INVESTIGATION OF STILBENE-FUSED CHALCONE AND FLAVANONE DERIVATIVES FOR THEIR CYTOTOXIC AND ANTI-CANCER PROPERTIES

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ABSTRACT

INVESTIGATION OF STILBENE-FUSED CHALCONE AND FLAVANONE DERIVATIVES FOR THEIR CYTOTOXIC AND ANTI-CANCER PROPERTIES

Stilbene, chalcone and flavanones are three major classes of molecules, which can be found in plants as secondary metabolites. Derivatives of those may possess variety of biological activities. In this study it is aimed to synthesize a hybrid molecule which may show the biological activities of both flavanone and stilbene, or chalcone and stilbene simultaneously. For this purpose previously synthesized 11 simple chalcone, flavanone and stilbene derivatives and 31 stilbene-fused chalcones and stilbene-fused flavanones were tested for their cytotoxic activities in prostate cancer cell line (PC-3) and breast cancer cell line (MCF-7) by using MTT assay. Then aromatase inhibition properties of simple chalcones, flavanones, stilbenes, stilbene-fused chalcones and stilbene-fused flavanones were studied.

Results of the study were evaluated in potential of the hybrid system to carry out more than one biological activity and mimicking performance of the simple ones. Results indicate that tested simple chalcone and flavanone derivatives are more cytotoxic than simple stilbenes in both cancer cell lines. On the contrary, simple stilbene structures were much more successful in aromatase inhibition assays. Cytotoxic activity profiles of stilbene-fused chalcones in cancer cells show that those molecules mostly mimic the simple chalcone structures. On the other hand, flavanones lost their cytotoxic activities when they were fused with stilbenes. In addition, aromatase inhibition assay showed that stilbene-fused chalcones again do mimic the simple chalcones but not simple stilbenes. In the same assays, stilbene-fused flavanones may mimic both simple flavanones and simple stilbenes by depending on the type and position of the substituent in terminal aromatic rings.

ÖZET

SİTİLBEN KAYNAŞTIRILMIŞ ÇALKON VE FLAVANON TÜREVLERİNİN SİTOTOKSİK VE ANTİ-KANSER ÖZELLİKLERİNİN İNCELENMESİ

Bitkilerde bulunan sekonder metabolitlerin üç temel sınıfını oluşturan sitilben, çalkon ve flavanonlar ve onların türevleri çeşitli biyolojik aktivitelere sahiptir. Bu çalışmada, flavanon-sitilben yapılarının veya çalkon-sitilben yapılarının biyolojik aktivitelerine aynı anda sahip hibrid moleküller sentezlemek amaçlanmıştır. Bunun için sentezleri daha önce gerçekleştirilen 11 basit çalkon, flavanon ve sitilben türevleri ile 31 sitilben-kaynaştırılmış çalkon ve sitilben-kaynaştırılmış flavanonların sitotoksik özellikleri prostat kanseri hücre hattı (PC-3) ve meme kanseri hücre hattında (MCF-7) MTT ile test edildi. Daha sonra bu maddelerin aromataz enzimi üzerindeki baskılayıcı etkileri araştırıldı.

Sonuçlar iki ana başlık altında değerlendirildi; birincisi hibrid moleküllerin birden fazla biyolojik aktiviteyi taşıma potansiyeli, ikincisi ise hibrid moleküllerin basit yapıların özelliklerini taklit etme yetenekleri. Sonuçlar gösteriyor ki, basit çalkon ve flavanon türevleri her iki kanser hücresi hattında da basit sitilbenlere göre daha yüksek sitotoksik etkiye sahiptirler. Bunun aksine basit sitilbenler aromataz enzimini inhibe etmede basit çalkon ve flavanonlardan daha başarılıdır. Sitilben-kaynaştırılmış çalkonların sitotoksik özellikleri de bu maddelerin çoğunlukla basit çalkonları taklit ettiğini göstermektedir. Diğer taraftan, flavanonlar sitilbenlerle kaynaştırıldığında sitotoksik aktivitelerini kaybetmektedir. Ayrıca aromataz inhibisyon çalışmalarında, sitilben-kaynaştırılmış çalkonların basit sitilbenleri değil basit çalkon yapılarını taklit ettiği görülmüştür. Sitilben-kaynaştırılmış flavanonlar ise terminal aromatik halkalarındaki substituentlerin türüne ve pozisyonuna bağlı olarak hem basit flavanonları hem de basit sitilbenleri taklit edebilmektedir.

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CHAPTER 1

INTRODUCTION

1.1. The Cell, Apoptosis and Cancer

The cell is the smallest functional unit of any living organisms which can be classified as unicellular or multicellular. Humans contain about 10 trillion (10^{13}) cells and the body makes up many types of cells. When the body is healthy cells grow and divide in a controlled way to produce more cells. In a normal process when cells become old or damaged, they die or are replaced with new cells. Cell death can be described as either apoptosis or necrosis. Apoptosis which is a normal physiological process of cell suicide is encoded in the chromosomes of all nucleated cells. Physiological cell death, which is a firmly controlled and finely organized event, plays a significant role in development, tissue homeostasis, and defense against viral infection and mutation by removing unnecessary and damaged cells. Apoptosis is regulated by a several range of cell signals, which may arise either extracellularly or intracellularly and may affect apoptosis positively or negatively. In an apoptotic process, the cell shrinks and separate from neighboring cells, thereby it dies with minimal harm to nearby cells compare to necrosis which is characterized by inflammation and widespread damage.[1]

The mutations, which occur through errors in the replication of deoxyribonucleic acid (DNA) during cell division or after exposure to a carcinogen, cause the loss of a cell's ability to undergo apoptosis and affect the normal cell growth and division. When this happens, cells continue to grow and form new and abnormal cells instead of dying. Thus, the balance between the rates of new cell growth and old cell death is disrupted.[2] This abnormality in cell growth and multiplication is called cancer, which is a general name for a more than 200 diseases in which cells in a part of the body begin to grow out of control, and occurs because of environmental factors (such as cigarette smoking or radiation exposure), viral factors, a genetic predisposition, "dumb luck", and as yet unexplained causes.[3]

Cancer cells can spread to other parts of the body by two mechanisms: invasion and metastasis. Invasion refers to the direct migration and penetration by cancer cells

into neighboring tissues. Metastasis refers to the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the blood stream, and then invade normal tissues elsewhere in the body.[4]

There are over 60 different organs in the body where a cancer can develop and each organ is made up of several different types of cells. For this reason, there are more than 200 different types of cancer. Breast cancer, the second leading cause of cancer deaths, is the most commonly diagnosed cancer among postmenopausal women.[5]

In general, breast cancer is classified as estrogen receptor positive breast cancer and estrogen receptor negative breast cancer. Roughly one-third of all breast cancer patients and two-third of postmenopausal breast cancer patients have estrogen receptor positive breast cancer. In this type of breast cancer, cells contain estrogen receptors which regulate gene expression as a DNA-binding transcription factor and require estrogen to grow and multiply.[6]

1.2. Estrogens

Estrogens are essential regulators of many physiological processes such as differentiation and development of reproductive tissues in both men and women, protection against osteoporosis via maintenance of bone density, reduction of lipid and cholesterol levels in blood acting as a cardioprotective hormone, regulation of reproductive behavior, homeostasis and general mood.[7] In addition to normal physiological roles of estrogen, the prevalence and progression of cancerous cells within the body has been linked to prolonged stimulation by higher than normal levels of estrogen.[8]

Estrogen receptors are normally in the nucleus. When an estrogen molecule enters into target cell by simple diffusion and passes into the nucleus, estrogen receptor which exists as monomer complexed with non-steroid-binding heat shock proteins binds to estrogen with high affinity and with a high degree of specificity, releasing the heat shock proteins. These estrogen-receptor complexes form homodimers, thus the affinity of receptors for DNA hormone response elements (HREs) in the regulatory regions of estrogen-responsive genes increases. The estrogen-receptor complex binds to DNA HREs and recruits coactivator proteins. In this way the target gene is activated to make

messenger RNA (mRNA) molecules. These messenger RNAs guide the synthesis of specific proteins to increase the cell proliferation.[9]

Estrogens, the primary female sex hormones, comprise a group of compounds, including estrone (**1**), estradiol (**2**) and estriol (**3**). They are the three major naturally occurring estrogens in women. Estradiol and estrone are the predominant estrogens during reproductive years and menopause, respectively. Estetrol which is another type of estrogen is produced only during pregnancy. All of the different forms of estrogen are synthesized from androgens. Estrone and estradiol which are the most important estrogens in humans are synthesized from androstenedione (**4**) and testosterone (**5**), respectively, by aromatase which is a unique member of a cytochrome P450 (CYP) superfamily of microsomal enzymes. (Figure 1.1) [7]

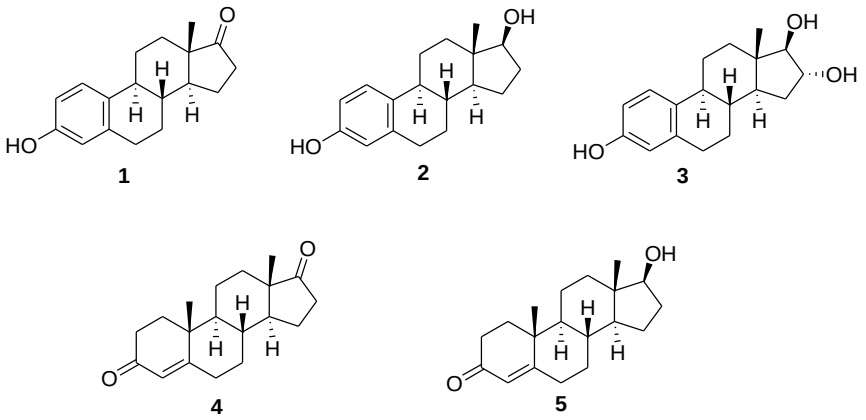

Figure 1.1. Estrone (**1**), estradiol (**2**), estriol (**3**), androstenedione (**4**), and testosterone (**5**) structures.

1.3. Aromatase Enzyme

Aromatase, also called estrogen synthetase or estrogen synthase, is an enzyme responsible for the last, rate-limiting step in the biosynthesis of estrogens. It is a member of the cytochrome P450 superfamily (E.C 1. 14. 14. 1), and is responsible for the aromatization of androgens into estrogens. The aromatase enzyme complex which is localized in the endoplasmic reticulum of estrogen producing cells is comprised of two polypeptides. The first is a specific cytochrome P450 heme protein. The second is a flavoprotein, NADPH-cytochrome P450 reductase. This enzyme complex catalyzes three consecutive hydroxylation reactions.[4]

In humans, the gene CYP19 encodes the aromatase enzyme. It contains nine coding exons from II to X. The expression of this gene is regulated by the alternative use of eight tissue specific promoters (PI.1, PI.3, PI.4, PI.6, PI.7, and PII) which employ different signaling pathways and different transcription factors. For example, as in healthy breast tissue aromatase expression is regulated by promoter I.4, in breast cancer tissue aromatase expression switches to promoter I.3 and II.[6] [10]

Aromatase enzyme has become a target for new drug synthesis to treat estrogen hormone dependent breast cancer due to its regulatory property in estrogen biosynthesis.[11] Approaches for hormonal therapy of estrogen dependent breast cancers are either blocking the mechanism of action of estrogens by selective estrogen receptor modulators (SERMs) or inhibiting estrogen biosynthesis by aromatase enzyme inhibitors (AIs).[4]

SERMs bind estrogen receptors and change their conformations, thus, simplify binding of co-regulatory proteins which activate or suppress transcriptional activation of estrogen target genes. SERMs can be properly divided into three main categories: (1) triphenylethylene derivatives like tamoxifen (**6**) (Figure 1.2), (2) other nonsteroidal compounds, and (3) steroidal compounds which possess more complete anti-estrogenic activity. Tamoxifen, which was approved by the United States Food and Drug Administration in 1977, like other SERMs, binds to estrogen receptors of breast cancer cells and antagonizes the effect of estrogen on various growth-regulatory genes. The dominant effect of tamoxifen is cytostatic with the induction of a G_1 cell cycle block by slowing cell proliferation.[12]

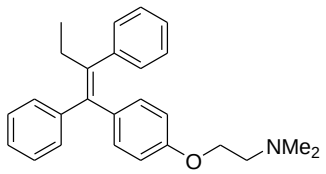

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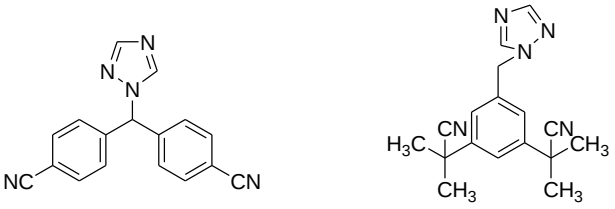
Figure 1.2. Structure of tamoxifen (**6**).

1.4. Aromatase Inhibitors

Aromatase inhibitors are classified as steroidal and nonsteroidal inhibitors based on their structures. Nonsteroidal inhibitors such as letrozole (IC_{50}=2 nM) (**7**) and

anastrozole (IC_{50}=8 nM) (**8**) which have the triazole functional group contain a heteroatom (usually nitrogen containing heterocyclic moiety) that interacts with the heme prosthetic group in the active site of aromatase enzyme and occupy its substrate binding site, thus preventing binding of androgens to the active site. (Figure 1.3) Heterocyclic moiety may interact with aromatase via hydrogen and/or van der Waals bonding. Since the binding is reversible, nonsteroidal inhibitors can be competitively displaced from the active site by endogenous substrates.



7 **8**

Figure 1.3. Letrozole (**7**) and anastrozole (**8**) structures.

Steroidal inhibitors such as exemestane (IC_{50}=15 nM) (**9**) (Figure 1.4) are recognized by the active site as an alternative substrate and compete rapidly with natural aromatase substrates for the active site of the aromatase enzyme initiating a time dependent reactive process resulting in either covalent or very tight binding of the inhibitor to the enzyme and causing its inactivation. This irreversible aromatase inhibitors are also known as inactivators or "suicide" inhibitors because aromatase is inactivated owing to its own mechanism of action.[13]

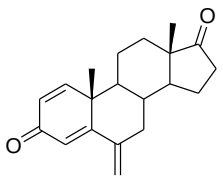


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Figure 1.4. Structure of exemestane (**9**).

Besides the large number of synthetic aromatase inhibitors, there is a continuous research for natural products to discover new breast cancer chemopreventive agents. Flavonoids attract the researchers as a new class of natural products due to their unique structures and aromatase inhibitory activities.[14]

1.5. Flavanoids

Flavonoids are a group of polyphenolic phytochemical compounds that occur ubiquitously in foods of plant origin. Polyphenols possess a basic structure that comprises one or more phenolic rings and often have several hydroxyl groups, which are highly correlated with their strong antioxidant capacity. Several studies have addressed the ability of flavonoids to interfere with the catalytic activity or expression of aromatase enzyme. Flavonoids which have considered as phytoestrogens may compete with endogenous steroids for enzyme active sites or interact with the estrogen receptors.[8]

Flavonoids are synthesized from 2'-hydroxy derivative of chalcones (**10**) (Figure 1.5) which are unique in the flavonoid family in lacking a heterocyclic ring and possess several biological activities such as estrogenic, antifungal, antibacterial, antiviral and anti-inflammatory.[15] Chalcones are also an important class of molecules among the currently identified antitumor agents because of being abundant in edible plants.[16]

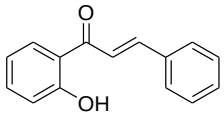

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Figure 1.5. Structure of 2'-hydoroxy chalcone (**10**).

Flavonoids are plant secondary metabolites [17] and are divided into six classes. Flavanones (**11**), flavones (**12**), and flavanols (**13**) are the common flavonoids. Isoflavonoids (**14**) which is a distinct class of flavonoids have estrogenic activity. Anthocyanins (**15**) are the charged and colored flavonoids. The last class of flavonoids is flavans (**16**) including catechin (**17**) and biflavan (**18**) which are complicated in structure and naming. (Figure 1.6) [18]

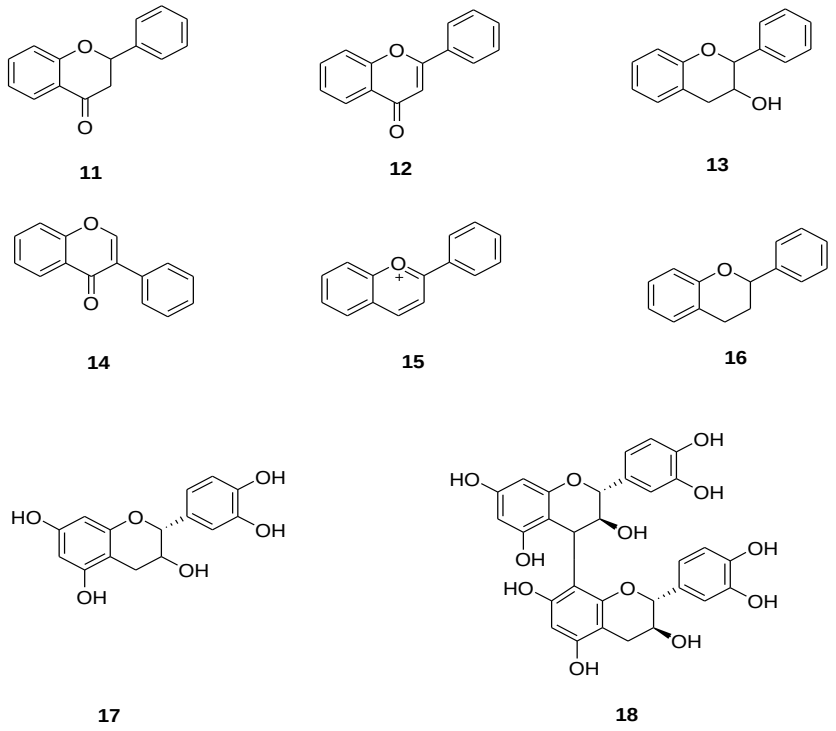

Figure 1.6. Structures of flavanone (**11**), flavone (**12**), flavanol (**13**), isoflavone (**14**), anthocyanin (**15**), flavan (**16**), catechin (**17**), and biflavan (**18**).

1.5.1. Chalcones

Chalcones are cancer preventive food components in a human diet that is rich in fruits and vegetables. In literature many chalcone derivatives were synthesized and investigated for their possible anticancer activity. As an example, Hsu and colleagues examined chalcone (1,3-diphenyl-2-propenone) (**10**) for its effect on proliferation in human breast cancer cell lines, MCF-7 and MDA-MB-231. Their data showed that chalcone exhibited significant inhibitory effect with IC_{50} values of 4.9 µg/mL and 6.0 µg/mL for MCF-7 and MDA-MB-231cell lines, respectively.[15]

In another example, a series of novel chalcones and bis-chalcones containing boronic acid derivatives were synthesized by Modzelewska and colleagues. Chalcones evaluated for their antitumor activity against the human breast cancer MDA-MB-231 and MCF7 cell lines at low micromolar to nanomolar concentrations. Compound **19** (Figure 1.7) had the most significant antiproliferative activity with an IC_{50} value of 0.35 µM.[16]

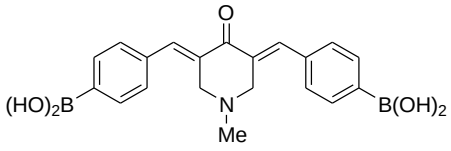

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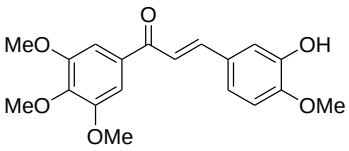
Figure 1.7. Structure of bischalcone (**19**).

Lawrence and research group investigated effects of α-substitutions on chalcone structure. The cell growth inhibitory properties of the substituted chalcones were determined in the K562 human chronic myelogenous leukemia cell line. The chalcones **20** and **21** (Figure 1.8) with the 3-hydroxy-4-methoxy ring showed the highest cytotoxicities with IC_{50} concentrations of 4.3 nM and 2.1 nM, respectively.[19]



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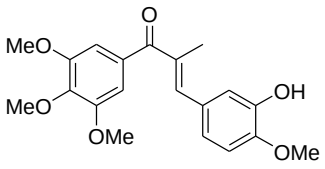

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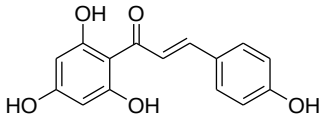
Figure 1.8. Structures of substituted chalcones **20** and **21**.

Bail and colleagues were evaluated different chalcone derivatives to better understand their anti-aromatase activity in human placental microsoms. They also used aminoglutethimide (IC_{50}=1.2 µM) as positive control for aromatase inhibition. Results show that treatment of human placental microsoms with 2',4',6',4-tetrahydroxychalcone (**22**) and 2',4',6',3,4-pentahydroxychalcone (**23**) exhibited more anti-aromatase activity compared to 2'-hydroxychalcone (**24**) and 4'-hydroxychalcone (**25**). (Figure 1.9) Their IC_{50} concentrations was found as 2.6 µM, 2.8 µM, 50 µM, and 30.6 µM, respectively.[8]



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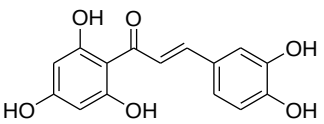

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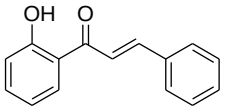

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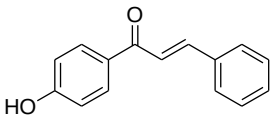

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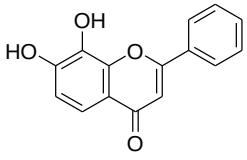
Figure 1.9. Structures of 2',4',6',4-tetrahydroxychalcone (**22**), 2',4',6',3,4-pentahydroxy-chalcone (**23**), 2'-hydroxychalcone (**24**), and 4'-hydroxychalcone (**25**).

1.5.2. Flavanones

A set of flavanone and flavone derivatives were synthesized and evaluated for their antiproliferative activity against MCF-7 human breast cancer cells by Pouget and colleagues. They discussed the structure-activity relationship of these compounds and found that unsubstituted flavanone was a weak inhibitor of MCF-7 cell growth as well as flavanones with hydroxy group. However, the methoxy group substitution increased the antiproliferative activity. On the other hand, flavones showed weak aromatase inhibitory effect except 7,8-dihydroxyflavone (**26**) which was found to be most potent (IC_{50}=27.5 µM) whereas 7,8-dihydroxyflavanone (**27**), the corresponding flavanone, was inactive. (Figure 1.10) [20]



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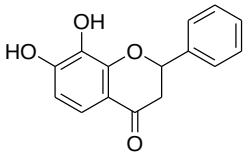

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Figure 1.10. Structures of 7,8-dihydroxyflavone (**26**) and 7,8-dihydroxyflavanone (**27**).

Fagnere and research team synthesized a series of flavanones by cyclization of 2'-hydroxy chalcones formed by Claisen-Schmidt condensation and investigated the aromatase inhibitory activity. It was observed that the substitution pattern was effective on the inhibitory activity. Hydroxyl group at position 3' and/or 4' increased the anti-aromatase activity; hence, 3',4'-dihydroxy-7-methoxyflavanone (**28**) (Figure 1.11)

(IC_{50}=2.5 µM) was found more potent than aminoglutethimide (IC_{50}=5.2 µM) which is the first aromatase inhibitor clinically used.[21]

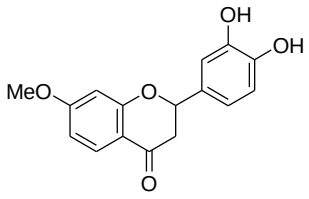

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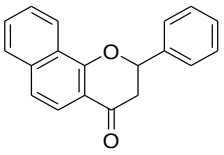
Figure 1.11. Structure of 3',4'-dihydroxy-7-methoxyflavanone (**28**).

Yahiaoui and colleagues synthesized new 7,8-benzoflavanones and compared them with hydroxy and methoxy substituted flavanones for their anti-aromatase activity. They found that 7,8-benzoflavanone (**29**) was twice more active than 7-methoxyflavanone (**30**) with IC_{50} values of 4.3 µM and 8.0 µM, respectively. They also demonstrated that introduction of an electron-donating group such as hydroxyl group increased the aromatase inhibitory effect. According to this, 3'-OH substituted 7-methoxhyflavanone (**31**) and 7,8-benzoflavanone (**32**) had more aromatase inhibitory activity with IC_{50} values of 3.5 µM and 0.61 µM, respectively. (Figure 1.12) [22]



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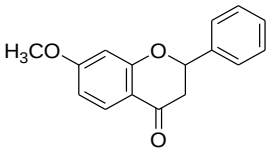

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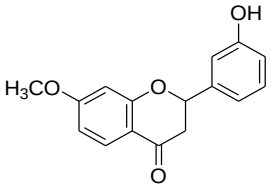

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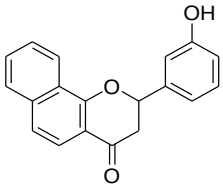
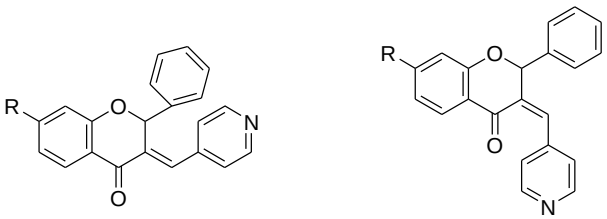


32

Figure 1.12. Structures of 7,8-benzoflavanone (**29**), 7-methoxyflavanone (**30**), 3'-hydroxy-7-methoxyflavanone (**31**), 3'-hydroxy-7,8-benzoflavanone (**32**).

Pouget and research team synthesized (*E*)- and (*Z*)-pyridinyl substituted flavanone derivatives. These compounds were tested for their cytochrome P450 aromatase inhibitory effect. It was observed that the presence of a pyridinylmethylene

group on flavanone significantly increased aromatase inhibition compared to unsubstituted flavanone. It was also showed that the presence of a methoxy group increased the aromatase inhibitory activity. IC_{50} values of (*E*)-isomers of compounds **33** and **34** (Figure 1.13) were 0.80 µM and 0.62 µM, respectively. The effect of configuration was also investigated and found that (*E*)-isomers had more anti-aromatase activity than (*Z*)-isomers with IC_{50} values of 3.3 µM and 1.6 µM, respectively.[23]



(E) 33, 34-

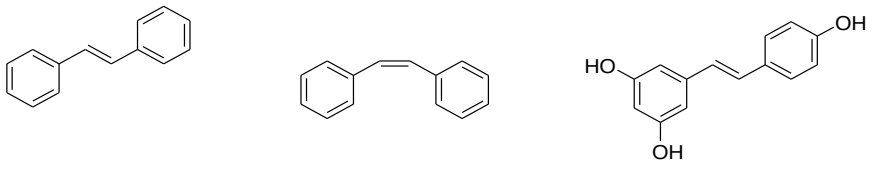
33: R=H; 34: R=OMe

(Z) 33, 34-

Figure 1.13. Structures of compounds (*E*)-**33**, **34** and (*Z*)-**33**, **34.**

1.6. Stilbenes

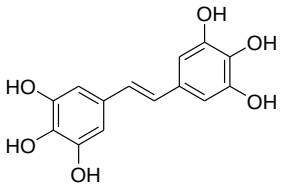
Stilbenes (1,2-diphenylethylene) are another class of plant secondary metabolites.[24] There are two isomeric forms of 1,2-diphenylethylene; (*E*)-stilbene (*trans*-stilbene) (**35**), which is not hindered by steric effect, and (*Z*)-stilbene (*cis*-stilbene) (**36**), which is hindered by steric effect, and therefore less stable. In nature, stilbenes overwhelmingly exist in the trans forms.[25] Most stilbenes are derived from the *trans*-resveratrol (**37**) which can prevent or slow the progression of a wide variety of illnesses, including cancer, obesity, senescence, and cardiovascular diseases. (Figure 1.14) [26]



35 **36** **37**

Figure 1.14. Structures of (*E*)-stilbene (**35**), (*Z*)-stilbene (**36**), and *trans*-resveratrol (**37**).

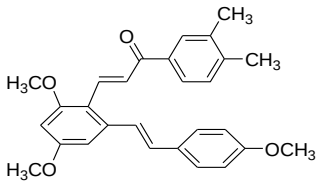
Murias and colleagues were synthesized five polyhydroxylated resveratrol analogues and evaluated for their cytotoxic activity against HL-60 leukemic cells and structure-activity relationships. It was demonstrated that resveratrol had higher growth inhibitory activity than gallic acid which is a commonly used reference anti-leukemic compound (IC_{50} values: 12.1±0.17 µM and 18.3±0.23 µM respectively). The presence of hydroxyl groups on resveratrol increased cytotoxic activity on HL-60 leukemic cells. In this study, compound **38** (Figure 1.15), substituted with six hydroxyl groups, was found the most active analogue (IC_{50}: 4.2±0.09 µM).[27]



38

Figure 1.15. Structure of compound **38**.

A group of resveratrol derivatives having chalcone moiety were synthesized and tested for their anti-proliferative activity by Ruan and colleagues. It was showed that compound **39** (Figure 1.16) had the most potent cytotoxic activity against HepG2 human liver carcinoma, B16-F10 melanoma, and A549 adenocarcinoma cells with IC_{50} values of 0.2 µg/mL, 0.1 µg/mL, and 1.4 µg/mL respectively.[28]

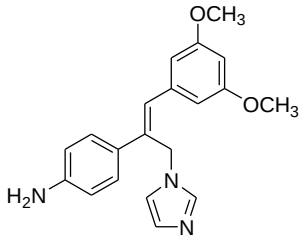


39

Figure 1.16. Structure of compound **39**.

Sun and colleagues designed and synthesized a series of new resveratrol analogues and evaluated their inhibitory activities against aromatase. Most of the resveratrol analogues which had an amino group on the para position of ring A consistently showed significant aromatase inhibition activity, with IC_{50} values in the range of 0.59-14.51 µM. When the para-amino group was substituted with nitro,

halogen, hydroxy, nitrile, acetyl, and aminomethyl groups, resulting compounds were inactive. Compound **40** (Figure 1.17) which had an IC_{50} value of 36 nM was the most potent aromatase inhibitor. Unsurprisingly, molecular modeling illustrated that one of the imidazole nitrogens coordinated with the iron atom of heme prosthetic group, which plays a key role in catalytic activity of aromatase.[26]

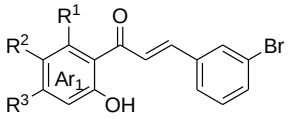


40

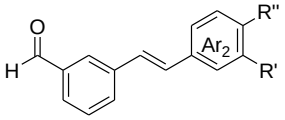
Figure 1.17. Structure of compound **40**.

In this study, it was aimed to investigate the cytotoxic and aromatase inhibition properties of previously synthesized simple chalcones (**C1-C5**), simple stilbenes (**S1-S4**), simple flavanones (**F1-F3**), stilbene-fused chalcones (**SC1-SC17**) and stilbene-fused flavanones (**SF1-SF17**) reported by Akçok et al. (Table 1.1) [29]

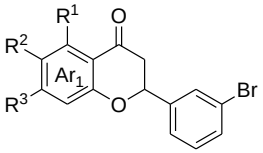
Table 1.1. Structures of simple-chalcones **C1-C5**, simple-stilbenes **S1-S4**, simple-flavanones **F1-F3**, stilbene-fused chalcones **SC1-SC17**, and stilbene-fused flavanones **SF1-SF17**.



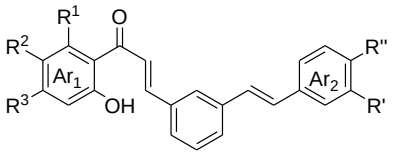
C1-C5



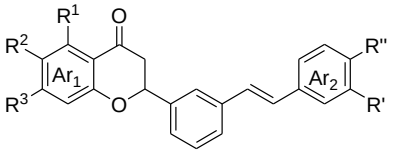
S1-S4



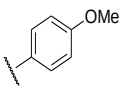
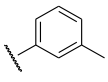
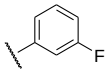
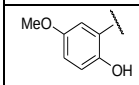
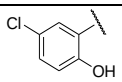
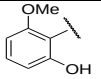
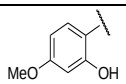
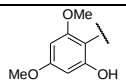
F1-F3



SC1-SC17



SF1-SF17

Ar_2 → Ar_1 ↓	Simple-Chalcones ↓	Simple-Flavanones ↓				
Simple-Stilbenes →			S1	S2	S3	S4
	C1	F1	SC1, SF1	SC2, SF2	SC3, SF3	SC4, SF4
	C2	F2	SC5, SF5	SC6, SF6	SC7, SF7	SC8, SF8
	C3	F3	SC9, SF9	SC10, SF10	SC11, SF11	SC12, SF12
	C4[a]		SC13, SF13	SC14[a], SF14	SC15[a]; SF15	SC16[a], SF16
	C5		SC17, SF17			

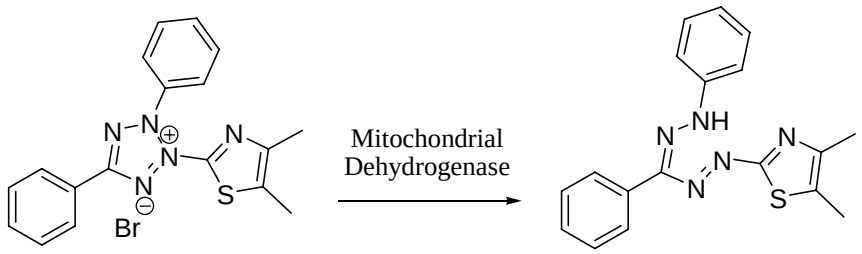
[a] could not be purified from starting materials.

CHAPTER 2

RESULTS AND DISCUSSION

2.1. Cell Viability Assay

In this study, the anti-proliferative activities of simple-stilbenes, simple-chalcones, simple-flavanones, and stilbene-fused chalcone and stilbene-fused flavanone derivatives were evaluated against MCF-7 and PC-3 cell lines at 50, 40, 30, 20, 10, 1, 0.5 µM concentrations by using MTT assay. The amount of formazan (**41**) crystals which are reduced from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (**42**) by mitochondrial dehydrogenase activity in living cells is directly proportional the cell number. (Figure 2.1)

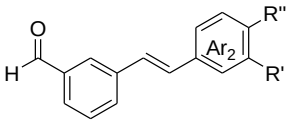


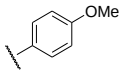
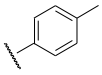
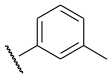
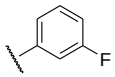
42 **41**

Figure 2.1. Conversion of 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl tetrazolium bromide (MTT) (**42**) to formazan (**41**) crystals.

The absorbance of formazan crystals was determined at 540 nm. IC_{50} values of cytotoxic compounds were calculated by nonlinear regression analysis of at least three separate triplicate experiments. Results of MTT assay were summarized in Tables 2.1-2.7.

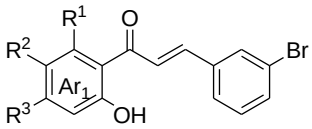
Table 2.1. Calculated IC_{50} values (μM) of simple-stilbenes **S1**-**S4** for cytotoxic activity on PC-3 and MCF-7 cell lines.

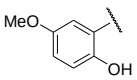
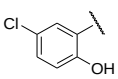
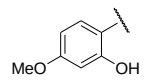
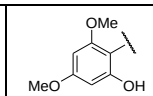


Ar_2 ⟶				
Simple-stilbenes	S1	S2	S3	S4
PC-3	_b	_b	_b	_b
MCF-7	_b	_b	_b	_b

[b] not active for all tested concentrations.

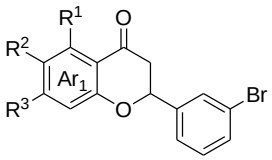
Table 2.2. Calculated IC_{50} values (μM) of simple-chalcones **C1**-**C5** for cytotoxic on PC-3 and MCF-7 cell lines.



Ar_1 ⟶					
Simple-chalcones	C1	C2	C3	C4[a]	C5
PC-3	33	27	3	nt	10
MCF-7	31	11	7	nt	6

[a] could not be purified from starting materials, **nt** not tested

Table 2.3. Calculated IC_{50} values (μM) of simple-flavanones **F1**-**F3** for cytotoxic activity on PC-3 and MCF-7 cell lines.



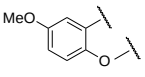
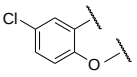
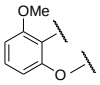
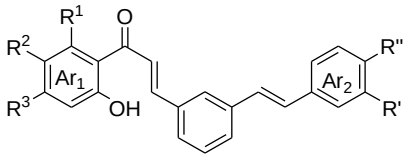
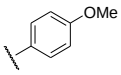
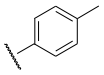
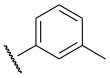
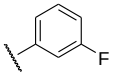
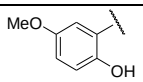
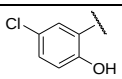
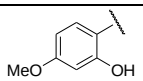
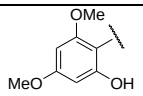
Ar_1 ⟶			
Simple-flavanones	F1	F2	F3
PC-3	21	23	17
MCF-7	20	14	17

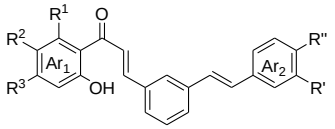
Table 2.4. Calculated IC_{50} values (μM) of stilbene-fused chalcones **SC1**-**SC17** for cytotoxic activity on PC-3 cell line.

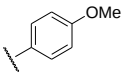
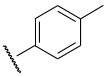
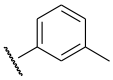
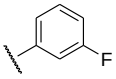
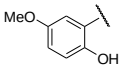
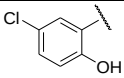
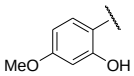
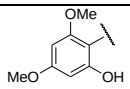


Ar_2 ⟶ Ar_1 ↓				
	15	12	12	11
	25	26	22	23
	12	12	16	8
	_b	nt	nt	nt
	_b			

[b] not active for all tested concentrations, **nt** not tested

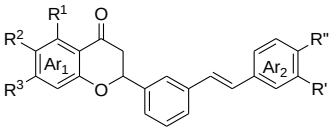
Table 2.5. Calculated IC_{50} values (μM) of stilbene-fused chalcones **SC1**-**SC17** for cytotoxic activity on MCF-7 cell line.

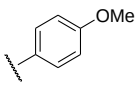
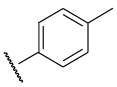
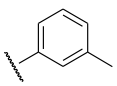
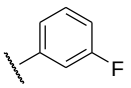
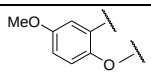
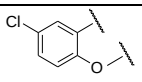
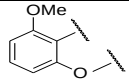
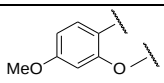
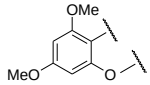


Ar$_2$ → / Ar$_1$ ↓				
	7	21	25	19
	21	23	27	20
	13	16	37	26
	_b	nt	nt	nt
	_b			

[b] not active for all tested concentrations, **nt** not tested

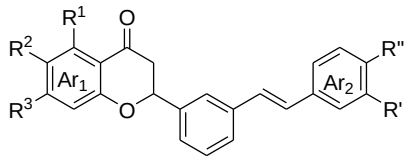
Table 2.6. Calculated IC_{50} values (μM) of stilbene-fused flavanones **SF1**-**SF17** for cytotoxic activity on PC-3 cell line.

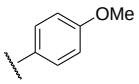
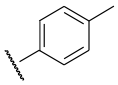
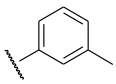
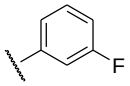
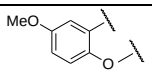
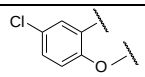
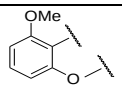
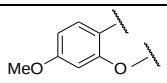
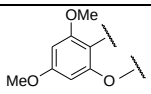


Ar$_2$ → / Ar$_1$ ↓				
	_b	_b	_b	_b
	_b	_b	_b	_b
	_b	9	22	_b
	_b	_b	_b	_b
	_b			

[b] not active for all tested concentrations.

Table 2.7. Calculated IC_{50} values (µM) of stilbene-fused flavanones **SF1-SF17** for cytotoxic activity on MCF-7 cell line.



Ar_2 → / Ar_1 ↓				
	_b	_b	_b	_b
	_b	_b	_b	_b
	_b	21	35	_b
	_b	_b	_b	_b
	_b			

[b] not active for all tested concentrations.

In this study, 42 compounds which were synthesized previously were tested for their cytotoxic activity. For this purpose the breast cancer cell line (MCF-7) and the prostate cancer cell line (PC-3) were used since both cell lines were hormone-dependent cancer cell lines.

When the compounds were classified for their cytotoxic properties it was seen that no simple-stilbene and stilbene-fused flavanone, except **SF10** and **SF11**, had cytotoxic activity against MCF-7 and PC-3 cells at tested concentrations. However, simple-chalcones, simple-flavanones, and stilbene-fused chalcones have cytotoxic activity.

When the structures of simple-chalcones were compared it was seen that although the simple chalcones **C1** and **C3** had the same substituent (methoxy group) at different positions on their Ar_1 rings their cytotoxic properties were fairly different. While the simple-chalcone **C3** had IC_{50} concentrations of 7 µM and 3 µM, the simple-chalcone **C1** had IC_{50} concentrations of 31 µM and 33 µM against MCF-7 and PC-3 cells, respectively. It seems the position of methoxy substituent on Ar_1 ring plays an important role especially methoxy substitution at 6' position of Ar_1 ring increases the

cytotoxic activity. A similar result was observed for the simple chalcone **C5** which had the comparable cytotoxic activity with that of **C3**.

When the structure-activity relationships of stilbene-fused chalcones were discussed it was seen that when the simple-chalcone **C1** was fused with stilbene structures their cytotoxic activities increased against both cell lines. Especially, the stilbene-fused chalcone **SC1** was more than four times cytotoxic against MCF-7 cells with an IC_{50} value of 7 µM compare to simple-chalcone **C1** with an IC_{50} value of 31 µM.

On the other hand, when the simple-chalcone **C2** was fused with stilbene-structures, the cytotoxic activity against PC-3 cells did not change while the cytotoxic activity against MCF-7 cells increased two times compare to simple one.

In addition, the fusion of simple-chalcone **C3** with stilbene structures decreased the cytotoxic activity against both cell lines. Nevertheless, all of the tested stilbene-fused chalcones, except **SC13** and **SC17**, had cytotoxic activity.

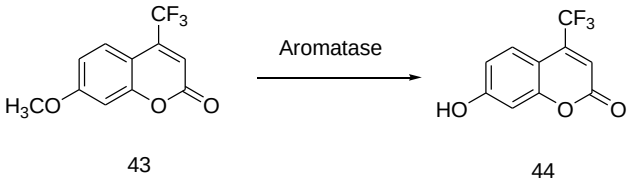
When the structures of simple-flavanones were compared; it was seen that the simple-flavanone **F3** with a methoxy substituent at position 5 was more effective than the simple-flavanone **F1** with a methoxy substituent at position 6. On the other hand, while the simple-flavanone **F2** with chlorine substituent was more cytotoxic against MCF-7 cells it was less cytotoxic against PC-3 cells compare to other simple-flavanones.

The stilbene-fused flavanones did not show any cytotoxic activity at all (except **SF10** and **SF11**) while the simple-flavanones were cytotoxic. Therefore, it is clear that stilbene fusion on simple-flavanones somehow was causing the loss of cytotoxic activity. It seems the stilbene-fused flavanones mimic the stilbene structures. In addition, when the structures of cytotoxic stilbene-fused flavanones, **SF10** and **SF11**, were thought; interestingly there is a methoxy substituent at the same position with the that of the most cytotoxic simple flavanone **F3** and simple chalcones **C3** and **C5**. Despite of this similarity it seems this is not the only requirement for a strong cytotoxic activity because there are two stilbene-fused flavanones having same substituent at ring Ar_1 but do not have cytotoxic activity **SF9** and **SF12**. Hence the substituent type at stilbene part of the molecule can also play an important role and it seems that existence of a nonpolar substituent at stilbene can be a good choice for increased cytotoxic activity. The stilbene-fused flavanone **SF10** with methyl substituent at para position was

more cytotoxic with IC_{50} concentrations of 9 µM and 21 µM against PC-3 and MCF-7 cells, respectively.

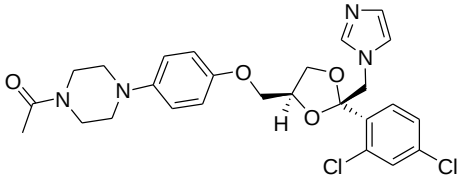
2.2. Aromatase Inhibition

In this study, in addition to cytotoxic activity, simple and fused compounds were tested for possible aromatase inhibition properties by CYP19/MFC High Throughput Inhibitor Screening Kit. The conversion of 7-methoxy-4-trifloromethyl coumarin (MFC) (**43**) substrate into fluorescent 7-hydroxy-4-trifloromethyl coumarin (HFC) (**44**) product was measured in the presence of different concentrations of tested compounds. (Figure 2.2) The intensity of the fluorescence of HFC is proportional the aromatase activity. IC_{50} values were calculated by nonlinear regression analysis of at least two separate experiments by "GraphPad Prism 5" software. Aromatase inhibitor ketoconazole (**45**) was used as positive control. (Figure 2.3) Results were summarized in Tables 2.8-2.12.



43 44

Figure 2.2. 7-Methoxy-4-trifloromethyl coumarin (**43**) is converted to 7-hydroxy-4-trifloromethyl coumarin (**44**) by aromatase activity.



45

Figure 2.3. Structure of ketoconazole (**45**).

Ketoconazole was tested at 40.00, 13.33, 4.44, 1.48, 0.49, 0.165, 0.055, and 0.018 µM concentrations. Its IC_{50} value was calculated as 2 µM. (Figure 2.4)

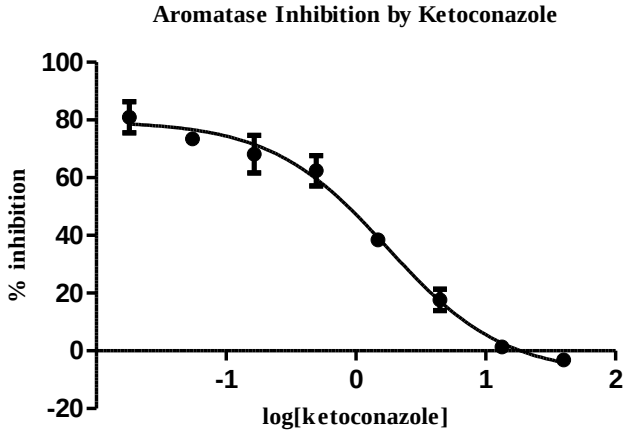
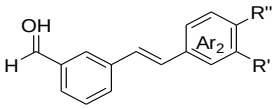
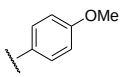
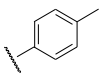
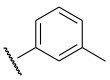
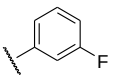


Figure 2.4. Dose dependent aromatase inhibition activity of ketoconazole (**45**).

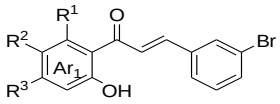
Table 2.8. Calculated IC_{50} values (µM) of simple-stilbenes **S1**-**S4** for aromatase inhibition.

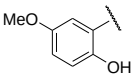
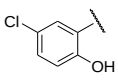
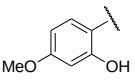
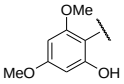


Ar_2 ⟶				
Simple-stilbenes	S1	S2	S3	S4
CYP19	7	_b	65	_b

[b] not active for all tested concentrations

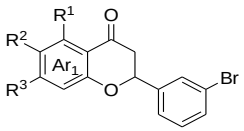
Table 2.9. Calculated IC_{50} values (μM) of simple-chalcones **C1**-**C5** for aromatase inhibition.

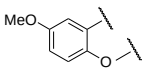
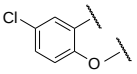
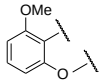


Ar_1 ⟶					
Simple-chalcones	C1	C2	C3	C4[a]	C5
CYP19	39	>300	>300	nt	>300

[a] could not be purified from starting materials, **nt** not tested

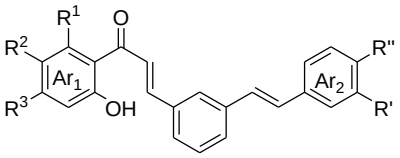
Table 2.10. Calculated IC_{50} values (μM) of simple-flavanones **F1**-**F3** for aromatase inhibition.

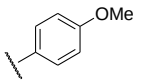
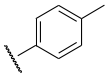
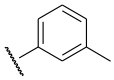
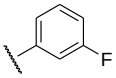
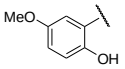
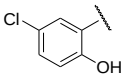


Ar_1 ⟶			
Simple-flavanones	F1	F2	F3
CYP19	_b	122	_b

[b] not active for all tested concentrations.

Table 2.11. Calculated IC_{50} values (μM) of stilbene-fused chalcones **SC1**-**SC17** for aromatase inhibition.



Ar_2 → / Ar_1 ↓				
	_b	223	>300	>300
	_c	>300	>300	_b
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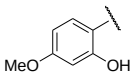
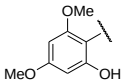
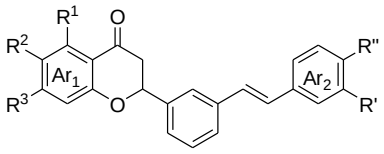
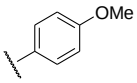
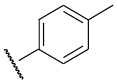
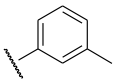
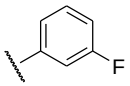
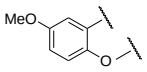
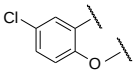
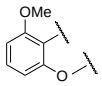
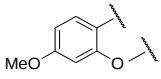
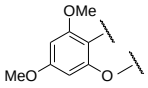
	_c	nt	nt	nt
	_b			

[b] not active for all tested concentrations, [c] gave higher fluorescent intensity compared to negative control, **nt** not tested.

Table 2.12. Calculated IC_{50} values (µM) of stilbene-fused flavanones **SF1-SF17** for aromatase inhibition.



Ar_2 → / Ar_1 ↓				
	_c	_c	_b	_c
	34	_c	_b	>300
	_c	_b	_b	_b
	>300	>300	39	>300
	_c			

[b] not active for all tested concentrations, [c] gave higher fluorescent intensity compared to negative control, **nt** not tested.

In this study, 42 compounds were tested for their aromatase inhibition activity. However, while twenty of them had anti-aromatase activity only seven of the twenty compounds (**S1**, **S3**, **C1**, **SC2**, **F2**, **SF5** and **SF15**) had significant activity with IC_{50} concentrations of less than 300 µM. Moreover, three of the seven compounds (**C1**, **F2** and **SC2**) also had cytotoxic activity at the same time.

Actually, there was not a common property in the structures of compounds which had anti-aromatase activity. Among these active compounds there were two simple-stilbene, one simple-chalcone, one simple-flavanone, one stilbene-fused chalcone, and two stilbene-fused flavanones.

When the structures of active simple-stilbenes were compared it was seen that while one simple-stilbene **S1** had a methoxy group the other one (**S3**) had a methyl group on their Ar_2 rings. The simple-stilbene **S1** which was substituted a methoxy group

was the most active compound with IC_{50} concentration of 7 µM for aromatase inhibition.

When the structures of simple-chalcone **C1** and stilbene-fused chalcone **SC2** were compared it was seen that these two compounds had the same methoxy group on their Ar_1 ring. However, it was clear that the fusion of chalcone structure with stilbene decreased the aromatase inhibitory activity more than five times. While simple-chalcone **C1** had IC_{50} concentration of 39 µM the stilbene-fused chalcone **SC2** had IC_{50} concentration of 223 µM for aromatase inhibition.

When the structures of simple-flavanone **F2** and stilbene-fused flavanone **SF5** were compared it was seen that they had the same Ar_1 ring with chlorine (Cl) substituent. However, IC_{50} concentrations of these compounds indicated that the fusion of flavanone structure with the stilbene increased the aromatase inhibition activity more than three times. The simple-flavanone **F2** had IC_{50} concentration of 122 µM and the stilbene-fused flavanone **SF5** had IC_{50} concentration of 34 µM for aromatase inhibition. In addition, the other active flavanone **SF15** had a stilbene-fused structure with IC_{50} value of 39 µM.

In this study it was aimed to investigate the compounds which possessed both cytotoxic activity and aromatase inhibition activity simultaneously. However, while twelve of the tested compounds had two activities at the same time only three of the twelve compounds (**C1**, **SC2** and **F2**) were significantly active. While these three compounds had average cytotoxic activities with IC_{50} concentrations between 12 and 33 µM the simple-chalcone **C1** had significant aromatase inhibition activity with IC_{50} concentration of 39 µM compare to other two compounds (**F2** and **SC2**) with IC_{50} concentrations of 122 µM and 223 µM, respectively.

The results were evaluated in two perspectives. The first one is the potential of hybrid system to carry more than one biological activity. In this perspective molecules were divided into four different subgroups. Accordingly the first group shows both cytotoxic and aromatase inhibitory activities. These molecules are **C1**, **C5**, **F2**, **SC2**, **SC3**, **SC4**, **SC6**, **SC7**, **SC10**, and **SC11**. The second group which exhibits only cytotoxic activity contains **C2**, **C3**, **SC1**, **SC5**, **SC8**, **SC9**, **SC12**, **F2**, **F3**, **SF10**, and **SF11**. In the third group there are compounds **S1**, **S3**, **SF5**, **SF8**, **SF13**, **SF14**, **SF15**, and **SF16** which have only aromatase inhibitory activities. The compounds in the fourth

group are **S2**, **S4**, **SC13**, **SC17**, **SF1-SF4**, **SF6**, **SF7**, **SF9**, **SF12**, and **SF17** which possess neither cytotoxicity nor aromatase inhibitory activity.

Although among the all test compounds only simple and stilbene-fused chalcones can carry two biological activities simultaneously and their aromatase inhibition properties only can be seen at above 300 µM concentrations. Conversely stilbene-fused flavanones cannot carry out cytotoxic and aromatase inhibition activities at the same time. There is only one compound (**C1**) which possess both cytotoxic and aromatase inhibitory activity at below 100 µM concentrations.

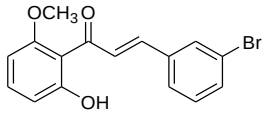
The second perspective is the mimicking performance of the fused system compared to simple ones. In aromatase inhibition experiments two of the simple-stilbenes **S1** and **S3** showed significant activity at lower µM concentrations (IC_{50}=7 µM, 65 µM respectively) without any cytotoxic activity. Conversely all of the simple-chalcones have cytotoxic activity at lower µM concentrations, however only the simple-chalcone **C1** inhibits aromatase significantly with IC_{50} value of 39 µM. In addition, two of the simple-flavanones (**F1** and **F3**) have not aromatase inhibitory activity although they are cytotoxic at lower µM concentrations. Only the simple-flavanone **F2** possesses two biological activities at the same time; however its aromatase inhibition concentration is above 100 µM. According to these results when the hybrid compounds are compared for their mimicking performances it was seen that stilbene-fused chalcones are mimicking only simple-chalcones rather than simple-stilbenes. On the other hand stilbene-fused flavanones have potency to show more than one trend because most of the stilbene-fused flavanones can mimic neither simple-flavanones nor simple-stilbenes depending on the substitution. Contrarily the fusion of stilbene structure to flavanone causes the missing of cytotoxic and aromatase inhibitory activities.

2.3. Apoptosis Analysis

Apoptosis, which is the process of programmed cell death in a normal cell growth, is characterized by exact morphologic properties. One of the earliest features is the loss of plasma membrane asymmetry. As in normal cells the membrane phospholipid phosphatidylserine (PS) is located inner side of the plasma membrane, in apoptotic cells it is translocated to the outer side of the plasma membrane. Annexin V-FITC Screening Kit was used in order to investigate the apoptotic effects of selected test compounds (**C3**, **F2**, **SC2** and **SF10**) on MCF-7 and PC-3 cancer cell lines. Annexin V, which is a Ca^{2+} dependent phospholipid-binding protein, has a great affinity for phospholipid phosphatidylserine (PS). When Annexin V is conjugated to fluorescein isothiocyanate (FITC), which is a fluorochrome, serves as a sensitive probe for flow cytometric analysis of apoptotic cells. Annexin V-FITC staining can recognize apoptosis at an earlier stage because externalization of phospholipid phosphatidylserine (PS) occurs in the earlier stage of apoptosis. In addition to Annexin V-FITC, propidium iodide (PI) was used to distinguish viable from nonviable cells. Propidium iodide is an intercalator and only the membranes of dead and damaged cells are permeable to it. If cells are undergoing apoptosis they stain positive for Annexin V-FITC and negative for PI. If cells stain positive for both Annexin V-FITC and PI they are either in the end stage of apoptosis, and are undergoing necrosis, or are already dead. Cells which stain negative for both Annexin V-FITC and PI are alive, and they are not undergoing apoptosis.[30]

The selected compounds (**C3**, **F2**, **SC2** and **SF10**) were analyzed for their dose-dependent apoptotic effects, since earlier studies have showed that a 48 hours exposure to the selected compounds reduced the viability of breast cancer cells (MCF-7) and prostate cancer cells (PC-3). Results were summarized in Figures 2.11-2.18.

The IC_{50} value of simple-chalcone **C3** was found 7 µM against MCF-7 cell line and 3 µM against PC-3 cell line by MTT assay. Results of apoptosis analysis also showed that while simple-chalcone **C3** decreased the percentage of live cells more than 50% at concentrations 10 µM and 20 µM in both MCF-7 and PC-3 cell lines, it increased the percentage of apoptotic cells. (Figure 2.5-2.6)



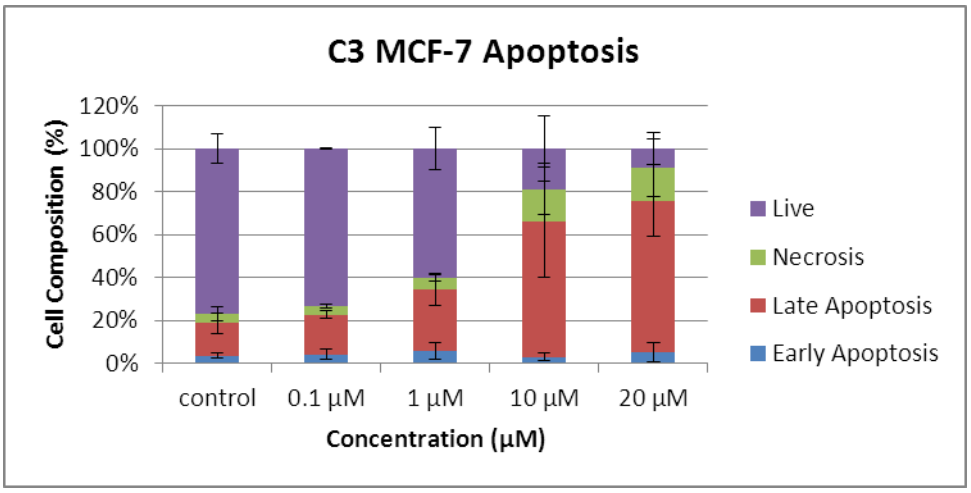


Figure 2.5. Apoptotic effect of simple chalcone **C3** on MCF-7 cells.

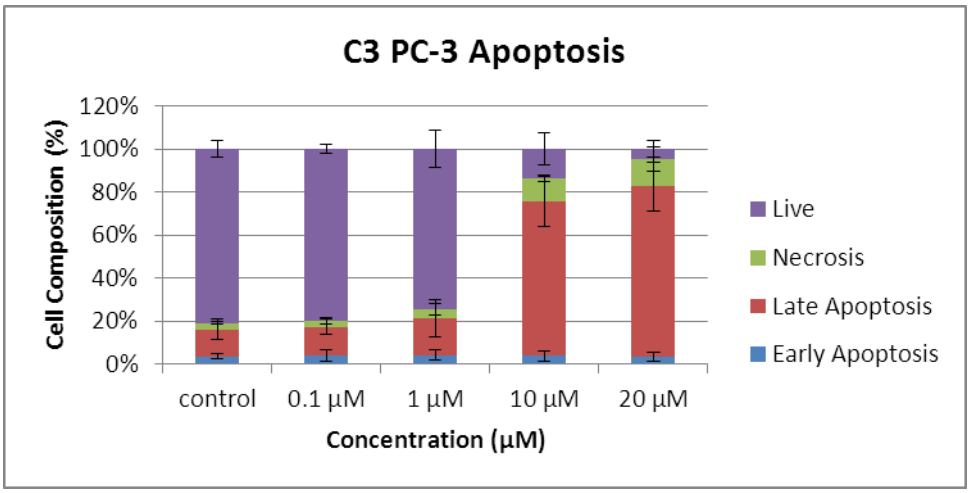
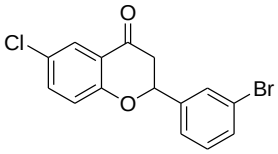


Figure 2.6. Apoptotic effect of simple chalcone **C3** on PC-3 cells.

The simple-flavanone **F2,** which had IC_{50} values of 14 µM and 23 µM against MCF-7 and PC-3 cell lines, respectively, increased the percentage of apoptotic cells in MCF-7 cells by 20% at 20 µM compare to control, however **F2** was ineffective against PC-3 cells. (Figure 2.7-2.8)



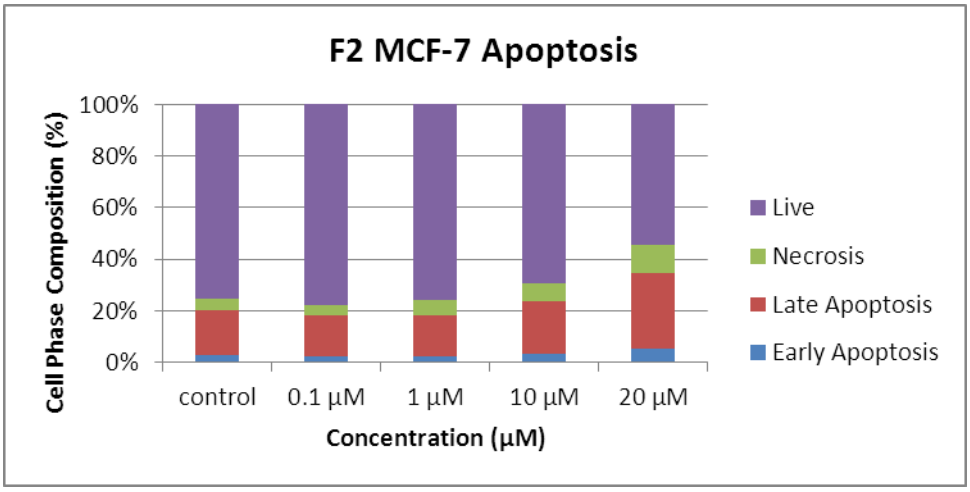


Figure 2.7. Apoptotic effect of simple flavanone **F2** on MCF-7 cells.

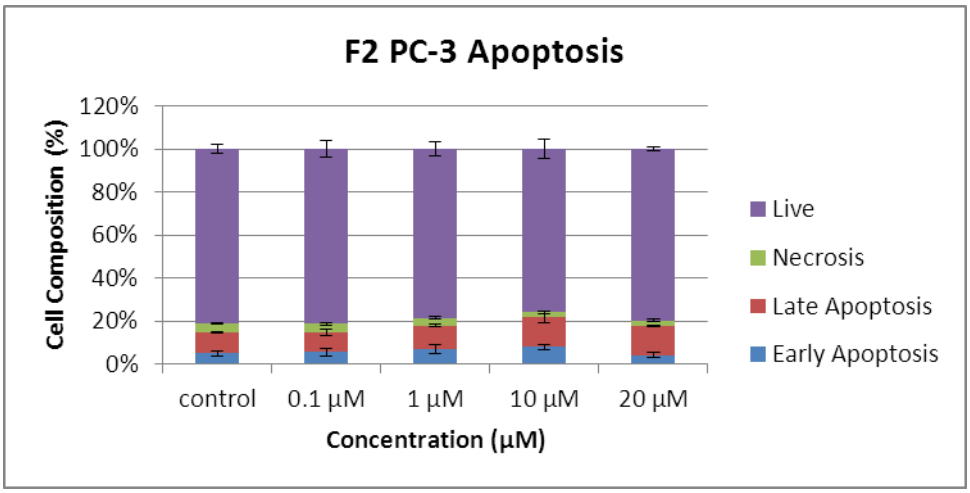
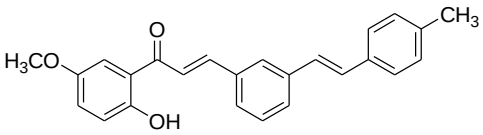


Figure 2.8. Apoptotic effect of simple flavanone **F2** on PC-3 cells.

The stilbene-fused chalcone **SC2** had cytotoxic activity against MCF-7 and PC-3 cancer cells with IC_{50} values of 21 µM and 12 µM, respectively. Results of apoptosis analysis showed that compound **SC2** can induce apoptosis on PC-3 cells than MCF-7 cells. **SC2** caused late apoptosis by 26% and necrosis by 24% in PC-3 cells at 20 µM; however, it was nearly ineffective at 10 µM and lower concentrations against PC-3 cells. On the other hand, **SC2** did not show significant apoptotic effect even at 20 µM although it had IC_{50} value of 21 µM against MCF-7 cells. (Figure 2.9-2.10)



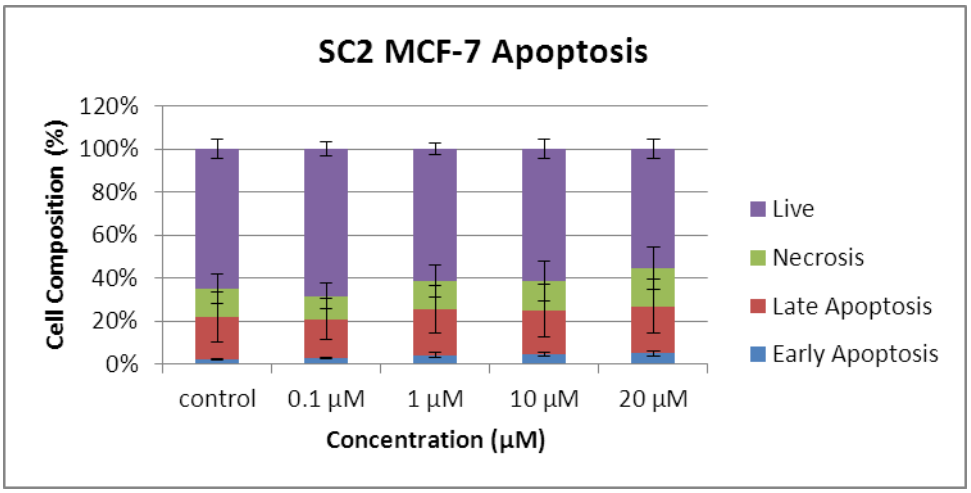


Figure 2.9. Apoptotic effect of stilbene-fused chalcone **SC2** on MCF-7 cells.

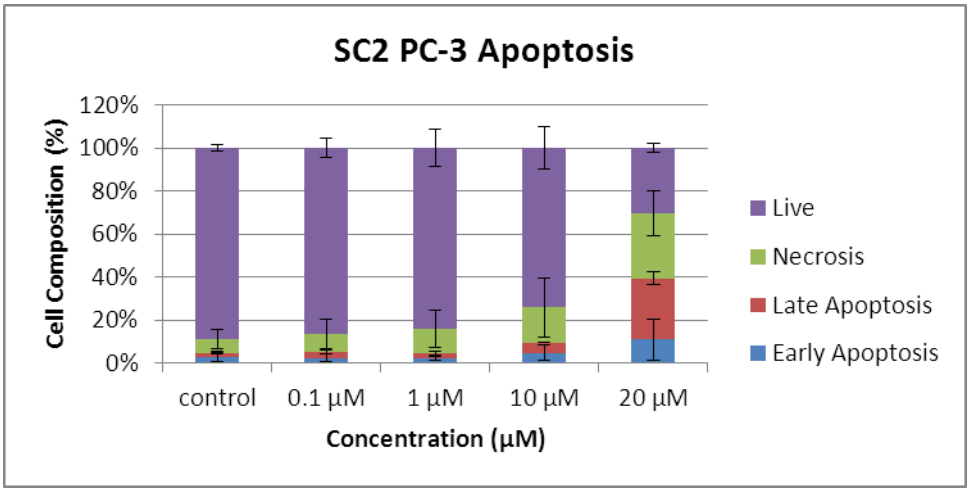
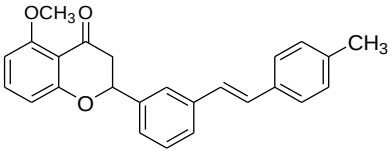


Figure 2.10. Apoptotic effect of stilbene-fused chalcone **SC2** on PC-3 cells.

The stilbene-fused flavanone **SF10** reduced the percentage of live cells by only 10% at 20 µM although it had 21 µM IC_{50} concentration against MCF-7 cells while it increased the percentage of apoptotic cells by 6% and 8% at 20 µM and 10 µM, respectively. In contrast to the behavior against MCF-7 cells, **SF10**, which had 9 µM IC_{50} value against PC-3 cells, decreased the percentage of live cells by 63% and 39% and increased the percentage of apoptotic cells by 53% and 32% in PC-3 cells at 20 µM and 10 µM, respectively. (Figure 2.11-2.12)



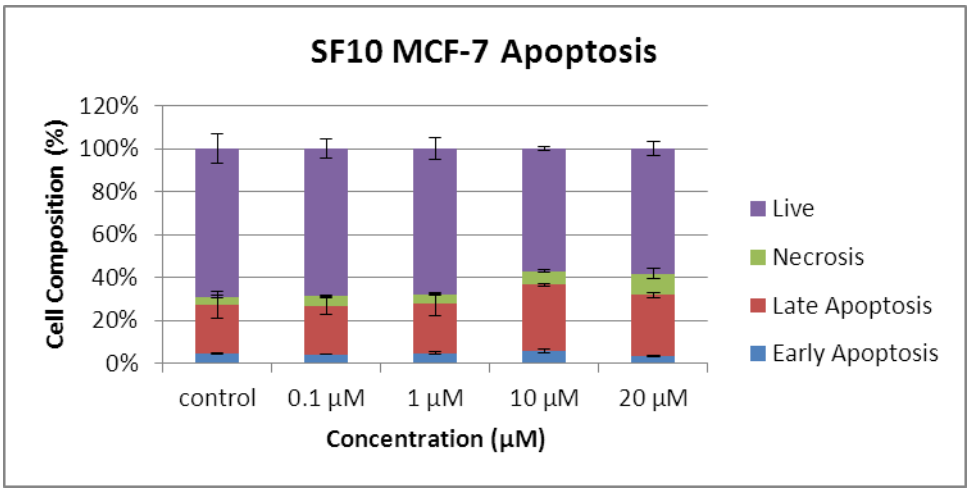


Figure 2.11. Apoptotic effect of stilbene-fused flavanone **SF10** on MCF-7 cells.

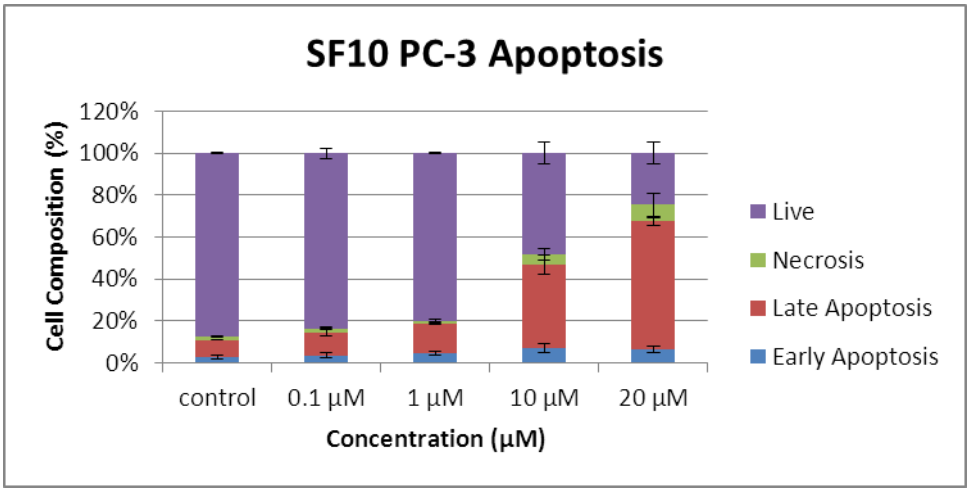


Figure 2.12. Apoptotic effect of stilbene-fused flavanone **SF10** on PC-3 cells.

2.4. Cell Cycle Analysis

The cell cycle has two major phases: interphase and mitosis. In mitosis, the mitotic phase, the mother cell divides into two genetically identical daughter cells. Interphase which is the phase between mitotic events has three distinct, successive stages. During the first stage which is called G_1, cells monitor their environment, and synthesize RNA and proteins to induce growth when the requisite signals are received.

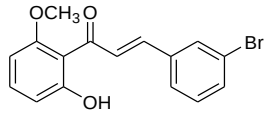
Cells enter the S phase when conditions are right and carry out to DNA synthesis and replicate their chromosomal DNA. In the G_2 phase, which is the final stage of the interphase, cells continue to grow and prepare for mitosis.

To investigate the effects of selected compounds (**C3**, **F2**, **SC2** and **SF10**) on the cell cycle of MCF-7 and PC-3 cancer cell lines cell cycle was analyzed by propidium iodide staining which is utilized as the DNA fluorochrome and requires blue light as the excitation source. The fluorescence intensity of the stained cells is correlated with the amount of cellular DNA content; however propidium iodide also stains double-stranded RNA. In order to prevent the interference of double-stranded RNA RNase A is added to the staining solution. This method provides the percentage of cells in G_1, S, and G_2/M phases.[31]

The dose-dependent effects on cell cycle parameters of breast cancer cells (MCF-7) and prostate cancer cells (PC-3) after exposure to selected compounds (**C3**, **F2**, **SC2** and **SF10**) were investigated by flow cytometer, since cell viability studies have demonstrated that a 48 hours exposure to these selected compounds (**C3**, **F2**, **SC2** and **SF10**) reduced the viability of MCF-7 and PC-3 cells.

Cells incubated with different concentrations (0.1 µM, 1 µM, 10 µM, and 20 µM) of these selected compounds for 48 hours were examined for their distribution in G_1, S, and G_2 phases of the cell cycle. Results were summarized in Figures 2.19-2.26.

The simple-chalcone **C3** did not significantly change the amount of cells in G_2 phase; however the cell composition in G_1 phase decreased by 15% and the cell composition in S phase increased by 12% at 10 µM in MCF-7 cells. On the other hand, in PC-3 cells **C3** decreased the percentage of cells in G_1 phase by 26% and increased the percentage of cells in S phase by 14% at 20 µM. **C3** also increased the cell composition in G_2 phase by 12% at 20 µM compare to control. (Figure 2.13-2.14)



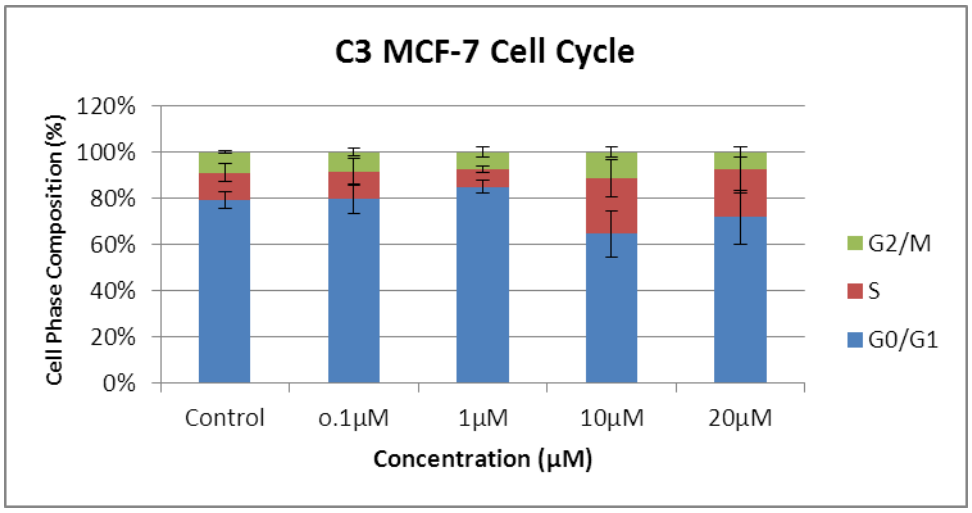


Figure 2.13. Effect of simple-chalcone **C3** on cell cycle in MCF-7 cells.

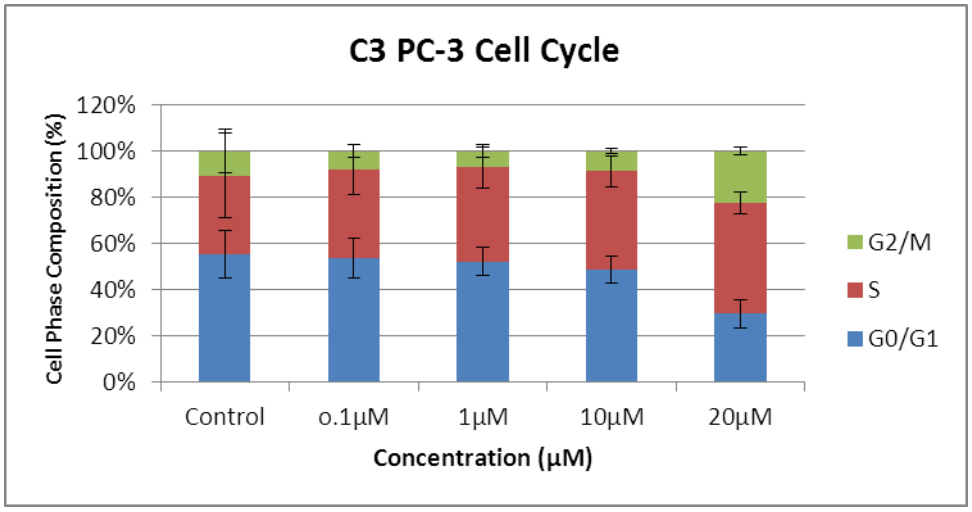
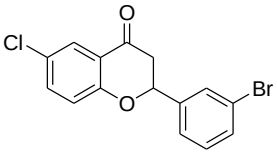


Figure 2.14. Effect of simple-chalcone **C3** on cell cycle in PC-3 cells.

The treatment with simple-flavanone **F2** caused a little change in the cell cycle distribution of MCF-7 cells, but it had no effect on the cell cycle distribution of PC-3 cells. (Figure 2.15-2.16)



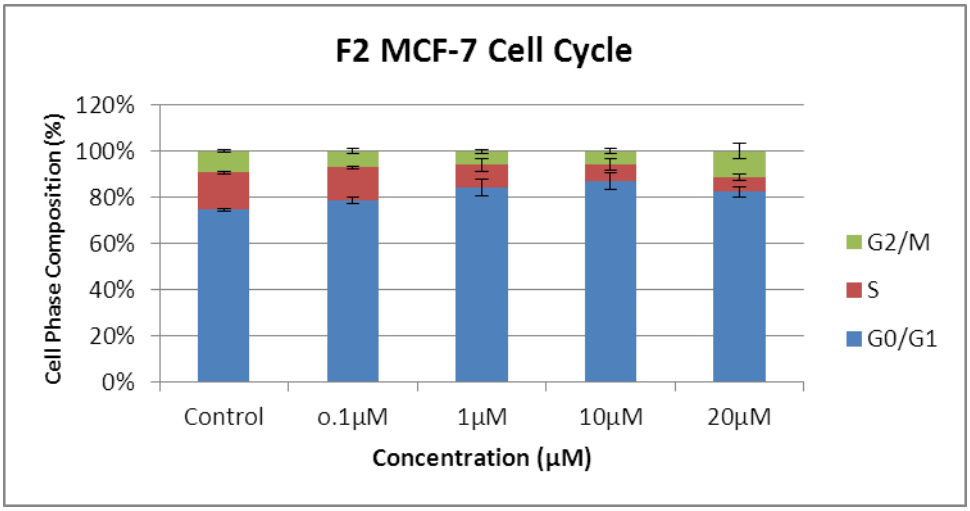


Figure 2.15. Effect of simple-flavanone **F2** on cell cycle in MCF-7 cells.

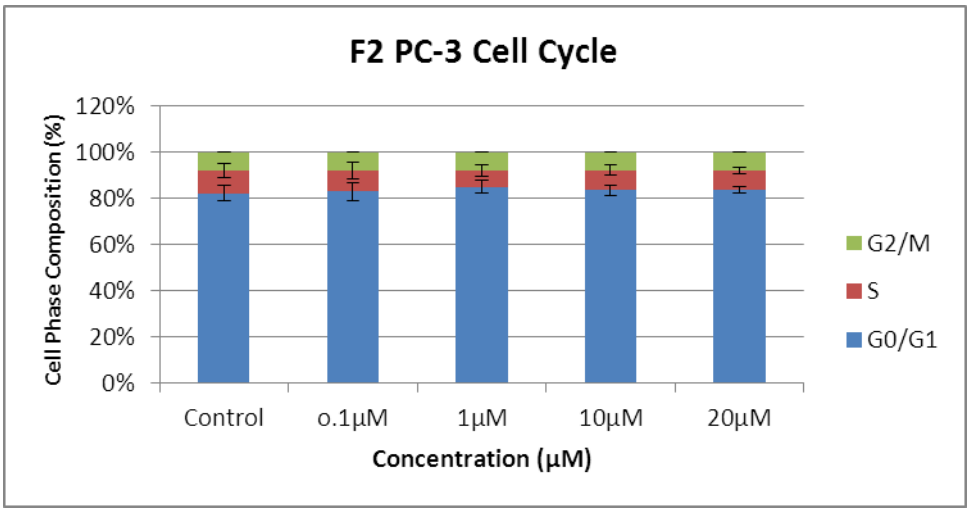
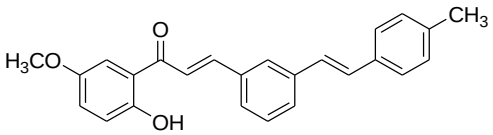


Figure 2.16. Effect of simple-flavanone **F2** on cell cycle in PC-3 cells.

The stilbene-fused chalcone **SC2** increased the percentage of cells in G_1 phase and decreased the percentage of cells in S phase in MCF-7 cells at 10 µM and 20 µM concentrations. There was not considerable change in the G_2 phase of MCF-7 cells. However, while the stilbene-fused chalcone **SC2** decreased the cell composition in G_1 phase by 21% and in G_2 phase by 5%, it increased the cell composition in S phase by 26% in PC-3 cells at 20 µM concentration. (Figure 2.17-2.18)



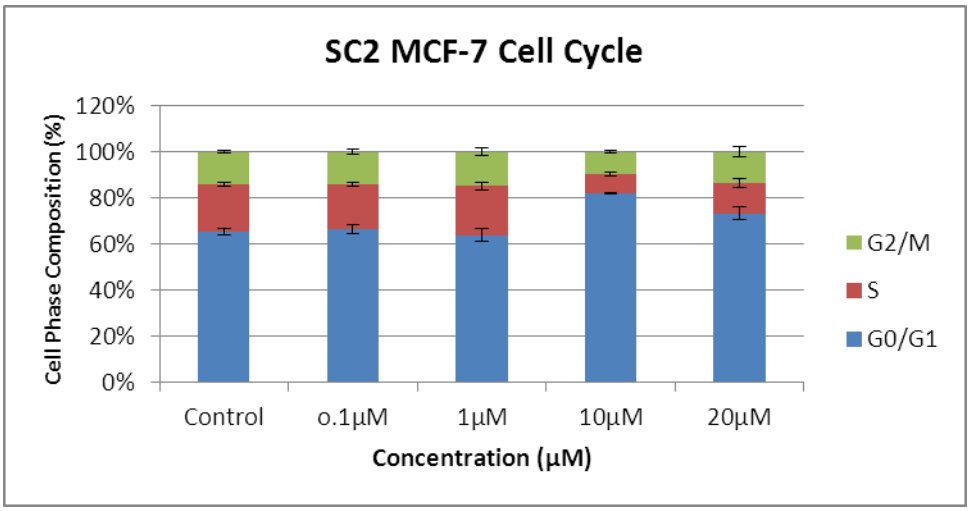


Figure 2.17. Effect of stilbene-fused chalcone **SC2** on cell cycle in MCF-7 cells.

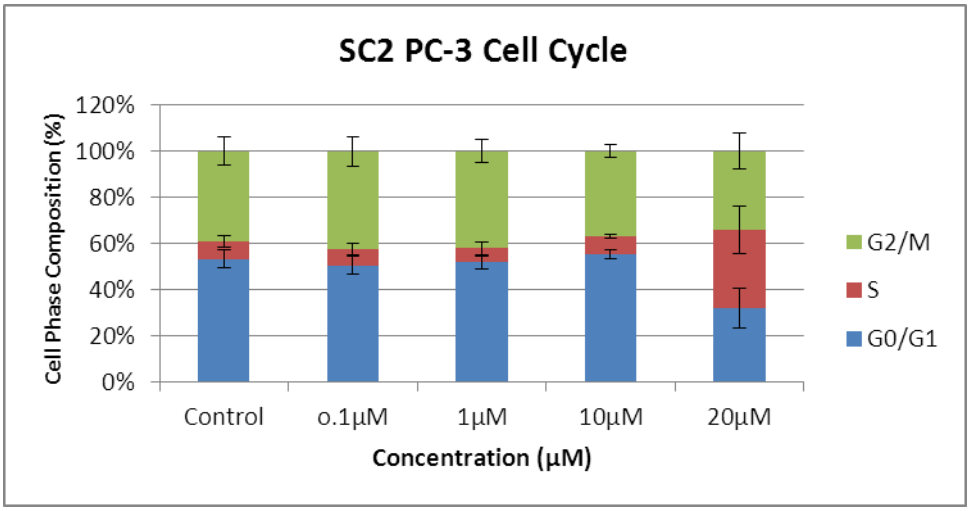
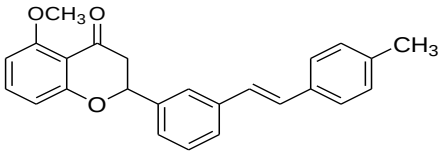


Figure 2.18. Effect of stilbene-fused chalcone **SC2** on cell cycle in PC-3 cells.

The stilbene-fused flavanone **SF10** decreased the percentage of cells in S phase by 10% and increased the percentage of cells in G_2 phase by 13% at 20 µM in MCF-7 cells, while it was ineffective on G_1 phase. **SF10** at 1 µM and lower concentrations were nearly ineffective on MCF-7 cell cycle distribution. However, while **SF10** decreased the cell composition in G_1 phase by 24% and increased the cell composition in S phase by 30% at 20 µM, it changed the amount of cells in G_2 phase by only 5% in PC-3 cells. (Figure 2.19-2.20)



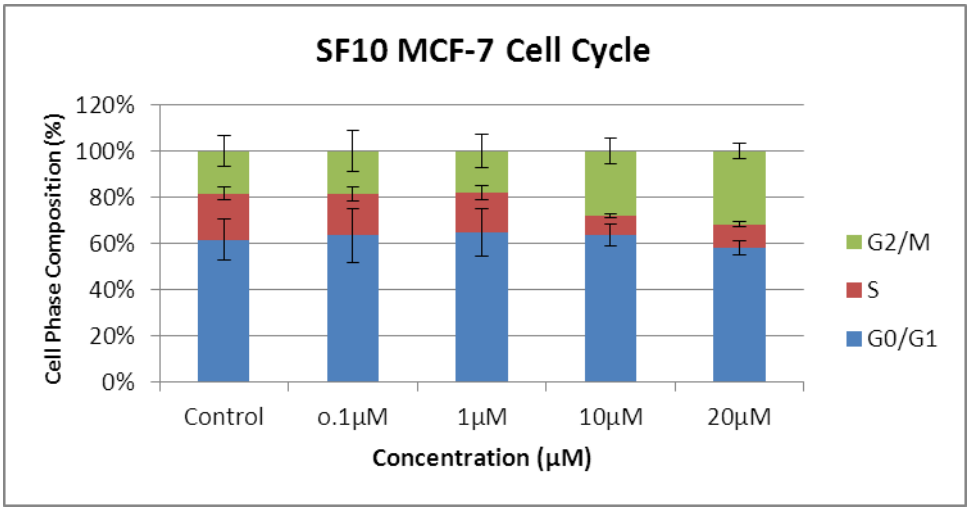


Figure 2.19. Effect of stilbene-fused flavanone **SF10** on cell cycle in MCF-7 cells.

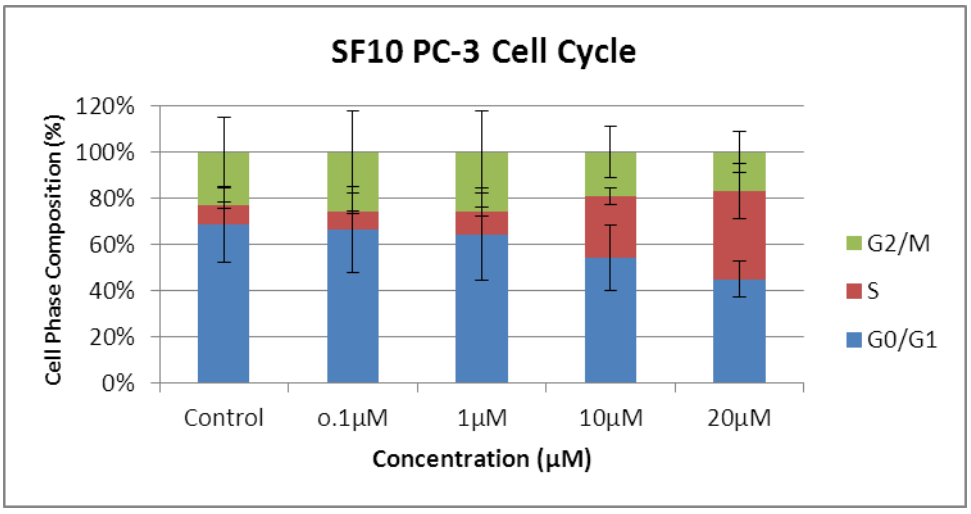


Figure 2.20. Effect of stilbene-fused flavanone **SF10** on cell cycle in PC-3 cells.

In this study, the most cytotoxic compound was simple-chalcone **C3** with IC_{50} value of 7 µM and 3 µM against breast cancer cells (MCF-7) and prostate cancer cells (PC-3), respectively. Additionally, the results of apoptosis analysis were compatible with IC_{50} concentrations. Results indicated that in both cell lines simple-chalcone **C3** decreased the percentage of viable cells by more than 50% at 10 µM and 20 µM. At

these concentrations both MCF-7 cells and PC-3 cells went to apoptosis; however simple-chalcone **C3** was ineffective on both cell lines at 1 µM and lower concentrations.

In addition, the results of cell cycle analysis showed that the percentage of cells in G_1 phase decreased and the percentage of cells in S phase increased in MCF-7 cells at higher **C3** concentrations especially at 10 µM. However, in the percentage of cells in G_2 phase did not change significantly. The simple chalcone **C3** caused the accumulation of cells in S phase at higher concentrations. In this case, **C3** might be cytotoxic during DNA synthesis.

On the other hand, the same compound **C3** caused the accumulation of PC-3 cells in S and G_2 phases while the percentage of PC-3 cells in G_1 phase decreased at 20 µM. The possible reason of the accumulation in G_2 phase might be blocking the formation of mitotic spindles. **C3** might be effective on tubulin polymerization.

The stilbene-fused chalcone **SC2** was the only compound which possessed both cytotoxic activity and aromatase inhibitory activity among the stilbene-fused chalcones. Its IC_{50} concentration was 12 µM against PC-3 cell line; however, it decreased the percentage of viable cells by 58% at 20 µM. At this concentration **SC2** caused the 26% of cells to go apoptosis and 24% of cells to go necrosis. But **SC2** was ineffective at 10 µM and lower concentrations. On the other hand, while the stilbene-fused chalcone **SC2** had an IC_{50} value of 21 µM against MCF-7 cells, it did not have apoptotic effect on MCF-7 cells.

The results of cell cycle analysis exhibited that in PC-3 cell line while **SC2** decreased the percentage of cells in G_1 and G_2 phases, it increased the percentage of cells in S phase. The accumulation in S phase indicated that **SC2** might be cytotoxic during DNA synthesis. In MCF-7 cells, **SC2** decreased the percentage of cells in G_1 phase by 17% and increased the percentage of cells in S phase and G_2 phase by 12% and 6% respectively.

The stilbene-fused flavanone **SF10** was one of two stilbene-fused flavanones which had cytotoxic activity against MCF-7 and PC-3 cells. It had cytotoxic activity against both cell lines at lower concentrations compare to other cytotoxic stilbene-fused flavanone **SF11**.

SF10 which had IC_{50} concentration of 9 µM against PC-3 cell line decreased the percentage of viable cells by 39% at 10 µM and 63% at 20 µM. While **SF10** caused the

cells to go apoptosis at higher concentrations it was ineffective at lower concentrations in PC-3 cells. On the other hand, in MCF-7 cells **SF10** decreased the percentage of live cells by only 10%.

According to the results of cell cycle analysis, **SF10** caused the accumulation of PC-3 cells in S phase. In contrast, **SF10** increased the percentage of MCF-7 cells in G_2 phase at higher concentrations. **SF10** might be effective on DNA synthesis and tubulin polymerization in PC-3 and MCF-7 cells, respectively.

CHAPTER 3

EXPERIMENTAL

3.1. Cell Viability Assay (MTT Test)

The human prostate cancer (PC-3) cell line was provided by Professor Doctor Kemal Sami Korkmaz (Ege University, Engineering Faculty, Department of Bioengineering), human breast cancer (MCF-7) cell line was obtained from Assistant Professor Oğuz Bayraktar (İzmir Institute of Technology, Department of Engineering, Chemical Engineering). The breast cancer cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and 1% gentamycin sulphate (50 mg/mL), prostate cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% gentamycin sulphate (50 mg/mL) in 5% CO_2 humidified incubator at 37 °C. Cells were passaged when they reached 80-85% confluence. The passage number range for both cell lines was maintained between 10 and 20.

In order to investigate the cytotoxic activity of test compounds, 95 µL of cell suspension was inoculated into 96-well microculture plates at $1x10^4$ cells density per well in culture media containing FBS, and gentamycin sulphate. Cells were incubated for 24 h to attach to wells before treatment with test compounds. The test compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA), filter sterilized, diluted at appropriate concentrations with the culture medium. After 24 h, 5 µL of diluted compounds were added to cells to maintain final concentrations 50, 40, 30, 20, 10, 1 µM and 500 nM for triplicate assay. In all wells, 1% DMSO final concentration was fixed. Cells treated with test compounds were incubated further for 48 h in CO_2 incubator at 37 °C. After the incubation, the medium was poured out and cell monolayers were washed with phosphate-buffered saline (PBS) to remove any trace of compounds and to prevent color interference while optical density determination. The stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) was made in PBS to obtain a concentration of 5 mg/mL. %10 MTT solutions were prepared with DMEM and RPMI for prostate and breast cancer cells,

respectively. 100 µL of diluted MTT solution was added to each well and plates were incubated at 37 °C for 4 h in dark. After 4 hours, plates were centrifuged at 1800 rpm for 10 minutes at room temperature to avoid accidental removal of formazan crystals. MTT solution was removed and 100 µL DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined on a plate reader. Each compound was assayed three times in triplicate. IC_{50} values of compounds were calculated by nonlinear regression analysis of these three separate triplicate experiments by "GraphPad Prism 5" software.[32] [33]

3.2. Aromatase Inhibition Assay

Inhibitory activity of compounds on aromatase was evaluated in vitro using CYP19/MFC high-throughput screening kit (BD Biosciences, Oxford, UK) according to the manufacturer's protocol. Ketoconazole was used as positive control and 7-methoxy-trifluoromethylcoumarin (MFC) was used as substrate. In this fluorescence-based assay it was measured the conversion rate of the non-fluorescent MFC substrate into fluorescence product 7-hydroxy-4-trifluoromethyl coumarin (HFC). Test compounds were dissolved in acetonitrile. Each compound was diluted in NADPH-cofactor mix (1.13 mL of Cofactors, 0.9 mL of G6PDH, 0.6 mL of Control Protein, 87.4 mL of 37°C water) for seven different concentrations (1200.00, 400.00, 133.33, 44.44, 14.81, 4.94, 1.65, and 0.55 µM) and placed in duplicate on a 96-well plate. The plate was then incubated at 37 °C for 10 minutes. After incubation, 100 µL of the enzyme/substrate mix (pre-warmed phosphate buffer (0.5 M, pH 7.4), 70.5 mL of 37°C water, 1.35 mL of HTS-760, and 180 µL of 25 mM MFC.) was added to the treated conditions and the plate was then incubated at 37 °C for 30 minutes. Concentrations of CYP19 and substrate were fixed to 7.5 nM and 25 µM respectively. After incubation, 75 µL of Stop Reagent (Tris Base, 0.5 M) was added to the entire plate and 100 µL of the enzyme/substrate mix was added in the non-treated blank columns. Background fluorescence which was observed from blank columns was subtracted from the fluorescence of the all reactions to find the fluorescence emitted only from HFC products. Fluorescence of HFC was detected by employing an excitation wavelength of 405 nm and emission wavelength of 520 nm. IC_{50} values for potential aromatase

inhibitors were calculated by nonlinear regression analysis of at least two separate experiments by “GraphPad Prism 5” software.

3.3. Apoptosis Analysis

In order to investigate the apoptotic effects of test compounds against breast cancer cells (MCF-7) and prostate cancer cells (PC-3) the selected compounds (**C3**, **F2**, **SC2** and **SF10**) were tested by using Annexin V- FITC Detection Kit. Cells were inoculated into 6-well plates and incubated in CO_2 incubator at 37 °C. After 24 hours 20 µL of test compounds, dissolved in DMSO, were added to incubated cells at final volume of 2 mL and final concentrations of 20, 10, 1, 0.1 µM. The treated cells were incubated for 48 hours in CO_2 incubator at 37 °C. After incubation, cells were harvested by trypsin and centrifuged at 800 rpm for 5 minutes. The pellet was dissolved in 5 ml of PBS and centrifuged again. The pellet was resuspended in 200 µL of binding buffer. 2 µL of Annexin V-FITC and 2 µL of PI were added. The stained cells were incubated for 15 minutes at room temperature. After incubation the apoptotic effects of test compounds were determined by flow cytometer.[34]

3.4. Cell Cycle Analysis

To investigate the effects of the selected compounds (**C3**, **F2**, **SC2** and **SF10**) on the cell cycle the most cytotoxic compounds were tested in breast cancer cell line (MCF-7) and prostate cancer cell line (PC-3) by propidium iodide staining. Into 6-well plates 1980 µL of cell suspension at a density of 5×10^5 cells per well were inoculated, and incubated for 24 hours. Test compounds were dissolved in DMSO and added to incubated cells to maintain the final concentrations of 20, 10, 1, and 0.1 µM. The cells treated with test compounds were incubated for 48 hours in CO_2 incubator at 37 °C. After incubation cells were harvested by trypsin and centrifuged at 1200 rpm for 10 minutes. The supernatant was poured and pellet was dissolved in 5 mL of PBS. The cell suspension was centrifuged again. The pellet was resuspended in 1 mL cold PBS and fixed by adding 4 mL of -20 °C ethanol (99.8%) on low speed vortex. The fixed cells at least 24 hours were incubated at -20 °C. After incubation fixed cell suspension was

centrifuged at 1200 rpm for 10 minutes at 4 °C. The pellet was dissolved in 5 mL of PBS and centrifuged again. The pellet was resuspended in 200 µL phosphate buffer including 0.1% Triton X-100. 20 µL RNase A (200 µg/mL) was added to cell suspension and cells were incubated in CO_2 incubator at 37 °C for 30 minutes. After incubation 20 µL PI (1 mg/mL) was added and cells were incubated at room temperature for 15 minutes. The cell cycle distribution was determined by flow cytometer, and data were analyzed by ModFit LT software; for each sample at least 20,000 events were collected.[31] [34] [35]

CHAPTER 4

CONCLUSION

Stilbenes, chalcones, and flavanones which are plant secondary metabolites have biological activities such as anti-viral, anti-inflammatory, anti-bacterial, anti-fungal, and anti-cancer activities.

In this study, previously synthesized 11 simple-stilbenes, simple-chalcones, simple-flavanones and 31 stilbene-fused chalcones and stilbene-fused flavanones were tested for their cytotoxic and anti-aromatase activities. The tested compounds were divided into four groups, according to their biological activities; the first group including **C1**, **C5**, **F2**, **SC2**, **SC3**, **SC4**, **SC6**, **SC7**, **SC10**, and **SC11** showed both cytotoxic and aromatase inhibition activities. While the second group including **C2**, **C3**, **SC1**, **SC5**, **SC8**, **SC9**, **SC12**, **F2**, **F3**, **SF10**, **SF11** showed only cytotoxic activity the third group including **S1**, **S3**, **SF5**, **SF8**, **SF13**, **SF14**, **SF15**, **SF16** showed only aromatase inhibition activity. The last group including **S2**, **S4**, **SC13**, **SC17**, **SF1**, **SF2**, **SF3**, **SF4**, **SF6**, **SF7**, **SF9**, **SF12**, **SF17** showed no activity.

In addition, when the relationship between the structure and cytotoxic activity was evaluated it was seen that the simple-stilbenes have not cytotoxic activity while the simple-chalcones and stilbene-fused chalcones are cytotoxic. Therefore, it is clear that the stilbene-fused chalcones mimic the simple-chalcones. However, while the simple-flavanones have cytotoxic activity, the stilbene-fused flavanones are not cytotoxic. This means that when the simple-flavanones are fused with stilbenes they loss their cytotoxic activity. In other words, the stilbene-fused flavanones mimic stilbenes.

In this study, while one of the goals was investigation of the mimicking performance of the hybrid compounds, the other one was the exploration of compounds which have both cytotoxic and aromatase inhibition activity. However, while the twelve of the tested compounds had both cytotoxic and aromatase inhibition activity, the relationship between cytotoxic activity and aromatase inhibition could not be explained. More investigations should be done to understand the relationship between cytotoxic activity and aromatase inhibition as a future work.

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