

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DETERMINATION OF ANTI-TUMOR ACTIVITY OF NOVEL
NAPHTHAZARIN DERIVATIVES

SEYHAN UNSAL GURSOY
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPERTMANT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
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2021

T.C.
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FEN BİLİMLERİ ENSTİTÜSÜ

YENİ NAPHTHAZARİN TÜREVLERİNİN
ANTI-TÜMÖR ETKİSİNİN BELİRLENMESİ

SEYHAN ÜNSAL GÜRSOY
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

One of the natural 4-naphthoquinone derivatives obtained from the roots of the Boraginaceae genus is naphthazarin. In cancer cells, naphthazarin derivatives have been shown to exhibit anti-tumor activity. However, no investigations on the anti-tumor activity of new naphthazarin derivatives modified by the addition of acetyl groups have been reported in the literature. In the female population, breast cancer is the most frequent cancer. Thus, the aim of this thesis is to study into the anti-cancer activity of naphthazarin derivatives 4-hydroxy-5,8-dioxo-dihydronaphthalen-1-yl acetate (Compound 1) and 5,8-dioxo-5,8-dihydroxynaphthalene-1,4-diyl diacetate (Compound 2) on breast cancer cell lines.

RTCA xCELLigence, MTT assay, flow cytometry, and Western blot analysis were used to analyse *in-vitro* cytotoxic, proliferative, apoptotic, and cell cycle arrest roles of naphthazarin derivatives on MCF-7, MDA-MB-231, SK-BR-3 breast cancer cells and breast epithelial cells (MCF-12A). Result showed that compound 2 suppressed proliferation in MDA-MB-231 and MCF-7 cells at 3 and 5 μ M concentrations compared to MCF-12A ($P<0.001$). Furthermore, while compared to MCF-12A, 7 μ M concentration of compound 2 had a cytotoxic effect on MCF-7 and SK-BR-3 cells ($P<0.05$). Compound 2 treatment caused cells dead in MCF-12A, according to apoptosis analysis. Lastly, compound 2 arrested the MCF-7 cell cycle in the G1 phase and the SK-BR-3 cell cycle in the G2/M phase.

Consequently, it was found that the modified naphthazarin derivative (compound 2) had anti-proliferative and cytotoxic effects on some of breast cancer cells, as well as causing cell cycle arrest.

TUBITAK provided support for this thesis (Project: 119Z092).

Key Words: Breast cancer cells, Breast epithelial cell, Naphthazarin derivatives, Anti-cancer, Apoptosis, Cell cycle arrest.

ÖZET

Naftazarin, Boraginaceae bitki cinsinin köklerinden elde edilen doğal 4-Naftakinon türevlerinden biridir. Naftazarinin kanser hücrelerinde anti-tümör etkileri olduğu gösterilmiştir. Bununla birlikte literatürde, asetil gruplarının eklenmesiyle modifiye edilen yeni Naftazarin türevlerinin anti-tümör aktivitesine ilişkin hiçbir çalışmaya rastlanmamıştır. Meme kanseri, kadın popülasyonunda en sık görülen kanser türüdür. Bu nedenle, bu tezin amacı 4-hidroksi-5,8-diokzo-5,8-dihidronaftalinil-1-asetat (Bileşik 1) ve 5,8-diokzo-5,8-dihidronaftalinil-1,4-diasetat (Bileşik 2) naftazarin türevlerinin meme kanseri hücre hatları üzerindeki hücresel antikanser aktivitesini incelemektir.

Naftazarin türevinin MCF-7, MDA-MB-231, SK-BR-3 meme kanseri ve meme epitel hücreleri (MCF-12A) *in-vitro* koşullarda sitotoksik, proliferatif, apoptotik ve hücre döngüsüne etkilerini analiz etmek için RTCA xCELLigence, MTT testi, akış sitometrisi kullanıldı. Sonuç, bileşik 2'nin, MCF-12A'ya kıyasla 3 ve 5 uM konsantrasyonlarda MDA-MB-231 ve MCF-7 hücrelerinde proliferasyonu baskıladığını gösterdi ($P<0.001$). MCF-12A hücreleriyle karşılaştırıldığında bileşik 2'nin 7 uM konsantrasyonu, MCF-7 ve SK-BR-3 hücreleri üzerinde sitotoksik etki gösterdi ($P<0.05$). Ayrıca, bileşik 2 uygulamasının MCF-12A hücresinde hücre ölümlerine sebep olduğu apoptoz analizleriyle belirlendi. Son olarak bileşik 2 MCF-7 hücre döngüsünü G1 fazında, SK-BR-3 hücre döngüsünü G2/M fazında durdurdu.

Sonuç olarak, modifiye edilmiş naftazarin türevinin (bileşik 2) bazı meme kanseri hücreleri üzerinde anti-proliferatif ve sitotoksik etkilerinin yanı sıra hücre döngüsü durmasına neden olduğu bulundu.

Bu tez TÜBİTAK tarafından desteklenmiştir (Proje: 119Z092).

Anahtar kelimeler: Meme kanseri hücreleri, Meme Epitel hücresi, Naftazarin türevleri, Anti-kanser, Apoptoz, Hücre döngüsü durması.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
3MA	: 3-methyladenine
ACS	: American Community Survey (ACS)
BRCA1	: Breast Cancer Type 1 Susceptibility Protein
BRCA2	: Breast Cancer Type 2 Susceptibility Protein
c- PARP	: Cleavage of Poly ADP-Ribose Polymerase
C1	: 4-hydroxy-5,8-dioxo-dihydronaphthalen-1-yl acetate, Compound 1
C2	: 5,8-dioxo-5,8-dihydroxynaphthalene-1,4-diyl diacetate, Compound 2
DCIS	: Ductal carcinoma in situ
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DMSOCtrl	: DMSO Control
EGF	: Epidermal Growth Factor
ER-	: Estrogen Receptor Negative
ER+	: Estrogen Receptor Positive
FBS	: Fetal Bovine Serum
GAPDH	: Glyceraldehyde 3-Phosphate Dehydrogenase
HATs	Histone Acetyl Transferases
HDACs	Histone Deacetylases
HER2-	: Human Epidermal Growth Factor Receptor 2 Negative
HER2, ERBB2	: Human Epidermal Growth Factor Receptor 2
HER2+	: Human Epidermal Growth Factor Receptor 2 Positive
IC50	: Half Maximal Inhibitory Concentration
MAPK	: Mitogen-Activated Protein Kinase
MCF-12A	: Mammary Epithelial Cell Line
MDA-MB-231, SK-BR-3, MCF-7	: Breast Cancer Cells
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Naph	: 5,8-dihydroxy-1,4-naphthoquinone, Naphthazarin
NCI	: National Cancer Institute

NEAA	: Non-Essential Amino Acid
PBS	: Phosphate Buffered Saline
PI3K	: Phosphatidylinositol-3 kinase protein
PR-	: Progesterone Negative
PR+	: Progesterone Positive
SE	: Standard Error
TNBC	: Triple Negative Breast Cancer
USA	: United State America

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1. INTRODUCTION

1.1. What Is the Cancer?

Cancer is based on the Latin "cancer" and the Greek word "carcinos" [1]. Cancer is the uncontrolled proliferation (divides) and growth (increases in mass) of aberrant cells in the body [2]. As a result, a structure known as a tumor is formed. The tumor is called benign if the tumor cells have not yet become invasive. True cancer cells, also known as malignant cells, must have acquired the ability to invade surrounding tissue in order to do so. Because cancer cells are characterized by their invasiveness. It causes them to split apart and enter blood or lymphatic vessels, where they can form additional tumors known as metastases in other body regions [3].

1.2. Cancer Types

Cancer can develop in any part of the body. Bladder cancer, colon cancer, liver cancer, thyroid cancer, endometrium cancer, lung cancer, bone tumors, stomach cancer, brain tumors, colon and rectal cancer, pancreatic cancer, lymphoma (lymphoma), childhood cancers, oral cancer, ovarian cancer, laryngeal cancer, skin cancers (melanoma), breast cancer, cervical cancer, and prostate cancer are just a few examples [4]. The most frequent malignancy among women is breast cancer. Prostate cancer is the most common cancer among men. Both men and women are affected by lung and colorectal cancer in large numbers. [5].

1.3. What Is the Breast Cancer?

Breast cancer is a malignant tumor that developed in breast cells (Figure 1.1 [6]). Breast cancer commonly starts in the milk-producing lobular cells or the ducts that transport milk from the lobular to the nipple. Breast cancer can start in the stromal tissues, which is uncommon [7].

It affects both men and women, albeit it is more frequent in women. However, because men are more likely to be diagnosed with breast cancer after it has spread, the consequences are worse [8].

Breast cancer is one of the most common cancers seen in women. The number of cancer patients is rising, with 2 million new cases diagnosed each year around the world. The National Cancer Institute (NCI) was foreseen that about 276,480 women will be diagnosed with breast cancer in 2020, and about 42,170 will die of the disease. The American Community Survey (ACS) also was foreseen that about 2,620 men will be diagnosed with breast cancer in 2020, and about 520 will die from the disease [9].

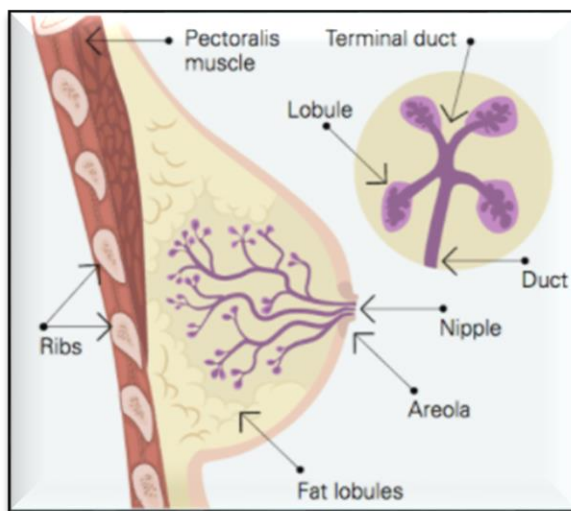


Figure 1.1: Breast structure and cells.

1.3.1. Types of Breast Cancer

Because cancer can begin in many regions of the breast, there are many types of breast cancer. Some cancers are more common than others, and some cancers can be combined (Figure 1.2 [10]). Types of breast cancer:

- Ductal carcinoma in situ (DCIS): The most frequent non-invasive type breast cancer develops in the milk duct. Because the cancer has not progressed to other parts of the breast, the cure rate is high at this stage.

- Lobular carcinoma in situ: Cancer develops in the glands that produce milk. It does not spread to cells in the surrounding tissues, like DCIS.
- Invasive ductal carcinoma: This form begins in the milk ducts and spreads to the surrounding tissue. Invasive ductal carcinoma accounts for around 80% of metastasizing breast cancer [10].
- Invasive lobular carcinoma: This type begins in the breast's milk-producing glands. It is less common than invasive ductal carcinoma [11], [12].

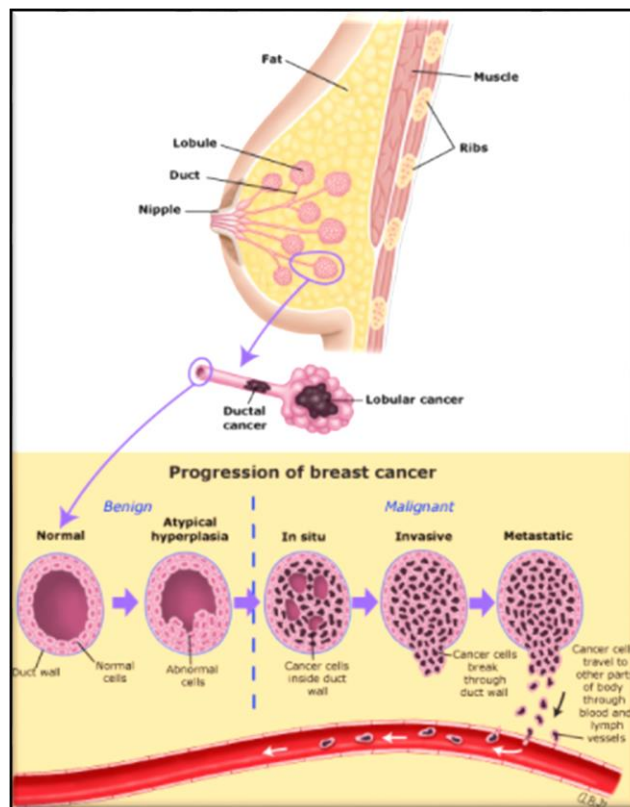


Figure 1.2: The figure shows that breast cancer cells spread to other parts of the body via blood and lymph vessels.

1.3.2. Causes of Breast Cancer

Breast cancer can be caused by a variety of factors. The most common reasons are as follows:

- Age: As you become older, your chances of developing breast cancer increase.

- Personal history: If one breast has cancer, the other is likely to have cancer as well.
- Menstruation: Women whose menstrual cycle begins at a young age and who enter menopause later are at a higher risk.
- Consuming alcohol, like any other cancer, raises the chance of breast cancer.
- BRCA1 and BRCA2 gene mutations are the most common cause of breast and ovarian cancer. It is well established that inheriting a mutant gene increases the risk of developing breast cancer [8], [13], [14].

1.3.3. Diagnosis of Breast Cancer

Breast cancer is diagnosed in a variety of ways:

- A manual breast examination can be performed by a doctor or by the women themselves.
- Mammogram: It is the most common test for examining the structure beneath the breast.
- Ultrasound: It's a test that creates a picture of the inside of the breast tissue using sound waves.
- If necessary, a biopsy is performed on the breast to obtain a cell or tissue sample, and a pathologist examines the sample under a microscope for aberrant cell shapes or growths [15], [16].

1.3.4. Treatment Methods of Breast Cancer

Initially, the size, stage, and extent to which the tumor can grow and spread, or grade, should be identified for breast cancer treatment. After that, therapy choices are discussed. Surgery is the most common treatment for breast cancer. Following that, further treatments such as chemotherapy, targeted therapy, radiation, hormone therapy, or plant medicine derivatives were used [16].

1.3.4.1. Chemotherapy

Chemotherapy is a treatment that kills cancer cells in the body. For this reason, choosing a breast cancer-specific chemotherapy treatment allows it to be controlled and its symptoms to be decreased. Chemotherapy medications are extremely strong. As a result, it dies in healthy cells with tumor cells [17].

1.3.4.2. Radiation

The main goal of treatment is to prevent cancer cells from growing and multiplying while minimizing damage to healthy tissues and organs. Normal cells are nonetheless harmed, despite the fact that it does not destroy healthy cells as much as chemotherapy [17].

1.3.4.3. Hormone Therapy (Targeted Therapy) and Medication

Estrogen and progesterone hormones connect with receptors on the inside and outside of breast cells. These hormones transmit information to the cell that encourage it to develop and function properly. These hormone receptors are found in the majority of breast cancer cells [18]. The estrogen hormone is used by some types of breast cancer to help them grow. This breast cancer is referred to as estrogen receptor positive (ER+). Hormone therapy blocks or stops the effect of estrogen on the cell in ER+ breast tumors. Because some kinds of breast cancer lack an ER receptor, estrogen cannot be used to stimulate them. These are referred to as estrogen receptor negative (ER-). They will not respond to estrogen-blocking medications. Some types of breast cancer are progesterone positive (PR+) whereas others are progesterone negative (PR-). Progesterone-suppressing hormone therapy can be used at that time for PR+ patients, but not for PR- patients [19].

The human epidermal growth factor receptor 2 (HER2, ERBB2) is a tyrosine kinase receptor that relates to the epidermal growth factor receptor family. Its proliferation triggers signaling pathways that promote cell proliferation and cancer [20]. Cancer cells have a high level of the HER2 protein, which causes them to proliferate and divide quickly. Hormone therapy and HER2 protein targeted

medicines inhibit the effects of estrogen, progesterone, and HER2 receptors, delaying or even preventing cancer growth [18]. Triple negative breast cancer (TNBC) accounts for 10-15% of all breast cancers, and TNBC patients lack estrogen, progesterone, and HER2 receptors. All three tests turn out to be negative. Hormonal treatment drugs like tamoxifen and aromatase inhibitors are ineffective since hormones do not cause cancer to spread. As a result, therapy options are extremely limited [21]-[23].

In addition, cancer cells may become resistant to targeted therapy. Combining hormone therapy medications or combining hormone therapy medications with conventional chemotherapy may be beneficial in treatment. Trastuzumab (Herceptin) targeted therapy, for example, may be more effective in treating metastatic breast cancer when combined with the chemotherapy drug docetaxel [24].

Chemotherapy, radiation, and hormone therapy are all unsuccessful treatments for many breast cancer patients [25], [26]. Furthermore, these treatments have numerous side effects; therefore, medicines must be developed to increase patient survival, symptom control, and quality of life. As a result, new and effective treatments for breast cancer are necessary.

Quinones can be found in animals, plants, and microbes. They play a significant function in the biochemistry of energy production on a regular basis [27]. Quinones contain a variety of biological activities, both synthetic [28] and natural quinones have proven anti-cancer activity [29] after more than 40 years of research. Quinones such as bleomycins, dactinomycin, and mitomycin-C are utilized in clinical chemotherapy [30]-[33]. Naphthazarin, a quinone with anti-tumor properties, is an interesting candidate for cancer treatment. Naphthazarin and its derivatives have successfully proven anti-cancer activities in previous research of colon cancer Chen *et al.* (2016) and prostate cancer Dyshlovoy *et al.* (2019).

1.4. Naphthazarin and Its Synthetic Derivatives

The 1,4- naphthoquinones are the most important chemical class in the quinone family. Naphthazarin (Figure1.4), also known as 5,8-dihydroxy-1,4-naphthoquinone (Naph) or 5,8-dihydroxy-1,4-naphthalenedione [34], is a lipophilic red pigment found in the roots of numerous Boraginaceae species. It is used in foods, cosmetics,

health foods, and textiles products [35], [36]. Naphthazarin is a bioactive molecule found in many plants that exhibits anti-tumor [37], anti-viral, anti-bacterial, anti-inflammatory [38], antiallergic, antithrombotic, antiplatelet, lipoxygenase, radical scavenging, and anti-ringworm properties [39]. It is also low in toxicity [40].

Half of the medicine used in oncology have been obtained by modification of natural substances [41]. With synthetic modifications and structure optimization, the effect of naphthazarin on cancer can be increased while hazardous side effects are minimized. In this study, 4-hydroxy-5,8-dioxo-dihydronaphthalen-1-yl acetate (Compound 1, C1) (Figure1:4) and 5,8-dioxo-5,8-dihydroxynaphthalene-1,4-diyl diacetate (Compound 2, C2) (Figure1:4) which are naphthazarin derivatives has been synthesized with synthetic chemistry by Prof. Dr. Devrim Atilla and Dr. Mehmet Fatih Sağlam from Gebze Technical University, Chemistry Department.

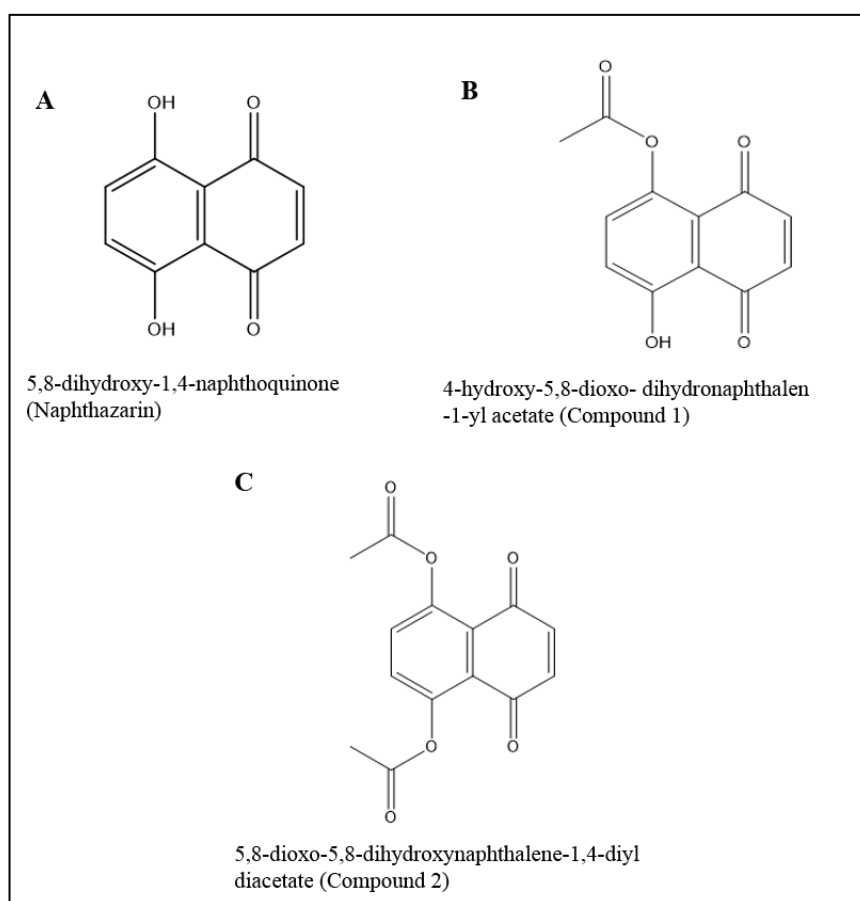


Figure 1.3: Naphthazarin (A) and its synthetic derivatives (B, C).

1.5. Purpose of The Thesis

The number of women diagnosed with breast cancer has risen dramatically in recent years. Breast cancer is responsible for 24% of all female cancers and 14% of cancer death. According to data from the United States, a woman's lifetime risk of breast cancer is 1/8. As you become older, your risk increases [42]. Breast cancer is not only the most frequent cancer in women, but it is also the second leading cause of mortality after lung cancer [43].

Naphthazarin impacts numerous mechanisms in the cell and causes cancer cells to apoptosis, according to the literature. As a result, it is considered that it can be useful in the treatment of metastatic cancer [44], [45].

The anti-proliferative, apoptotic, and anti-tumor effects of synthetically produced derivatives of naphthazarin on breast cancer were explored and compared to naphthazarin in this thesis.

Besides from the effects of naphthazarin on cancer cells, no research on the anti-tumor activity of newly synthesized naphthazarin derivatives has been found in the literature. Therefore, this thesis is the first to applications of newly synthesized naphthazarin derivatives in the treatment of breast cancer.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Cell Lines

MDA-MB-231 is an immortal human cell line originally derived from a patient with metastatic breast cancer. This cell line lacks the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HEGF) (HER2-). As a result, MDA-MD-231 cells are extremely malignant, invasive, and aggressive [46]. The *Her2* proto-oncogene product is overexpressed in SK-BR-3, a human cancer cell. It's a cell line that's utilized to test therapeutic compounds for HER2-targeted treatments [47]. MCF-7 is a non-invasive breast cancer cell line that is ER+ and PR+. It suggests it has a metastatic potential [48]. MCF-12A is a mammary epithelial cell line that is non-tumorigenic and estrogen-independent [49].

2.1.2. Cell Culture Solutions

Table 2.1: List of cell culture solutions.

Solution	Company
Dulbecco's Modified Eagle Medium 1X (DMEM) with L-glutamine, pyruvate and 4,5 g/L Glucose	Gibco
Fetal Bovine Serum (FBS)	Gibco
Penicillin, Streptomycin (10.000 µg/ml Penicillin, 10.000 µg/ml Streptomycin)	Gibco
Non-Essential Amino acid (NEAA) 100X	Gibco

Table 2.1: Continue.

Phosphate Buffered Saline (PBS)	Gibco
0,05 % Trypsin-EDTA Solution 1X	Gibco
Dimethyl sulfoxide (DMSO)	Fischer Scientific
Epidermal Growth Factor (EGF) (100µg/ml)	Santa Cruz Biotechnology
Hydrocortisone (1mg/ml)	Santa Cruz Biotechnology
Insulin (10mg/ml)	Santa Cruz Biotechnology

2.1.3. Colorimetric Assay Solution and Materials

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Thermo Fisher Scientific, Lot: 2295018) prepared with distilled water.
- 0,2 µM filter (VWR)

2.1.4. Cytotoxic Effects Assay Material

- xCELLigence System RTCA DP Instrument System E-Plate 16 PET (Agilent, CST: 300600890)

2.1.5. Apoptosis and Cell Cycle Analysis Solution

- Annexin V Reagent (BD Biosciences, Cat. No: 556421)
- 7-amino-actinomycin (7-AAD) Staining Solution (BD Biosciences, Cat. No: 559925)
- Binding Buffer Solution (10X):
 - 0,1 M HEPES pH 7,4 (Fischer Scientific)
 - 1,4 M NaCl (Fischer Scientific)

- 25 mM CaCl_2 (Fischer Scientific) dilute with distilled water and use 1X
- Ethenol 96 % (Riedel de Haen) dilute with distilled water and use 70%
- RNase A (20 mg/ μl) (Hibrogen, Lot: 647-1)

2.1.6. Western Blot Solutions

- Qubit protein assay kit (Thermo Scientific, Cat.No: Q33212).

Table 2.2: 5X SDS Lysis Buffer.

Solutions	Quantity
Distilled Water	3,8 ml
0,5 M Tris pH 6,8	1 ml
Glycerol	0,8 ml
10 % SDS	1,6 ml

Table 2.3: 5X Loading Buffer.

Solutions	Quantity
Distilled Water	3,8 ml
0,5 M Tris pH 6,8	1 ml
Glycerol	0,8 ml
10 % SDS	1,6 ml
2-Mercaptoethanol	0,4 ml
Bromophenol Blue	0,4 ml

Table 2.4: Resolving Gel (%10).

Solutions	Quantity
Distilled Water	4,9 ml
1,5 M Tris pH 8,8	2,5 ml
10 % SDS	100 µl
AC/BIS	2,5 ml
10 % APS	100 µl
TEMED	6 µl

Table 2.5: Stacking Gel (%4).

Solutions	Quantity
Distilled Water	3,15 ml
0,5 M Tris pH 6,8	1,25 ml
10% SDS	50 µl
AC/BIS	0,5 ml
10 % APS	50 µl
TEMED	3 µl
10 % SDS	50 µl

Table 2.6: 10X Running Buffer.

Solutions	Quantity
Distilled Water	1000 ml
250 mM Tris	30,3 gram
1,4 M Glycine	144 gram
1 % SDS	10 gram

Table 2.7: 10X TBS Buffer pH 7,6.

Solutions	Quantity
Distilled Water	1000 ml
Tris	30 gram
NaCl	80 gram
KCl	2 gram

Table 2.8: 10X Transfer Buffer.

Solutions	Quantity
Distilled Water	1000 ml
250 mM Tris	30,3 gram
1,4 M Glycine	144 gram

Table 2.9: 1X Blocking Buffer.

Solutions	Quantity
TBS-T	250 ml
Milk Powder Free Fat	12,5 gram

Primer Antibodies:

- c-PARP (cleavage of Poly ADP-ribose polymerase) rabbit 1:2000 (Cell Signaling, CST: 9541S)
- GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase) rabbit 1:5000 (Cell Signaling, CST: 5174S)

Secondary Antibodies:

- Goat- α rabbit IgG (H+L) HRP (Advansta, 210323-77)
- Broad Range Protein Ladder (BioLegend, Lot: B295981)
- WesternBright ECL HRP substrate (Advansta, Cat. No: K-12045-D50, Lot: 190905-87)

2.1.7. Material, Equipments and Devices

- 60 mm, 100 mm tissue culture plate (TRP)
- 6-well, 96-well culture plate (TRP)
- 15 ml and 50 ml Falcon (CAPP)
- 5 ml, 10 ml ,25 ml, 50 ml serological pipettes (CAPP)
- -80°C freezer (Thermo, Forma -86 °C ULT Freezer)
- Western Blot System (Bio- Rad)
- CO2 incubator- Stericycle (Labogene, Scancell)
- Laminar Flow Cabinet (Labconca, Logic)
- Micro pipettes (Gilson)
- Microcentrifuge tubes (0,5 ml, 1,5 ml, 2ml)
- Varioskan Flash (Thermo Scientific)
- Water Bath (Techne-TE-10ATempette)
- Autoclave (Hirayama, HiclVE hve-50)
- Centrifuge (Nüve, NF400)
- Refrigerated centrifuge (Hitachi Himac CT-RE15)
- Vortex (DragonLabs, MX-S)
- Light microscope with fluorescent attachment (Nikon)
- Heat Blok (denville Scientific)
- Precision balance (Sartorius BL210S)
- Rechargeable pipettor (Fisher Scientific)
- Shaker (Denville Scientific)
- Liquid nitrogen tank (Arpege 140)
- Accuri C6 (Becton Dickinson)
- Membrane imaging system (Biorad ChemiDOC XRS)
- Qubit 3.0 Fluorometer (Thermo Scientific)
- xCELLigence System RTCA DP (ROCHE/ACEA Biosciences)
- Tween-20 (Thermo Scientific)
- Digital Sonifier (BRANSON)

2.2. Methods

2.2.1. Cell Culture

The MDA-MB-231, SK-BR-3, and MCF-7 cell lines were melted at room temperature after being taken from the liquid nitrogen tank. The cells are then centrifuged at 1800 rpm for 5 minutes in DMEM complete medium containing high glucose supplemented with 10 % FBS, 1 % penicillin-streptomycin, and 1 % non-essential amino acid (for MCF-12A, complete DMEM supplemented with EGF (100 g/ml), insulin (10 mg/ml), and hydrocortisone (1 mg/ml)). Fresh complete media was added with the cell pellet and seeded into 60 mm culture petri dishes. The cultivated cells are incubated at 37 °C and 5 % CO₂.

When cells filled 80-90 percent of 100 mm or 60 mm culture petri dishes, they were sub-cultured via passage twice a week. The following is how subcultured was accomplished: The media was aspirated and washed with 1X PBS to remove dead, unbound and debris cells. After which 2X Trypsin-EDTA was added, and the cells were allowed to detach from the surface of the culture petri dish for 3 minutes in the incubator. Depending on the cell type, passages were made at 1/2 - 1/7 dilutions.

Cryotubes and DMSO are used to freeze cells and store them at -80 °C or liquid nitrogen. After eliminating the cells with trypsin, the cells were centrifuged for 5 minutes at 1800 rpm. The supernatant was removed and the cryotube was added with cell freezing solution. The cells were then frozen at -80 °C before being stored in liquid nitrogen tanks.

2.2.2. Cell Viability Assay

Naph, C1 and C2 substances were dissolved in DMSO to make a main stock at a concentration of 50 mM. The main stock was then diluted in DMEM media and treated to MCF-7, MDA-MB-231, MCF-12A cells at concentrations of 1, 3, 5, 7, 10, 12 µM and SK-BR-3 cells at concentrations of 1, 3, 5, 7, 10, 12, 15, 20 µM.

MCF-7 and MDA-MB-231 with a density of 7.5×10^3 cells/well; SK-BR-3 and MCF-12A with a density of 1×10^4 cells/well were seeded in triplicate in a 96-well plate an atmosphere containing at 37 °C. The medium was removed after 24

hours and replaced with 90 µl medicated medium in various concentrations for another 24 hours. The controls were given only 0.02 % DMSO (DMSOctrl). At the end of the treatment, 10 µl of MTT (5 mg/ml) was added to a final volume of 100 µl and continued incubated for 4h. After the MTT solution was removed, 100 µl DMSO was added to each well and shaken for 15 minutes. The absorbance was measured with photometric at 570 nm using a microplate reader (Thermo Scientific, Varioskan Flash). Cell viability is used to calculate the data.

2.2.3. Cell Proliferation Assay

C2 from the main stock was diluted in DMEM media and applied to MCF-7, MDA-MB-231, MCF-12A cells at concentrations of 0.1, 0.5, 1, 3, 5, 10 µM and SK-BR-3 cells at concentrations of 5, 7, 10, 12, 15, 20 µM.

Firstly, for blank, 50 µl of empty DMEM was added to E-plate wells and measured using the xCELLigence RTCA DP instrument system. Then, MCF-7 and MDA-MB-231 with a density of 7.5×10^3 cells/well; SK-BR-3 and MCF-12A with a density of 1×10^4 cells/well were seeded duplicate 100 µl in a E-plate in xCELLigence RTCA DP instrument system an atmosphere containing at 37 °C. After 24h, a final volume of 200 µl of media with various concentrations of substance was added to each well, and cell growth was measured for 24 hours. It was detected in real-time based on impedance. In this system, sensors on microplates detect cell proliferation and data are recorded in real-time for 24 - 48 - 72 hours or longer. The resistance of the cell sensor in each well is called the cell index. The cell index is calculated by subtracting the resistance of the control well electrode (Rb) from the electrode resistance (Rn) of the experimental wells of the cell and dividing by 15 (Cell index = $(R_n - R_b)/15$). As a result of these measurements, the half maximal inhibitory concentration (IC₅₀) for each naphthazarin derivative cell line was calculated using the 'normalized cell index' values at various times (Table 2.10).

Table 2.10: Cell IC₅₀ concentrations were calculated.

Cell	MCF-12A	MDA-MB-231	MCF-7	SK-BR-3
IC ₅₀ concentrations	7,06 µM	4,41 µM	4,99 µM	7,18 µM

2.2.4. Determination of Apoptosis

2.2.4.1. Flow Cytometry

As shown below, each cell was separated into four groups. Control, DMSOctrl, C2 and Naph from the main stock were diluted in DMEM media and applied to MCF-7, MDA-MB-231, and MCF-12A cells at IC50 concentrations (Table 2.10).

MCF-7 and MDA-MB-231 with a density of 1.2×10^5 cells/well; SK-BR-3 and MCF-12A with a density of 1.6×10^5 cells/well were seeded in a 6-well plate an atmosphere containing at 37 °C. After 24 hours, cells were treated with IC50 concentrations (Table 2.10) in a final volume of 1.5 ml for 24 hours. Supernatant was collected in 15 ml falcon and washed with 1X PBS before cells were 2X trypsinized and collected same 15 ml falcon. Falcons were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. Cells were washed twice in 1.5 ml eppendorf with 1 ml ice cold 1X PBS and centrifuged at 2500 rpm for 5 minutes at 4 °C. Then 200-300 µl of ice cold 1X binding buffer was added, depending on the cell density, and 100 µl of samples were taken, divided into two groups: stained and unstained. The stained sample group was then incubated at room temperature for 30 minutes with 5 µl of Annexin V (BD Biosciences, 556419) and 7-AAD (BD Biosciences, 559925). After incubation, each sample was given 300 µl of 1X binding buffer, and the percentage of apoptotic and live cells was determined using an Accuri C6 flow cytometer.

2.2.4.2. Western Blot

As shown below, each cell was separated into four groups. Control, DMSOctrl, C2 and Naph from the main stock were diluted in DMEM media and applied to MCF-7, MDA-MB-231, and MCF-12A cells at IC50 concentrations (Table 2.10).

MCF-7 and MDA-MB-231 with a density of 1.2×10^5 cells/well; SK-BR-3 and MCF-12A with a density of 1.6×10^5 cells/well were seeded in a 6-well plate an atmosphere containing at 37 °C to examine the apoptotic effects of Naph and C2

substances. After 24 hours, cells were treated with IC₅₀ concentrations (Table 2.10) in a final volume of 1.5 ml for 24 hours. The top medium was collected in 1.5 mL eppendorf tubes and centrifuged for 5 minutes at 2500 rpm. 100-300 µl of 2X SDS lysis buffer was added to a 6 well plate, eppendorf, and incubated at -20 °C for 1 hour, depending on the cell density. The plates were scraped with a scraper after 1 hour, and the pellets were collected in the same eppendorf tube. A digital sonifier (BRANSON) was used to sonicate the samples in two 15-second cycles. The amount of protein in the lysates was measured in the Qubit 3 Fluorometer using the Qubit protein assay kit (Thermo Scientific, USA). To prepare it for loading, 40 µg of protein was mixed with 5X loading buffer and incubated at 95 °C for 10 minutes. After loading the samples onto a 10 % SDS-PAGE gel in 1X running buffer, they were run at 80 V until they passed through the stacking gel, then at 130 V until the stain was removed entirely from the gel. 1X transfer buffer was prepared and run for 2 hours at 70 V to transfer the proteins to the PVDF membrane. Membranes were blocked for 1 hour at room temperature using a solution made from milk powder free fat dissolved in TBS containing 5 % Tween-20. Primary antibodies were incubated overnight at +4 °C on blocked membranes. The next day, it was incubated with secondary antibody for 1 hour at room temperature after being washed three times with TBS-T for five minutes. The membranes were washed three times with TBS-T for five minutes after the secondary antibody application. The proteins were imaged with Biorad ChemiDOC and Advansta WesternBright ECL HRP (Lot: 190905-87) substrate on membranes.

2.2.5. Cell Cycle

As shown below, each cell was separated into four groups. Control, DMSOctrl, C2, Naph and C2 from the main stock were diluted in DMEM media and applied to MCF-7, MDA-MB-231, and MCF-12A cells at IC₅₀ concentrations (Table 2.10).

MCF-7 and MDA-MB-231 with a density of 1.2×10^5 cells/well; SK-BR-3 and MCF-12A with a density of 1.6×10^5 cells/well were seeded in a 6-well plate an atmosphere containing at 37 °C. After 24 hours, cells were treated with IC₅₀ concentrations (Table 2.10) in a final volume of 1.5 ml for 24 hours. Supernatant was

collected in 15 ml falcon and washed with 1X PBS before cells were 2X trypsinized and collected same 15 ml falcon. Falcons were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. Cells were washed twice in 1.5 ml eppendorf with 1 ml ice cold 1X PBS and centrifuged at 2500 rpm for 5 minutes at 4 °C. After the second wash, 100 µl of 1X PBS was aspirated and added to the pellet. 1.400 ml ice cold 70 % ethanol was added for fixation and incubated at + 4 °C for at least 1 hour. The ethanol was then removed by centrifugation at 3000 rpm for 5 minutes before being washed with 1X PBS again. Pellets were stained for 1 hour at 37 °C with 1 µl of RNase A (20 mg/l), 5 µl 7-AAD, and 500 µl PBS per sample. After that, the stain was removed by centrifugation for 5 minutes at 2500 rpm. Each sample had 400 µl of 1X PBS before being analysed on an Accuri C6 flow cytometer.

2.2.6. Statistical Result

All data from three to five independent experiments were provided as mean SE (standard error). Two-way ANOVA was used to analyse the differences between sets of data. GraphPad Prism was used to compare the means of the control and treatment groups. The statistical significance criterion was used at a P value < 0.05.

3. RESULTS

3.1. Identification of Cytotoxic Effects of Naphthazarin Derivatives

Breast cancer cells were treated for 24 hours with Naph, C1, and C2 at the indicated concentrations, and the cytotoxic effect of the cells was measured using the MTT test. While Naph had no cytotoxic effect in any cells when compared to MCF12A cells (Figure 3.1), C1 had a cytotoxic effect on SK-BR-3 cells when compared to MCF12A cells at a concentration of 3 μ M (Figure 3.2). In comparison to MCF-12A cells, a 7 μ M concentration of C2 had a markedly cytotoxic effect on MCF-7 and SK-BR-3 cells ($P<0.05$) (Figure 3.3). C2 was also found to be cytotoxic in SK-BR-3 cells when compared to MCF12A cells at 10 μ M and 12 μ M concentrations ($P<0.05$) (Figure 3.3). The MTT assay revealed that C2 treatment was more powerful than Naph and C1 treatment for 24 hours. C2 was chosen for the follow-up experiments due to its comparative cell viability.

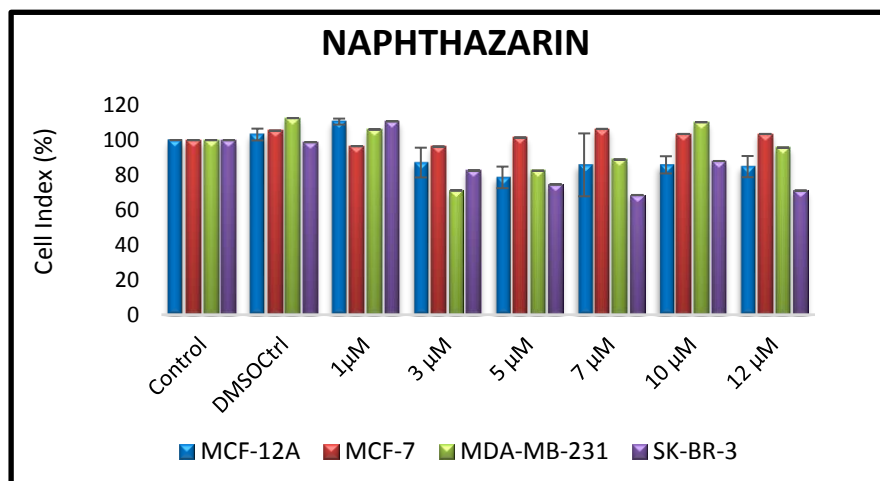


Figure 3.1: Cell cytotoxicity results of naphthazarin for 24h.

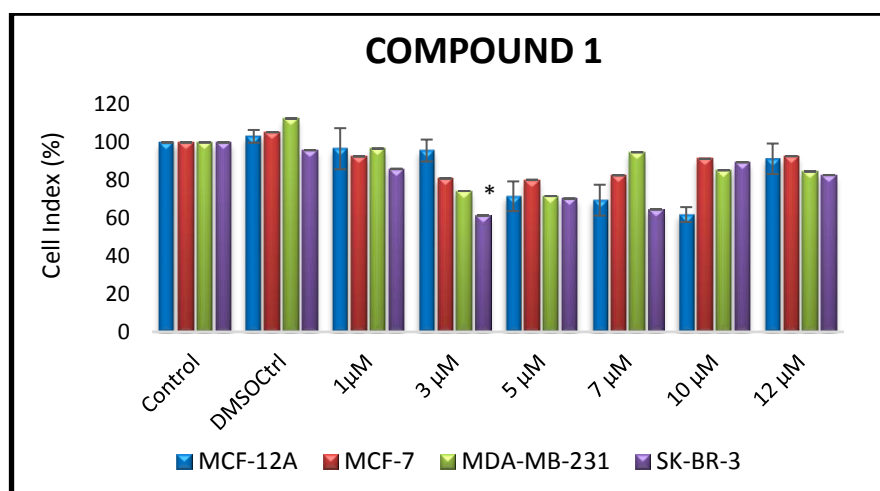


Figure 3.2: Cell cytotoxicity results of compound 1 for 24h. Statistical significance: * $P < 0.05$.

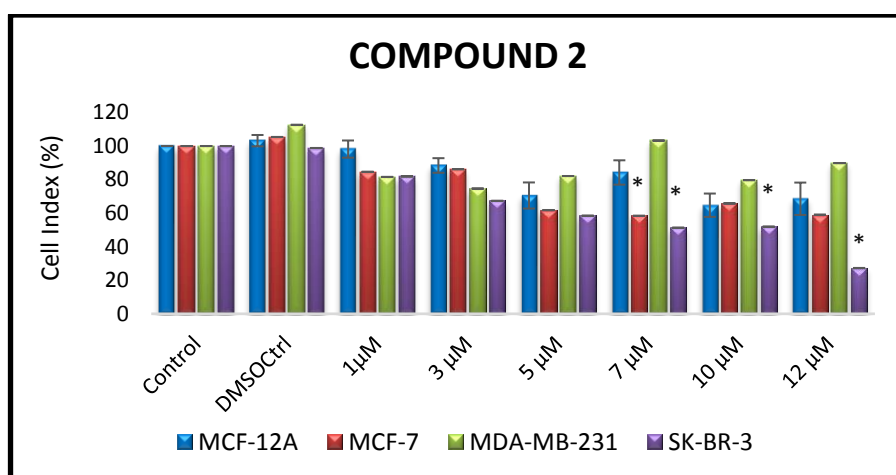


Figure 3.3: Cell cytotoxicity results of compound 2 for 24h. Statistical significance: * $P < 0.05$.

3.2. *In Vitro* Determination of Proliferation Effect of Compound 2

MDA-MB-231, MCF-7, and MCF-12A were treated with C2 concentrations 0.1 to 10 μM during a proliferation assay according to the dose-response cytotoxicity study. MDA-MB-231 and MCF-7 cells were considerably decreased in proliferation by C2 at 3 μM and 5 μM concentrations when compared to MCF-12A cells ($P < 0.001$) (Figure 3.4, 3.5). Because a dose of less than 7 μM did not suppress the proliferation of SK-BR-3, concentrations of between 5-20 μM were used, and when compared to the control group, proliferation was significantly suppressed starting at

7 M concentrations and gradually increasing ($P<0.01$), ($P<0.001$) (Figure 3.6). In comparison to MCF12A, C2 did not show significant suppression in SK-BR-3, but at 10 μM concentrations of C2, the cell index decreased by up to 40 % (Figure 3.7). Even at low concentrations, C2 decreased proliferation in MCF-7 and MDA-MB-231 cells in a time-dependent manner, confirming cell cytotoxicity. Overall, the results demonstrate that C2 has potential cytotoxicity and inhibits the proliferation of some breast cell lines. In future investigations, the IC₅₀ concentrations of C2 calculated using the cell proliferation assay and consistent with the cytotoxicity assay were applied.

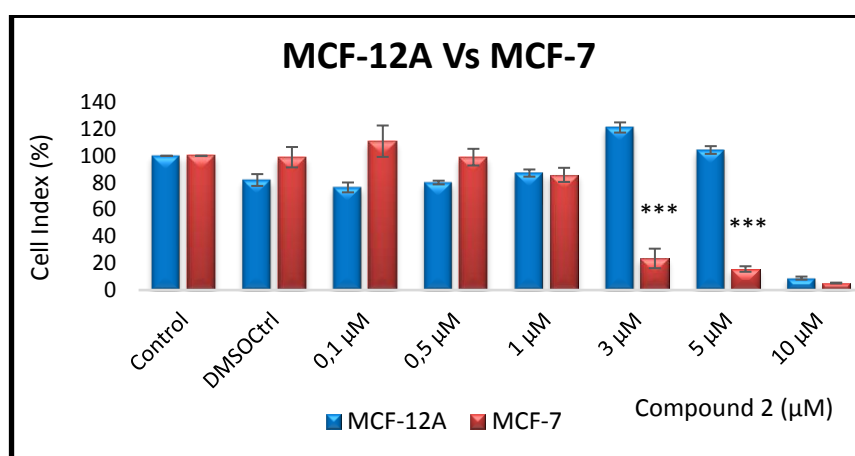


Figure 3.4: Comparison of cell proliferation of MCF-7 with MCF-12A cell proliferation for the compound 2 (24h). Statistical significance: *** $P<0.001$.

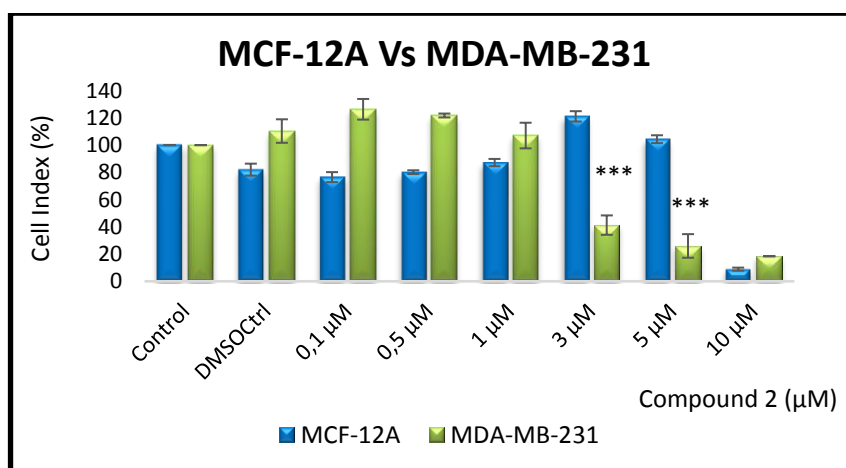


Figure 3.5: Comparison of cell proliferation of MDA-MB-231 with MCF-12A cell proliferation for the compound 2 (24h). Statistical significance: *** $P<0.001$.

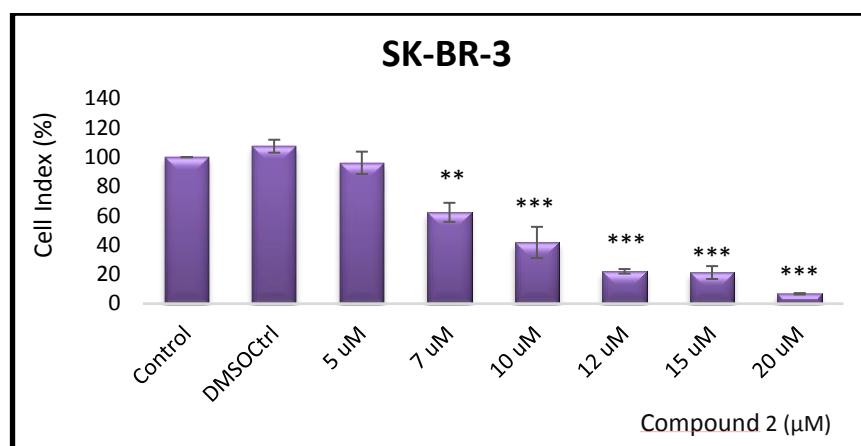


Figure 3.6: Proliferation of SK-BR-3 cells after treatment with C2 for 24h. Statistical significance: ** $P < 0.01$, *** $P < 0.001$.

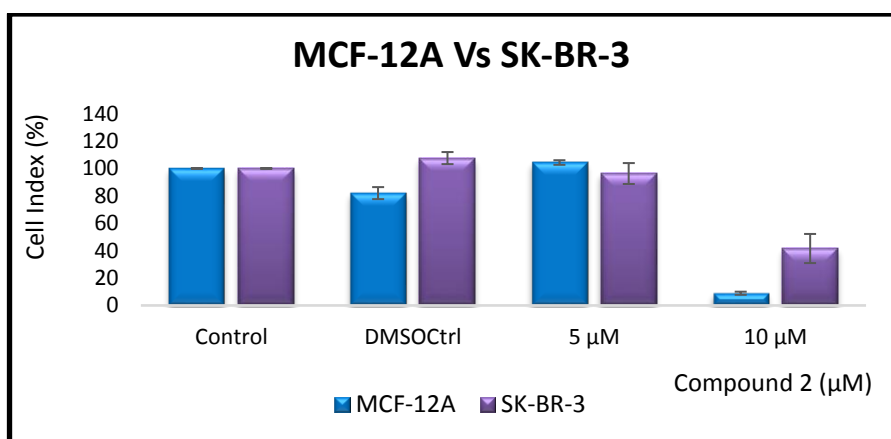


Figure 3.7: Comparison of cell proliferation of SK-BR-3 with MCF-12A cell proliferation for the compound 2 (24h).

3.3. Determination of Effect of Compound 2 on the Apoptosis

3.3.1. Determination of Cell Death by Flow Cytometry

C2 caused cell cytotoxicity and decreased proliferation in breast cancer cells, according to MTT and eXCELLigence results. Afterwards, using the Annexin V and 7-AAD (BD Bioscience) double staining analysis on an Accuri C6 flow cytometer, an apoptosis detection assay was performed to see if it causes breast cell death. C2 and Naph (IC₅₀ concentration) were applied to cells for 24 hours. Cells negative for 7-AAD and positive for Annexin V were classified as early apoptotic cells

(Annexin V+, 7-AAD-) in Quadrant LR; 7-AAD and Annexin V positive cells were classified as late apoptotic cells (Annexin V+, 7-AAD+) in Quadrant UR; 7-AAD positive and Annexin V negative cells were defined as necrotic cells (Annexin V-, 7-AAD+) in Quadrant UL. Figures 3.8A, 3.8B, and 3.8C show the flow cytometer data. The mean and standard deviations of two times repeated flow cytometer values were calculated and presented as a quantification graph in Figure 3.8D. When compared to DMSOCntrl, MCF-12A cells treated with C2 showed an increased late apoptotic population (41 %) and a decrease in the population of live cells (51.5 %). When compared to DMSOCntrl, MCF-12A cells treated with Naph showed an increased early apoptotic population (22.15 %) and a decrease in the population of live cells (63.1 %) (Figure 3.8D).

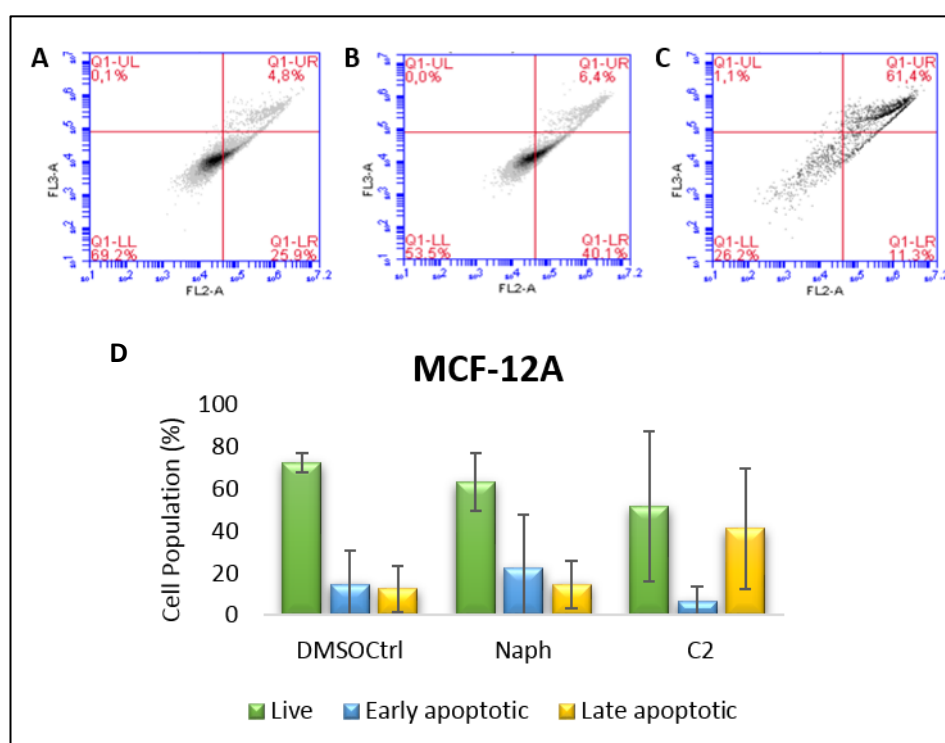


Figure 3.8: Staining assay in MCF-12A cells. (A) DMSOCntrl, (B) Naph, (C) C2. (D) Quantification graph of Annexin V and 7-AAD assay.

Flow cytometer results were obtained as in Figures 3.9A-3.9C. Means and standard deviations of flow cytometer results of two times repeated were calculated and shown as a quantification graph as in Figure 3.9D. While the live cell population was decreased in MCF-12A treated with C2, the live cell population in MDA-MB-231 treated with treated Naph and C2 (86.75 %; 88.9 %, respectively) was nearly the

same as in DMSOCtrl (86.35 %). MDA-MB-231 with treated C2 (5.8 %) had a lower population of late apoptotic cells than DMSOCtrl (7.55 %) (Figure 3.9D).

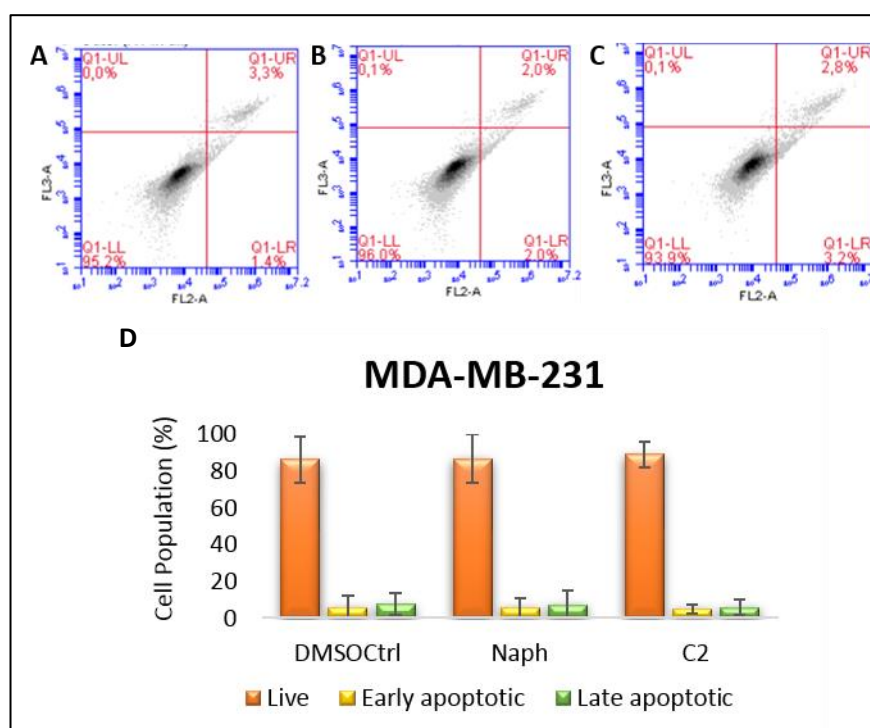


Figure 3.9: Staining assay in MDA-MB-231 cells. (A) DMSOCtrl, (B) Naph, (C) C2. (D) Quantification graph of Annexin V and 7-AAD assay.

Figures 3.10A-3.10C show the flow cytometer data. The mean and standard deviations of two times repeated flow cytometer values were calculated and presented as a quantification graph in Figure 3.8D. When compared to DMSOCtrl (73.15 %), the live cell population of MCF-7 treated with Naph and C2 (74.75 % and 72 %, respectively) showed no significant differences. On the other hand, cells treated with Naph and C2 had a slightly higher population of early apoptotic cells (12.5 %; 12.7 %, respectively) than with DMSOCtrl (11.05 %) (Figure 3.10D).

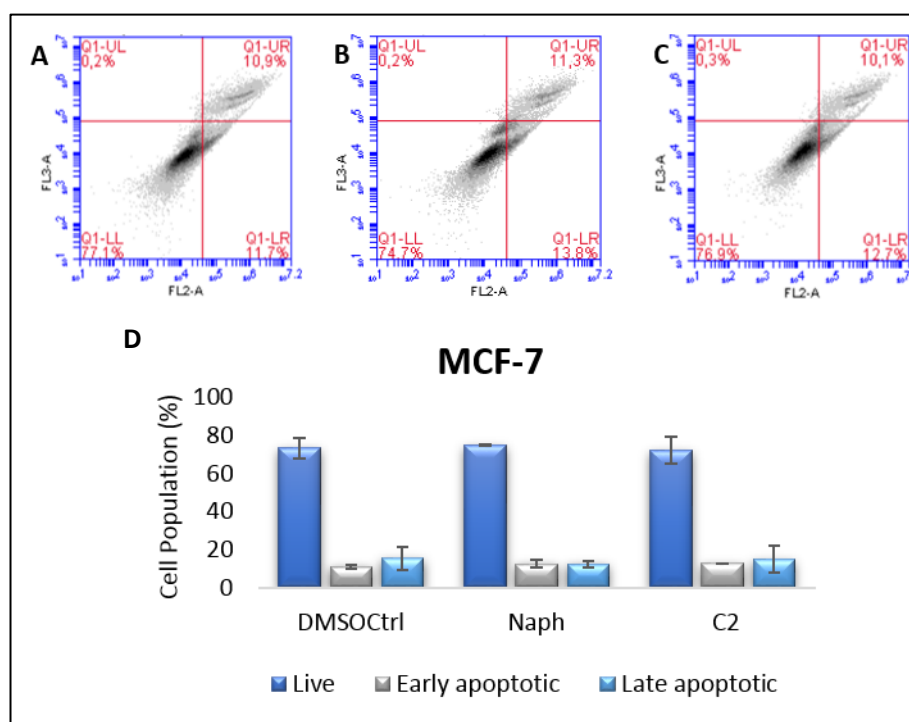


Figure 3.10: Staining assay in MCF-7 cells; (A) DMSOctrl, (B) Naph, (C) C2. (D) Quantification graph of Annexin V and 7-AAD assay.

Figures 3.13A-3.13C show the cell cycle results. The means and standard deviations of two times repeated cell cycle findings were calculated and displayed as a quantification graph in Figure 3.13D. SK-BR-3 cells treated with Naph (67.45 %) showed significant no difference live cell population when compared to DMSOctrl (66.85 %), whereas cells treated with C2 (59.7 %) had a lower population of live cells when compared to DMSOctrl (66.85 %). Moreover, cells treated with Naph and C2 (26.45 %; 28.75 %, respectively) had a slightly much population of early apoptotic cells than with DMSOctrl (25.2 %), also when compared to DMSOctrl (7.2 %), late apoptotic cells population was decreased in cells treated with Naph (5.5 %) while late apoptotic cells population was increased in cells treated with C2 (11.05 %) (Figure 3.11D).

In addition, necrosis was around 0.2 % in all cell groups.

Taken together, our results demonstrated that C2 did not significantly elevated the apoptotic effect in MCF-7, MDA-MB-231, SK-BR-3 cells but increased the apoptosis in MCF-12A. Statistical results (Figure 3.8D, 3.9D, 3.10D, 3.11D) showed that C2 and Naf treatment of the cells had no significant effect.

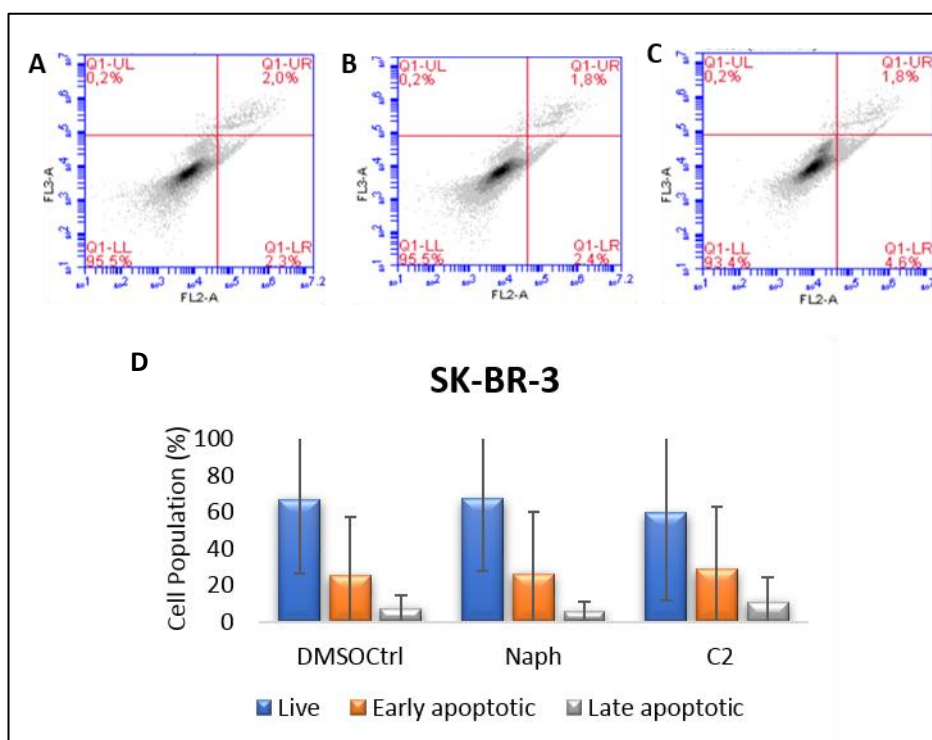


Figure 3.11: Staining assay in SK-BR-3 cells; (A) DMSOctrl, (B) Naph, (C) C2. (D) Quantification graph of Annexin V and 7-AAD assay.

3.3.2. Determination of Cell Death by Apoptosis

The levels of apoptosis-related proteins such c-PARP (89 kDa) were evaluated using Western blot analysis to detect apoptosis following Naph and C2 treatment to confirm flow cytometry results. Except for MDA-MB-231, Naph activated PARP cleavage in all cells, however C2 only cleaved PARP protein in MCF-12A cells (Figure 3.12A). In MCF-12A (Figure 3.12B) ($P<0.01$), MCF-7 (Figure 3.12C) ($P<0.05$), and SK-BR-3 (Figure 3.12D) ($P<0.001$), the statistical result of western blot demonstrated that Naph significantly increased c-PARP protein level compared to DMSOctrl. While Naph induces apoptosis, C2 has almost no effect, according to the western blot results and the flow cytometry results.

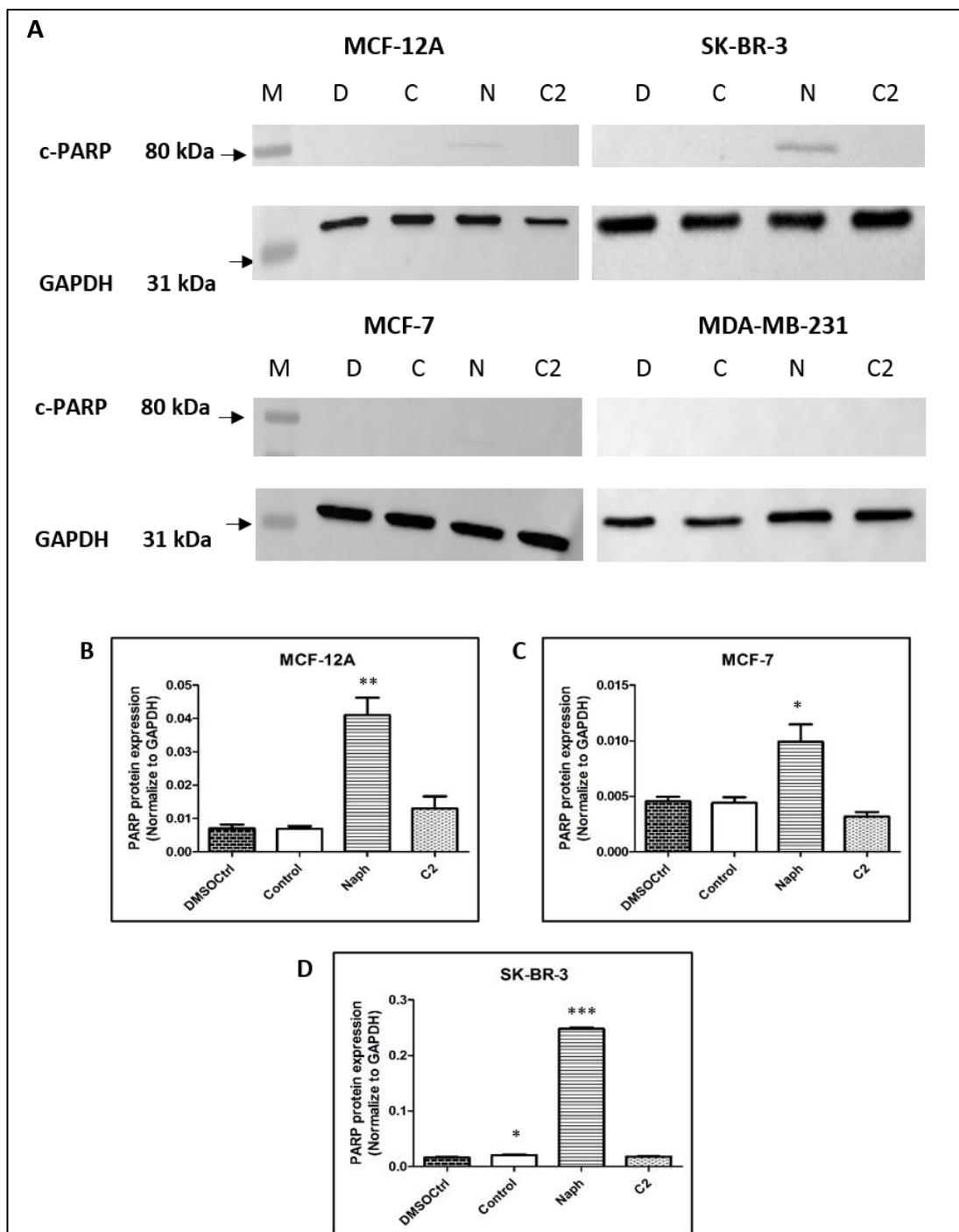


Figure 3.12: c-PARP protein expression patterns in all cells were analyzed using Western blots. As a loading control, GAPDH (37 kDa) was used. M, Marker; D, DMSO control; C, untreated condition; N, naphthazarin; C2, compound 2 (A).

Quantification graph of MCF-12A (B). Quantification graph of MCF-7 (C). Quantification graph of SK-R-3 (D). Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4. Determination of Effect of Compound 2 on the Cell Cycle

Cell cycle progress is linked to cell proliferation and apoptosis. The next step was to see if C2 could cause cell cycle arrest in breast cancer cells. The cell cycle of all cells was analyzed using Accuri C6 flow cytometry after they were treated with IC50 concentrations of Naph and C2 for 24 hours. Figures 3.13A-3.13C show the cell cycle results. The means and standard deviations of two times repeated cell cycle findings were calculated and displayed as a quantification graph in Figure 3.13D. Flow cytometry analysis of DMSOctrl of MCF-12A found that 76.9% of cells were in the G1 phase, 3.6 % were in the S phase, and 14.35 % were in the G2/M phase. In contrast, the ratios of cells in G1, S, and G2/M phases in cells treated with Naph were 75.25, 4.5, and 14.45 %, respectively, and the proportions in G1, S, and G2/M phases in cells treated with C2 were 77.4, 3.15, and 14.45 %, respectively (Figure 3.13D). C2 and Naph did not cause cell cycle arrest in MCF-12A, according to these findings. On each substance, it had no statistically significant effect.

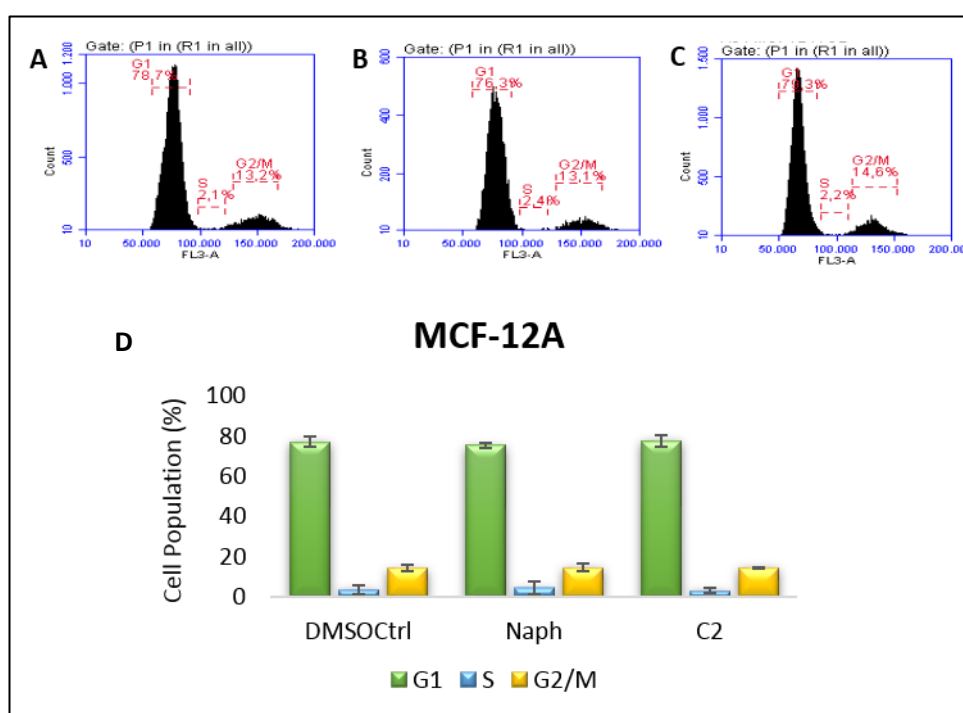


Figure 3.13: The MCF-12A cell cycle result for 24 h. (A) DMSOctrl, (B) Naph, (C) C2, (D) Statistical quantity graph.

The cell cycle data are shown in Figures 3.14A-3.14C. Figure 3.14D shows the means and standard deviations of two times repeated cell cycle results, as well as a quantification graph. DMSOCtrl indicated that 36.2 % of cells were in the G1 phase, 18.7 % were in the S phase, and 36.4 % were in G2/M phase. The proportions of cells in G1, S, and G2/M phases in cells treated with Naph were 33.35, 17.3, and 41.85 %, respectively, while the proportions of cells exposed to C2 in G1, S, and G2/M phases were 24.8, 13.15, and 34.1 %, respectively (Figure 3.14D). The results suggest that Naph treatment causes MDA-MB-231 to induce the G2/M phase arrest. C2, on the other hand, failed to provide any noticeable results. Statistically, neither chemical has a significant effect.

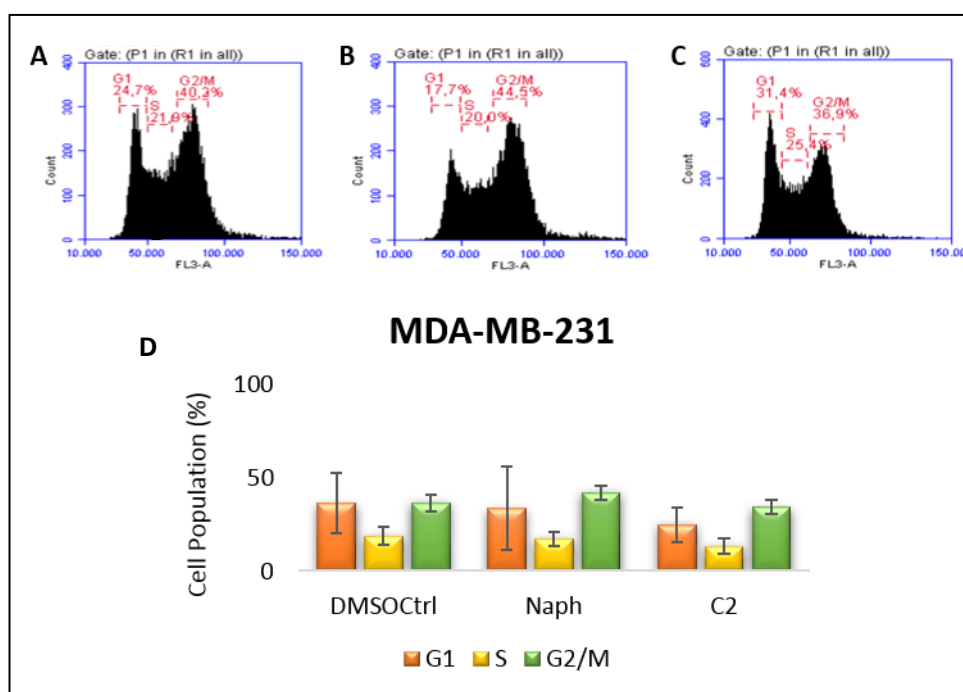


Figure 3.14: The MDA-MB-231 cell cycle result for 24 h. (A) DMSOCtrl, (B) Naph, (C) C2, (D) Statistical quantity graph.

Figures 3.15A-3.15C show the results of the MCF-7 cell cycle. The means and standard deviations of two times repeated cell cycle results were analysed and presented as a quantification graph in Figure 3.15D. DMSOCtrl showed that 43.25 % of cells were in the G1 phase, 13.05 % were in the S phase, and 37.7 % were in the G2/M phase. The proportions of cells in G1, S, and G2/M phases in Naph treated cells were 46.55, 13.25, and 32.5 %, respectively, while the proportions of cells in G1, S, and G2/M phases in C2 treated cells were 50.85, 13.65, and 27.35 %, respectively.

respectively. The findings suggest that C2 and Naph treatment caused MCF-7 cells to induce the G1 phase arrest. Neither Naph nor C2 have a statistically significant effect on cells.

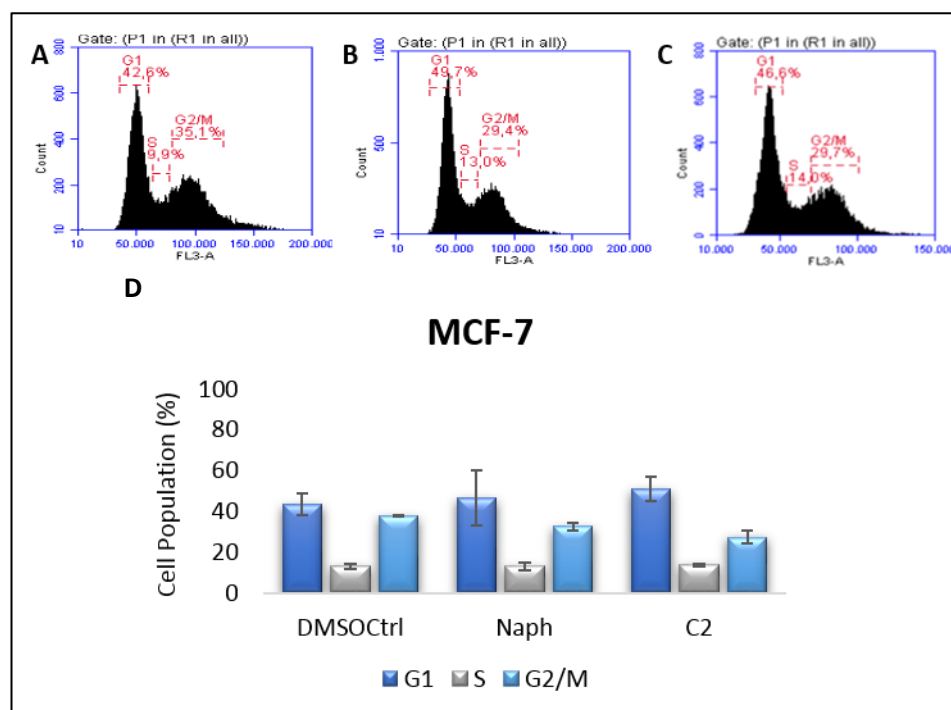


Figure 3.15: The MCF-7 cell cycle result for 24 h. (A) DMSOctrl, (B) Naph, (C) C2, (D) Statistical quantity graph.

Figures 3.16A-3.16C show the results of the SK-BR-3 cell cycle. The means and standard deviations of two times repeated cell cycle data were calculated and presented as a quantification graph in Figure 3.16D. DMSOctrl indicated that 69.55 % of cells were in the G1 phase, 9.1 % were in the S phase, and 17.2 % were in the G2/M phase. The percentage of cells in G1, S, and G2/M phases in cells treated with Naph were 59.45, 8.15, and 12.1 %, respectively, and the percentages in G1, S, and G2/M phases in cells treated with C2 were 58.25, 11.25, and 18.7 %, respectively. The results show that C2 treatment caused G2/M phase arrest in SK-BR-3 cells, but Naph had no effect. Naph and C2 have no effect on cells that is statistically significant.

Despite the fact that Naph caused cell death in some breast cancer cells, it had no effect on cell cycle arrest. In both apoptosis and the cell cycle, C2 did not have a statistically significant effect.

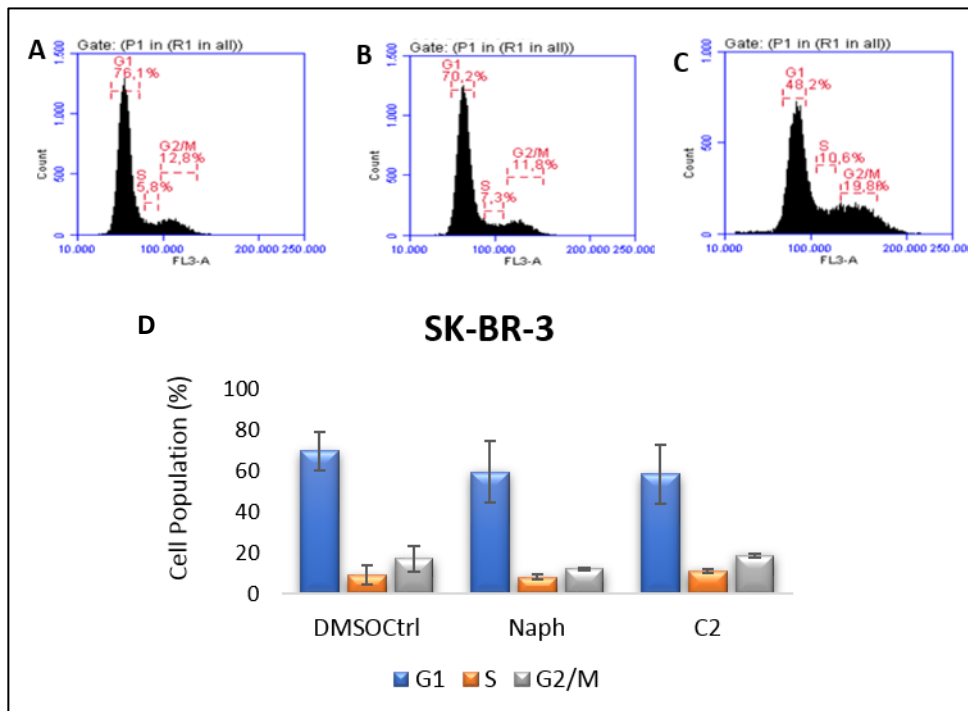


Figure 3.16: The SK-BR-3 cell cycle result for 24 h. (A) DMSOCtrl, (B) Naph, (C) C2, (D) Statistical quantity graph.

4. DISCUSSION and CONCLUSION

The human breast has a complicated structure that responds to hormones and changes morphologically and structurally as a reproductive age. In addition, breast carcinoma is recorded in about 12% of all new cancer patients each year [50]. Breast cancer is divided into subgroups, and its complicated biological structure makes treatment more difficult. Breast cancer subtypes are generally classified according to the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [51], [52].

Plant-derived compounds have been shown to have anti-cancer effects in several investigations. In clinical trials for new anti-tumor medicines, 1,4 naphthoquinones and their derivatives have been utilized often [53], [54]. Naphthazarin which is a the 1,4- naphthoquinones [34] and known to have an anti-tumor effect therefore very suitable for cancer treatment [37]. The effect of several naphthazarin derivatives on cancer, as well as their combined effect with other cancer therapy approaches, has been investigated in prior studies [55], [56]. However, in breast cancer, the biological roles and cytotoxic mechanism of our newly synthesized naphthazarin derivatives have yet to be determined. In this study, naphthazarin derivatives were investigated to have cytotoxic, proliferative, apoptotic, and cell cycle arrest effects in breast cancer cells *in-vitro*.

We initially investigated at how Naph, C1, and C2 affected the viability of breast cancer cells compared to epithelia breast cells in a dose-dependent manner. In comparison to MCF12A cells, Naph did not have a notable cytotoxic effect on breast cancer cells (Figure 3.1). C1; 3 μ M for SK-BR-3 cells (Figure 3.2), C2; at 7 μ M for MCF-7 cells (Figure 3.3), and 7, 10, 12 μ M for SK-BR-3 cells (Figure 3.3) exhibited a substantial cytotoxic effect for 24 hours when compared to MCF-12A.

As C2 had a more substantial and effective cytotoxic effect than Naph and C1, real-time cell growth was conducted only using C2 substance concentrations. MCF-7 (Figure 3.4) and MDA-MB-231 (Figure 3.5) were shown to suppress cell growth by more than 60% C2 at 3 and 5 μ M concentrations when compared to MCF-12A. We used naphthazarin to explain the newly synthesized compound (C2) because there is no cancer research on it in the literature. They stated in a recent literature study that employing 1 μ M Naph concentration with radiotherapy was effective in MCF-7 cells

[55], nevertheless the effect of Naph on normal breast cells was not analyzed. In comparison to MCF-12A, SK-BR-3 did not have a statistically significant result at common concentrations (Figure 3.7). Consistent with the IC₅₀ value of C2 on SK-BR-3 cells we observed with antiproliferative effect starting with 7 μ M concentrations progressively increased. (Figure 3.6). It's possible that the fact that C2 had no effect on SK-BR-3 cells at concentrations lower than 7 μ M may be due to that tumors develop, or cell have naturally own mechanism to avoid HER2 (ERBB2) inhibition [57]. The RAS/Raf/Mitogen-Activated Protein Kinase (MAPK) pathway (related with proliferation) and the phosphatidylinositol-3 kinase protein (PI3K) / Akt pathway (related with cell survival) are both activated when HER ligand binds to its receptor. Increased proliferation, decreased apoptosis, angiogenesis, and invasiveness can all result from abnormal HER-family member expression or activation [58], [59]. Resistance to HER2 inhibitors is caused by a variety of mechanisms. First, a mutation in the *Her2* gene causes a change in medicines binding [60]. Second, signaling via other receptor tyrosine kinases can transactivate HER2 and enhance signal transduction downstream, bypass the inhibitory impact of medicines [61]. Third, PI3K pathway activating mutations can provide resistance to HER2 inhibitors [62]. In brief, we may conclude from this thesis that C2 treatment dramatically slowed the growth of MDA-MB-231 and MCF-7 cells, as well as being less toxic in MCF-12A. The xCELLigence System RTCA program determined IC₅₀ concentrations of C2 for each cell line (Table 2.10), and these concentrations were used in subsequent experiments.

The newly synthesized naphthazarin derivatives were added various numbers of acetyl groups. We observed that C2 suppressed cell growth and cytotoxicity better than Naph and C1. The reason for this could be the addition of two acetyl groups to C2. As the number of acetyl groups increases, the substance's efficiency could rise. As a result of acetylation, these acetyl groups can be utilized in the cell. Histone acetyl transferases (HATs) and histone deacetylases are post-translational modifications that alter the mantle of histone and nonhistone proteins (HDACs) [63]. These enzymes work together to control the balance between acetylation and deacetylation [64], and they're important in regulation of genes controlling cellular proliferation, differentiation, cell cycle delay, DNA repair, and apoptosis. Furthermore, many of the non-histone proteins that are acetylated are products of oncogenes or tumor suppressor genes [63]. In based on previously mentioned, C2's

acetyl groups may have been involved in the transcription of genes required for cell proliferation suppression.

Apoptosis is cancer cells response to anticancer medicines that causes cytotoxicity [65]. As a result, two distinct methods of apoptosis analysis were used. First, the apoptosis-inducing activity of cells was examined by flow cytometry. MCF-12A cells treated with C2 had a significantly higher number of late apoptotic cells and a lower number of live cells than those treated with Naph (Figure 3.8D). In contrast, at IC₅₀ concentrations, MDA-MB-231 cells showed essentially negligible apoptosis (Figure 3.9D). We found a relatively increased number of apoptotic cells in MCF-7 and SK-BR-3 cells treated with C2 and Naph compared to the control groups of cells (Figure 3.10D, 3.11D). Our MCF-7 finding is consistent with prior reports of Naph's effect on MCF-7. In a previous study, 1 μ M Naph had the same effect on human gastric cancer cells, and Naph did not cause effective apoptosis [66]. Another critical fact to note is that the C2 substance did not cause necrosis in any of the breast cell lines studied. Our findings showed that C2 did not efficiently promote apoptosis in MDA-MB-231, MCF-7, or SK-BR-3 cells, but it did induce apoptosis in MCF-12A cells.

Second, we investigated at changes in the level of c-PARP proteins in response to C2 treatment to learn more about the mechanism underlying C2's anti-proliferative effect. Consistent with the flow cytometry method, C2 treatment of MCF-12A cells increased the expression of cleaved PARP, however C2 treatment of MDA-MB-231 cells did not detect the expression of cleaved PARP (Figure 3.12A). These findings suggested that the c-PARP signaling pathway is unlikely to be implicated in C2's anticancer effects on TNBC. MCF-12A (Figure 3.12B), MCF-7 (Figure 3.12C), and SK-BR-3 (Figure 3.12D) cells treated with Naph showed considerably higher levels of c-PARP protein expression than C2 treated cells. It has been proven in previous studies that naphthazarin induces apoptosis in different cancer cells [55], [56], [66]. Caspases-3 cleaves PARP during apoptosis, suppressing PARP activity. Because PARP is involved in the repair of DNA damage. [67]. Apoptosis is also regulated by anti-apoptotic proteins like Bcl-2 and pro-apoptotic proteins like Bax [68]. To be certain of the results, the levels of cleavage caspase-3, Bax/Bcl-2 proteins should also be analyzed. Our findings showed that C2 treatment of MDA-MB-231, MCF-7 cells decreased cell viability and suppressed cell growth but did not actually cause apoptosis. MCF-12A treatment of C2 did not cause toxicity, but it did cause

apoptosis. Instead of inhibiting HER2 at the IC₅₀ concentration, SK-BR-3 treated with C2 demonstrated resistance.

C2 substance decrease the population of some breast cancer cells, according to MTT and proliferation analysis results, however this may not be due to apoptosis. It could be caused by autophagy, a type of programmed cell death. Autophagy can cause cell death if caspase proteins are blocked in apoptosis by cancer cells [69] and agents that cause tumor cell death can also induce autophagy. Autophagy and apoptosis may work synergistically, or one mechanism may be antagonistic to the other, or they may be mutually incompatible. Sphingosine 1phosphate (S1P), ceramide, and sphingosine are lipid messengers that control cell death, growth, and survival. Ceramide and S1P provide autophagy regulation [70]. S1P and its producing enzyme, sphingosine kinase 1 (SK1), were demonstrated to activate autophagy in MCF-7 cells in a previous study [71]. In addition, autophagy is inhibited by substances such 3MA (3-methyladenine), wortmannin, and LY294002 [72]. With the application of 3MA in MCF-7 cells, in previous research have demonstrated that lysosomal activity increases, autophagic vesicles accumulate, and autophagy reduces. Hence this pathway is independent of caspase proteins [73]. Taking all of this into consideration, autophagy in breast cancer cells can be investigated using 3MA autophagy inhibitors or autophagy regulators such as Beclin1, LC3, and the pro-death Bcl2 family member BNIP3 and the autophagic cell death kinase DAPk [70].

The cell cycle and apoptosis are frequently disrupted during cancer development. As a result, we investigated whether C2 induce cell cycle arrest in the breast cancer cells using flow cytometry. The cell cycle of MCF-12A was not arrested by C2, indicating that C2 had no effect on the mechanisms of normal breast cells (Figure 3.13D). In MCF-7 cells, C2 and Naph compounds caused G1 phase arrest (Figure 3.15D). Because MCF-7 cells are more sensitive than MDA-MB-231 or SK-BR-3 cells, they may have been arrested at the first checkpoint. Naph compounds induced G2/M phase arrest in MCF-7 cells in previous research (maximum concentration 1.5 μ M) [55], [56], although the concentration was dramatically lower than ours (4.99 μ M). As a result of the high concentration, the cell cycle may have been arrested in the G1 phase. MDA-MB-231 cells treated with C2 showed no substantial cell cycle arrest, whereas Naph caused a G2/M phase arrest (Figure 3.14D). The cell cycle arrest of SK-BR-3 cells treated with Naph was

not notable, however C2 was arrested in the G2/M phase (Figure 3.16D). C2 and Naph may cause DNA damage or stress in cells, leading in G2/M cell cycle arrest. To obtain more high accuracy, Western blot analysis of the cyclin protein, which controls the cell cycle, and the cell cycle regulatory proteins Cdc25 phosphatase or p27 are required. Because the amount of cyclin proteins varies depending on the stage of the cell cycle. For example, the levels of cyclin E in the G1 phase and Cyclin B1 in the G2/M phase can be investigated [3].

As a conclusion, in MCF-7 and MDA-MB-231 cells low concentrations of C2 decreased proliferation and had a significant toxic effect but were ineffective in cell death and cell cycle arrest. Because of the SK-BR-3's HER2 resistance mechanisms, meaningful outcomes in the IC50 concentration of C2 may not have been obtained. Lastly, C2 did not cause toxicity or cell cycle arrest in MCF-12A cells, but it caused apoptosis. C2 does not cause apoptosis in breast cells, although it is possible that it stimulates autophagy, like Naph did in the literature by Acharya *et. al.* (2011). Thus, the newly synthesized modified naphthazarin derivative could be utilized as a candidate medicine in the treatment of some type breast cancer. However, more research into the mechanisms underlying C2's anticancer effects on breast cancer is required. Furthermore, similar C2 derivatives or the same compounds can be investigated in different types of cancer in the future, or more precise results can be acquired by expanding the research.

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