

Isoverbascoside and verbascoside showed fairly weak AChE inhibitory activity but showed cytotoxic activity against MCF-7 cells strongly. This result supports the results of our study. Because in our study, it was found that 100 μM verbascoside has the highest cytotoxic effect on MCF-7 breast cancer cells only after 72h exposure. This shows that 100 μM and higher concentrations of verbascoside have cytotoxic effect on MCF-7 cells. Liao et al. (2012) examined the effects of acteoside extracted from *A. indica* in 12-O-tetradecanoylphorbol-13-acetate-treated MCF-7 cells *in vitro*. Cell viability was measured by MTT assay and the cells were treated with test agents for 24 h. Acteoside at 30 μM didn't cause significant change in MCF-7 cell viability. This result supports the results of our study. In our study, 25 and 48 μM concentration of verbascoside did not show any significant effect on MCF-7 breast cancer cells. Research about the anticancer and cytotoxicity effect of verbascoside on MCF-7 is very scarce. Verbascoside in each study was extracted from a different plant species in that case cytotoxicity effects and IC50 values determined in each study are different.

IC50 values for MDA-MB 231 cell line after 24 hour, 48 hour and 72 hour of exposure to different concentrations of verbascoside were found as 0.1597, 0.2584 and 0.2563 μM respectively. R^2 values for 24h,48h and 72h exposure to verbascoside were calculated as 0.8438, 0.5107 and 0.9203 respectively. Two way Anova multiple comparisons test was performed for MDA-MB 231 cell line to determine the significance of mean difference between control and other concentrations of verbascoside for 24h, 48h and 72h respectively. 48, 25,10,1, 0.5 and 0.1 μM concentrations of verbascoside are not effective on MDA-MB 231 breast cancer cells after 24h and 48h and 72h exposure. 100 μM verbascoside has the highest cytotoxic effect on MDA-MB 231 breast cancer cells after 24h , 48h and 72 h exposure. There are few studies about the cytotoxic effects of verbascoside on MDA-MB 231 breast cancer cell line in literature. Rahmat et.al., (2006) evaluated antiproliferative effect of Strobilanthes crispus containing verbascoside on MDA-MB 231 cells using MTT assay and found IC50 value of methanolic extract as 27.2. μgmL^{-1}. Ramos et.al. (2013) studied effect of dry olive mill residue water containing verbascoside and found that dry olive mill residue water inhibited MDA-MB 231 cell growth by EC value of 57.15 ± 1.04 c. Both of these studies supported the idea

that plant extracts containing verbascoside have cytotoxic effects on MDA-MB 231 breast cancer cell line but researchers used plant extract containing any other chemicals in these studies, not pure verbascoside like in our study.

In conclusion, this is the first study which examined the cytotoxic effects of verbascoside extracted from *Phlomis nissolii* L. plant on MCF-7 and MDA-MB 231 breast cancer cell lines. This adds importance to this study. Detailed, further studies can be done to evaluate the underlying mechanisms for apoptotic induction of verbascoside extracted from *Phlomis nissolii* L. Additionally, studies which investigate the synergic effects of verbascoside extracted from *Phlomis nissolii* L. plant with verbascoside extracted from other species of plants such as *Scrophularia subaphylla L., Pseuderanthemum carruthersii (Seem.) Guill. var. atropurpureum (Bull.) Fosb.* (Acanthaceae), *A. indica, Strobilanthes crispus* and dry olive mill residue water are also recommended.

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CV

EDUCATION LEVEL	NAME OF THE INSTITUTION GRADUATED	THESIS TOPIC	ADVISOR/S	YEAR OF GRADUATION
Doctorate Degree	Near East University, Educational Administration, Evaluation, Planning and Economy	Quality improvement in secondary schools: developing school self-evaluation scale	Prof. Gökmen Dağlı	2017
Master Degree	Eastern Mediterranean University, Educational Sciences	A Comparative Study: Science Lessons Taught in Turkish versus Science Lessons Taught in English	Prof. Hüseyin Yaratan Assoc.Prof.Hamit Caner	2006
Bachelor Degree	Marmara University Biology			1991

WORK EXPERIENCE

ROLE	INSTITUTE	DURATION
Instructor	Eastern Mediterranean University,TRNC Biological Sciences	2014-ongoing
Founder-Montessori Instructor	Green Island Montessori Pre-School,TRNC	(2014-2018)
Founder- A level Biology Instructor	Yeni Dönem Dersanesi,TRNC	(2011-2015)
Deputy	Eastern Mediterranean College,TRNC	(2008-2011)
ISO 9001:2000 Committee Member	Eastern Mediterranean College,TRNC	(2008-2011)
A level Biology Teacher, Head Of Science Department	Eastern Mediterranean College,TRNC	(2000-2011)

Biology and Science Teacher	Istanbul Ar-el College,Turkey	(1995-2000)
Biology and Science Teacher	İstanbul Tercüman College,Turkey	(1991-1995)

FOREIGN LANGUAGE	**READING COMPREHENSION**	**SPEAKING**	**WRITING**
English	Very good	Very good	Very good

FOREIGN LANGUAGE EXAM	**RESULT**
Nearest University English Exam 2017	Successful
Nearest University English Exam 2014	Successful

ALES RESULT	**NUMERIC**	**EQUAL WEIGHT**	**VERBAL**
5.5. 2019	56.99	62.32	66.03
4.6.2003	50.74	51.32	51.89

PROGRAMME	**ABILITY TO USE**
Word,Excel, Power Point	Very good
SPSS	Very good
Graph Pad Prism 8	Good

CURRICULUM DEVELOPMENT

- 2014-2018, Green Island Montessori Pre-School, Montessori curriculum development

- 2008 ,TRNC secondary school science curriculum development with collaboration of Prof. Ali Yıldırım, Assist.Prof Ercan Kırmızı

PUBLICATIONS (ARTICLES)

1. Lesinger,F., Şenol,H. & Akyürek,S. (2019). Okul Öncesi Eğitim Kurumlarindaki Öğretmenlerin Psikolojik Sermaye ve Okul Kültürü Özellikleri Arasindaki İlişkinin İncelenmesi**,** Journal of Folklor/Edebiyat, cilt: 25,sayı: 97-1,pp. 201-232. Doi:10.22559/folklor.937 (indexed in ULAKBIM)

2. Akyürek,S., Şenol,H. & Lesinger,F.Y. (2018). The Impact of Psychological Capital Levels of Preschool Teachers on Their Life Satisfaction and

Individual Performance Perceptions. Modern Journal of Language Teaching Methods, Vol. 8, Issue 10, pp. 218-233, Thomson Reuters ESCI, Web of Science, ISSN: 2251-6204

3. Senol, H. & Dagli,G. (2017). Increasing Service Quality in Education: Views of Principals and Teachers. EURASIA Journal of Mathematics Science and Technology Education, 13(8): 4857-4871. DOI: 10.12973/eurasia.2017.00969a

4. Senol H & Dagli G (2016). Quality Improvement in Secondary Schools: Developing a School Self-evaluation Scale. *International Journal of Educational Sciences,* 15(1,2):53-65.

PUBLICATIONS (BOOK CHAPTERS/BOOKS)

1. **BOOK EDITOR**: Educational Leadership , Intechopen, London:United Kingdom. ISBN 978-1-83880-487-9 https://www.intechopen.com/welcome/387155f6ce16da4065319ac18ef9bf1f (this book will be published in September 2019)

2. **BOOK CHAPTER:** "Professional Development Needs of Educational Leaders" in book entitled Educational Leadership, Intechopen, London:United Kingdom. ISBN 978-1-83880-487-9 , https://www.intechopen.com/welcome/387155f6ce16da4065319ac18ef9bf1f (this book chapter will be published in September 2019)

3. **BOOK CHAPTER:** Senol,H.(2019). Evaluation of Service Quality in Education: A Case of North Cyprus. Scientific Developments,2019, Gece Yayınevi,International, Ankara, ISBN: 978-605-78521-20. Access: file:///C:/Users/NewTech/Desktop/New%20folder%20(3)/YAYINLANAN%20MAKALELER/BOOKS/CHAPTER%20GECE%20YAYINLARI%202019/e42d97_7c27fba534104e8a91e795777efbc7f6.pdf

4. **BOOK CHAPTER:** Senol, H. & Lesinger, F. (2018)"The Relationship between Instructional Leadership Style, Trust and School Culture in book titled as "Leadership, ISBN" 978-1-78923-685-9. http://dx.doi.org/10.5772/intechopen.75950, London:United Kingdom. Access: https://www.intechopen.com/books/leadership/the-relationship-between-instructional-leadership-style-trust-and-school-culture (September, 2018)

ABSTRACTS PUBLISHED IN INTERNATIONAL SCIENTIFIC CONFERENCES, MEETINGS (Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabındabasılan bildiriler)

1. ICOPFE "INTERNATIONAL CONFERENCE ON PERSPECTIVES IN FUTURE EDUCATION, ABSTRACT BOOK, CYPRUS INTERNATIONAL UNIVERSITY, May 2-4, 2018, Kyrenia, http://icopfe.ciu.edu.tr/pdf/abstract_book.pdf

2. International Congress of Science, Education and Technology Research, BOOK OF ABSTRACT, 10-12 August 2018, Odessa / Ukraine, Yayın No.: 01, ISBN: 978-605-81236-01, Yayımlanmatarihi:30Ağustos2018. file:///C:/Users/NewTech/Downloads/%C3%96zet%20Kitab%C4%B1%20-%20G%C3%BCncel.pdf

PARTICIPATION IN CONFERENCES, CONGRESS AND SYMPOSIUMS

1. Participation, oral presentation and full paper submission:Reading Habits and Communication Skills of Students. International Congress of Science, Education and Technology Research, 10-12 August,2018. Mechnikov University, Odessa, Ukraine.

2. Oral presentation and full paper submission: The Relation Between School Culture and Organizational Cynicism . International Teacher Education Conference, August 8-10, 2018. Indiana University College of Education Bloomington, United States of America (organized by Sakarya University and TASET)

3. Participation, oral presentation and full paper submission: okul öncesi eğitim kurumlarindaki öğretmenlerin psikolojik sermaye ve okul kültürü özellikleri arasindaki ilişkinin incelenmesi, Journal of Folklor/Edebiyat (indexed in ULAKBIM).ICOPPE International Conference on Perspectives for Future Education,2-3-4 May 2018,North Cyprus

4. Meeting the needs and expectations of students in pre-university education: A Literature review and a guide for future research, ICES 17 Environmental Sciences and Educational Studies, 18-20 October 2017, Kyrenia, North Cyprus

5. Quality Improvement in Secondary Schools: Developing School Self-Evaluation Scale-presented at 6th World Conference on Learning, Teaching and Educational Leadership, University of Descartes, Paris, France, 2015

ARTICLES THAT I REVIEWED

1. "Education Quality Challenges in Ethiopian Secondary Schools",2019, Journal of Education, Society and Behavioural Science

2. "Cultural Implications for Learners' Effectiveness as Governors of Schools in Rural South Africa,2018, South African Journal of Education

3. "Assessment of student satisfaction model: Evidence of Western Balkans" 2018, Total Quality Management and Business Excellence

4. "Developing scientific literacy through multimodal activities that integrate science and literacy instruction" 2007, Journal of Research in Science Teaching

5. "Does foundation training effect on trained college teachers' class management?" 3rd SSPHE conference 2019, China.