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ORIGINAL ARTICLE

A mixture of potent polyphenolic anticarcinogens: Microarray analysis of their efficacy in MCF-7 and MCF-10A cell lines

Mehmet Fatih Seyhan[1,3*], Allison Pinar Eronat[2], Ayca Diren[3], Mete Bora Tuzuner[4], Ayse Begum Ceviz[5], Hulya Yilmaz-Aydogan[3], Tulin Ozturk[6] and Oguz Ozturk[3]

[1]Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Istanbul Yeni Yuzyil University, Istanbul, Türkiye
[2]Department of Molecular Biology and Genetics, Faculty of Arts and Sciences Halic University, Istanbul, Türkiye
[3]Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Türkiye
[4]Department of Biochemistry, Faculty of Pharmacy, Istanbul Health and Technology University, Istanbul, Türkiye
[5]Department of Medical Genetics, Faculty of Medicine, Istanbul Health and Technology University, Istanbul, Türkiye
[6]Department of Pathology, Faculty of Medicine, Istanbul University-Cerrahpasa, Istanbul, Türkiye

Abstract: Polyphenols are abundantly available through diet and show great promise with their safe use against cancer. In our previous studies, we have shown cytotoxic effects of more than 30 polyphenols in breast cancer. In this study, we have aimed to investigate the effects of various polyphenols upon MCF-7 breast cancer and MCF-10A normal epithelial cells. To this end, we have designed a polyphenolic mixture based on the most effective concentrations of seven compounds, which is a first according to literature in terms of polyphenolic combination studies. Cell proliferation experiments were executed utilizing WST-1 and apoptotic status by Annexin V-PI. After determining most effective concentrations, we operated whole genome microarray analysis. Based on microarray data, the best effective concentration for both cell lines is 75% of polyphenolic mixture. In MCF-7 cell line, this dosage induced in down regulation of cell cycle, cell division, DNA repair and some genes linked to breast cancer. In contrast, no remarkable effect was observed in MCF-10A cell line. The designed polyphenolic mixture was demonstrated to inhibit breast cell division in multiple pathways. Our findings on cell division in MCF-7 cell line were found similarly with the study based on polyphenol-rich propolis which inhibited cell division remarkably in SK-BR-3 cells. This mixture, which is shown to have highly effective anticarcinogenic effects, can be considered as a prototype of natural prescription design for our future research.

Keywords: Polyphenols, breast cancer, whole genome expression, cell proliferation, cell division, microarray

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1 Introduction

Polyphenols, namely flavonoids and phenolic acids, are plant secondary metabolites with important roles such as regulating growth, metabolism, and protection against UV radiation or diverse pathogens (Niedzwiecki et al., 2016). Various potential health benefits of polyphenols have been studied against various conditions such as cardiovascular disease, asthma, diabetes, aging, neurodegenerative disease, oxidative stress and cancer (Scalbert et al., 2005; Pyo et al., 2024). Recently, several studies have focused on these substances in order to develop novel approaches that are more powerful and less toxic than conventional therapies (Niedzwiecki et al., 2016). To this end, several polyphenols have been investigated to clarify their effects on tumor growth, metastasis, cell proliferation, angiogenesis, apoptosis and inflammation (Fantini et al., 2015; Farhan et al., 2023).

It has been shown that polyphenols can be effective in various cancer preventive systems such as detoxification of xenobiotics, inhibition of oxidation, induction of apoptosis, anti-inflammatory features, prompting effects on immune system and estrogenic/anti-estrogenic activity (Niedzwiecki et al., 2016). Recently, various *in vitro* and *in vivo* studies have focused on the use of polyphenols to prevent cancer by employing them in combinations. Specifically created combinations of distinct polyphenols can be utilized to expand metabolic properties of these mixtures or mixtures in controlled and reproducible ways. Furthermore, accurate combinations of polyphenols may help to use lower doses of individual substance without sacrificing their effectiveness (Vladu et al., 2022).

*Corresponding Author: Mehmet Fatih Seyhan.
Email: mehmetfatih.seyhan@yeniyuzyil.edu.tr

Various *in vitro* and *in vivo* studies have indicated that combinations of two or three polyphenols inhibit cancer growth more effectively than exposure to a single substance (Niedzwiecki et al., 2016). Several studies have focused on different combinations such as quercetin, epigallocathechin gallate (EGCG) and genistein on cancer cell lines modeling prostate cancer (Hsieh & Wu, 2009; Tang et al., 2010); EGCG and resveratrol on prostate cancer (Ahmad et al., 2007) and breast cancer (MCF-7) (Hsieh & Wu, 2008); and also curcumin, EGCG and epicathechin on lung cancer cells (Saha et al., 2010; Zhou et al., 2013); and curcumin and EGCG on human B-cell chronic leukemia (Ghosh et al., 2009). Additionally, researches have been combining the polyhenols with chemotherapeutics as curcumin with dasatinib on colon cancer (Nautiyal et al., 2011) and resveratrol with 5-flurourasil on skin and esophaegel cancer (Dun et al., 2015), which have shown that combinations of polyphenols with chemotherapeutics result with a preferred anticarcinogenic effect (Vladu et al., 2022).

As of 2024, breast cancer remains the most diagnosed cancer among women globally and continues to have a significant impact on public health worldwide. In 2022, approximately 2.3 million new cases of breast cancer were diagnosed, and it caused around 670,000 deaths among women globally (https://www.wcrf.org/cancer-trends/breast-cancer-statistics/). Current breast cancer treatment modalities include chemotherapy, radiotherapy, surgical intervention, and hormone therapy, each targeting distinct aspects of tumor biology to optimize patient outcomes (Carter et al., 2016). As in many cancer types, development of resistance and side effects due to chemotherapeutic drugs remains to be a significant problem in breast cancer treatment (Vladu et al., 2022). Hence, alternative or adjuvant approaches need to be developed. Therefore, researches have turned to natural products such as flavonoids and phenolic acids to limit the side effects of highly toxic chemotherapy approaches and develop/establish novel complementary therapy agents.

The use of polyphenolic substances against cancer is very promising, since they are considered safe and widely available via diet. Regarding their ability to impact several biological pathways involved in the initiation and progression of cancer, they provide more comprehensive therapeutic effects than individual drugs. Combination treatments with natural compounds from the same or different chemical class may enhance the absorption of nutrients as well as their therapeutic and prophylactic effects. For example, combining polyphenols with micronutrients required for maintaining the integrity and stability of the extracellular matrix can yield increased anti-cancer benefits in order to target complimentary metabolic pathways that are essential for limiting the invasion and spread of cancer (Mocanu et al., 2015; Cojocneanu-Petric et al., 2015). Therefore, future research topics should include the use of common substances as safe, effective, and rational cancer therapy methods, especially when used in mixtures (Niedzwiecki et al., 2016).

Here we aimed to understand the combined and synergistic effects of several polyphenols on cell lines modeling hormone responsive breast cancer, MCF-7, and fibrocystic breast epithelium, MCF-10A, as control. MCF-7 is an ER+, PR+, Her2/neu-invasive ductal breast carcinoma (IDC) cell line with caspase-3 mutation and no p53 mutation, modeling the most common with an incidence of 60–70% amongst all breast cancers. This study pioneer in demonstrating the comprehensive effects of polyphenolic combinations on the MCF-7 breast cancer cell line, utilizing whole genome expression analysis via microarray technology to uncover intricate molecular interactions and pathways for the first time.

In the presented study, MCF-7 and MCF-10A cells were treated with various polyphenols and the most effective concentrations. In this mixture, seven polyphonenols were selected including quercetin, apigenin, caffeic acid phenyl ester (CAPE), luteolin, chrysin, galangin and pinostrobin based on our previous studies focused on the anticancer effects of various propolis samples and polyphenolic compounds (WST-1 cell proliferation results of all the tested polyphenols separately were given in the Supporting Information) (Seyhan et al., 2019). The polyphenolic mixture contains seven compounds and this design has been done for the first time according to literature in terms combination studies of polyphenols. Thereon, from this point this mixture have expanded to the Polyphenolic Mixture (PFK5120; Turkish patent reference number: 20355/26, International patent application number: PCT/TR2023/051027. https://worldwide.espacenet.com/patent/search/family/090608764/publication/WO2024076325A1?q=PCT%2FTR2023%2F051027).

2 Materials and Methods

2.1 Polyphenolic Standards

Polyphenolic compounds were purchased from Sigma Aldrich, Merck. Purities and catalog numbers of polyphenolic compounds are: apigenin (≥95%), 10798; chrysin (≥96.5%), C80105; galangin (≥97%), 92342; CAPE (≥97%), C8221; quercetin (≥95%), Q4951; luteolin (≥90%, Sigma-Aldrich), 440025; pinostrobin (≥99%), 38790. All polyphenolic compounds were solved as a 0.5 mg/mL in ethanol/water solution (60% ethanol: 40% water) as main stock solution, then diluted solutions were prepared for the experiments. The established concentrations of each compound regarding the IC_{50} values for MCF-7 were collected in a tube and referred as 100% concentration of polyphenolic mixture (per one well). Subsequently, 10, 20, 35, 50, 65, 75, 90 and 100% of this polyphenolic mixture were applied to MCF-7 and MCF-10A cells. IC_{50} concentrations of individual polyphenolic compounds in the mixture were given in Table 1. Also, chemical structures of the selected polyphenols were given in Figure 1 (The chemical structures were drawn using https://moldraw.com/ website).

2.2 Cell Culture

Every cell culture related commercial products were acquired from Biochrome, located in Berlin, Germany and cell lines modeling ER/PR+, HER2-, and nontumoregenic epithelial cell lines, MCF-7 (HTB-22) and MCF-10A (CRL-10317),

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Table 1. IC_{50} concentrations of individual polyphenolic compounds in the mixture

No	Compound	IC_{50} (µg/mL)	% Composition in mixture
1	Apigenin	10	5.6%
2	Chrysin	25	13.9%
3	Galangin	15	8.3%
4	CAPE (Caffeic acid phenethyl ester)	40	22.2%
5	Quercetin	45	25.0%
6	Luteolin	15	8.3%
7	Pinostrobin	30	16.7%
	Total phenolic content	**180**	**100%**

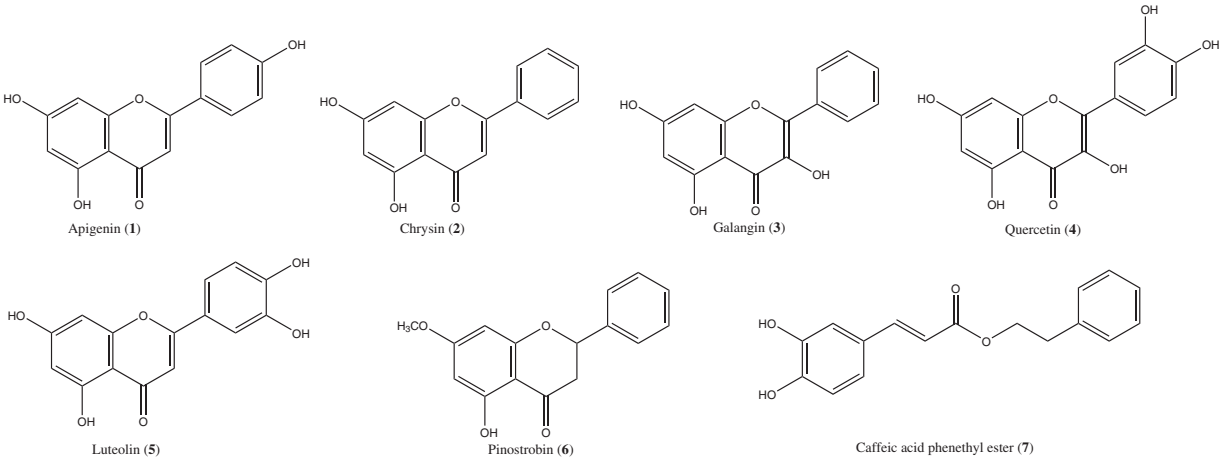

Figure 1. The chemical structure of selected polyphenols in polyphenolic mixture [1: apigenin, 2: chrysin, 3: galangin, 4: quercetin, 5: luteolin, 6: pinostrobin, 7: caffeic acid phenethyl ester (CAPE)]

from The American Type Culture Collection (ATCC) (Rockville, MD, USA). MCF-7 and MCF-10A cell lines were kept in Dulbecco's Minimum Essential Medium (DMEM) and Mammary Epithelial Cell Growth Medium (MEGM) and Bullet Kits (Lonza Group Ltd, Basel, Switzerland), respectively, containing 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin. Every cell line was cultivated in 75 cm^2 flasks and kept at 37°C in a humidified environment with 5% CO_2. Standard mycoplasma controls were carried out regularly.

2.3 WST-1 Cell Proliferation Assay

WST-1, a cell proliferation detection reagent, was acquired from Roche (Mannheim, Germany). The Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA) was used to count the cells. Cells were seeded at a density of 1×10^4 cells per well in 96-well plates (Greiner Bio-one, Austria). After letting the cells adhere for one night, the medium was changed with a 3% FBS containing medium. Cells were then given predetermined concentrations to measure the impact of the polyphenolic mixture at various points in time (24, 48, and 72 hours). WST-1 of 10 µL/well was added at these intervals and following incubation about 4 hours at 37°C with 5% CO_2, measured at the absorbance of 450 nM, with 620 serving as the reference wavelength with Multiscan ELISA Reader (Thermo Fisher Scientific, Germany). Data is given as percentages of absorbance readings from control wells on a relative proliferation index scale (mean ± SD). An estimated sigmoidal curve was used to determine the IC_{50} values. Each test was run in four duplicates.

2.4 Annexin V-PI Apoptosis Detection Analysis

Cells were cultivated at a density of 3×10^5 cells/well in 6-well plates (Greiner Bio-one, Austria). Following one-night adherence, the medium was changed with that contained 3% FBS. The cells were then given specific concentrations so that the effects of the polyphenolic mixture could be observed at predetermined intervals. Annexin V-PI Apoptosis Detection Kit 1 (BD PharmingenTM, San Diego, CA, USA) was utilized to ascertain the induction of apoptosis, and the procedure was carried out in accordance with the manufacturer's instructions. A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was then used to measure the cells to detect apoptosis. Based on their size and granularity, cells were gated independently for forward scatter (FSC) versus side scatter (SSC). Then were evaluated as intact cells (Annexin V-PI-), early apoptotic cells (Annexin+PI-), late apoptotic cells (Annexin V+PI+) and necrotic cells (Annexin V-PI+). All tests were performed in triplicate.

2.5 RNA Isolation and Whole Genome Expression

Cells were cultured in T75 flask at the density of 1×10^6 cells and treated with different concentrations (35% and 75%) of polyphenolic mixture for 48 hours as 4 replicates. RNA was collected utilizing RNeasy Plus Mini Kit (Qiagen) and the RNA integrity was assessed via the Bioanalyzer (Agilent) with all RIN values were greater than 7. As instructed by the manufacturer, the Custom Gene Expression Microarray, 8 × 60 K (Agilent, CA, USA) was used to analyze overall changes in gene expression. All tests were performed in quadruplicate.

2.6 Validation of Microarray Data via Real Time PCR Analysis

To validate the microarray data, the extracted total RNA, which was treated with varying concentrations, was amplified using Brilliant III Ultra-Fast QRT-PCR Master Mix (Agilent, CA, USA) with particular primers and probes of chosen genes from Qiagen library. Selected genes are cyclin B2 (CCBB2) (NM_004701), Cyclin-dependent kinase inhibitor 3 (CDKN3) (NM_005192), Cyclin-dependent kinase 1 (CDK1) (NM_001786), Baculoviral IAP Repeat Containing 5 (BIRC5) (NM_001168), E2F Transcription Factor 1 (E2F1) (NM_005225), Cell Division Cycle 25C (CDC25C) (NM_001790). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_002046) is selected as a housekeeping gene.

2.7 Statistical Analysis

All statistical analysis was conducted using GraphPad Prism 6 (GraphPad Prism Software, San Diego, CA, USA) and presented as mean ± standard deviation performing One-Way ANOVA and Dunnett's test, with $p < 0.01$ being considered significant. In addition, IC_{50} values were calculated. Quantile Normalization was used to normalize the raw microarray study data, and GeneSpring software version 7.0 (Silicon Genetics, Redwood City, CA, USA) was used for analysis. Excluded data had a coefficient of variation (CV) greater than 50%. After conducting a moderated t-test and applying Bonferroni correction for family-wise error rate (FWER), we included changes that exceeded 2-fold as well as results with p-values of less than 0.05 and less than 0.01 in the analysis. In addition, the logFC (log fold change) values of altered genes were admitted to Bioinformatics website platform (http://www.bioinformatics.com.cn/srplot) in order to perform GO pathway enrichment analysis.

3 Results and Discussion

3.1 Cell Proliferation Experiment

MCF-7 and MCF-10A cells were exposed to the polyphenolic mixture in different time intervals and concentrations. It was observed that polyphenolic mixture suppressed the cell proliferation of MCF-7 cells, starting from the dosage of 35% of the polyphenolic mixture at all-time intervals (Figure 2 and Table 2). The most effective time interval was estimated as the 48th hour (IC_{50} value 53.74). In contrast, no inhibitory effect of polyphenolic mixture was detected in MCF-10A epithelial cells (Figure 2). In addition, the IC_{50} value for MCF-10A cells could not been calculated (Table 2). The 35% and 75% concentrations of polyphenolic mixture were determined to be the most effective concentration at the 48th hour for further experiments in both cell lines. The concentration of 75% was selected in order to examine the effect of highly concentrated polyphenols.

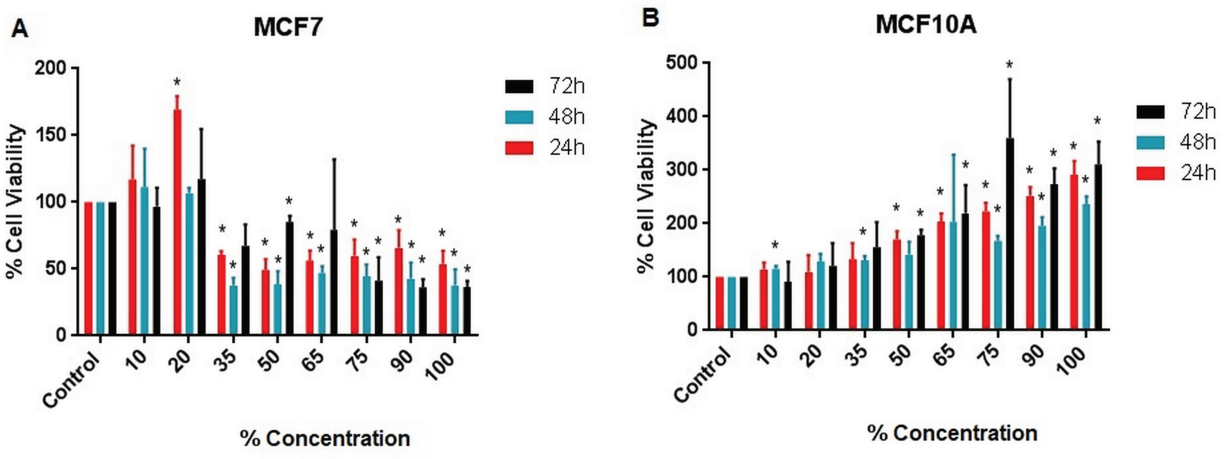

Figure 2. WST-1 Cell proliferation results. *p value is lower than 0.05

Table 2. IC_{50} values of WST-1 experiment results

IC_{50} (µg/mL)	24 h	48 h	72 h
MCF-7	91, 97 (59.65 to 141.8)	53, 74 (41.51 to 69.57)	78, 68 (65.20 to 94.94)
MCF-10A	Not converged	Not converged	Not converged

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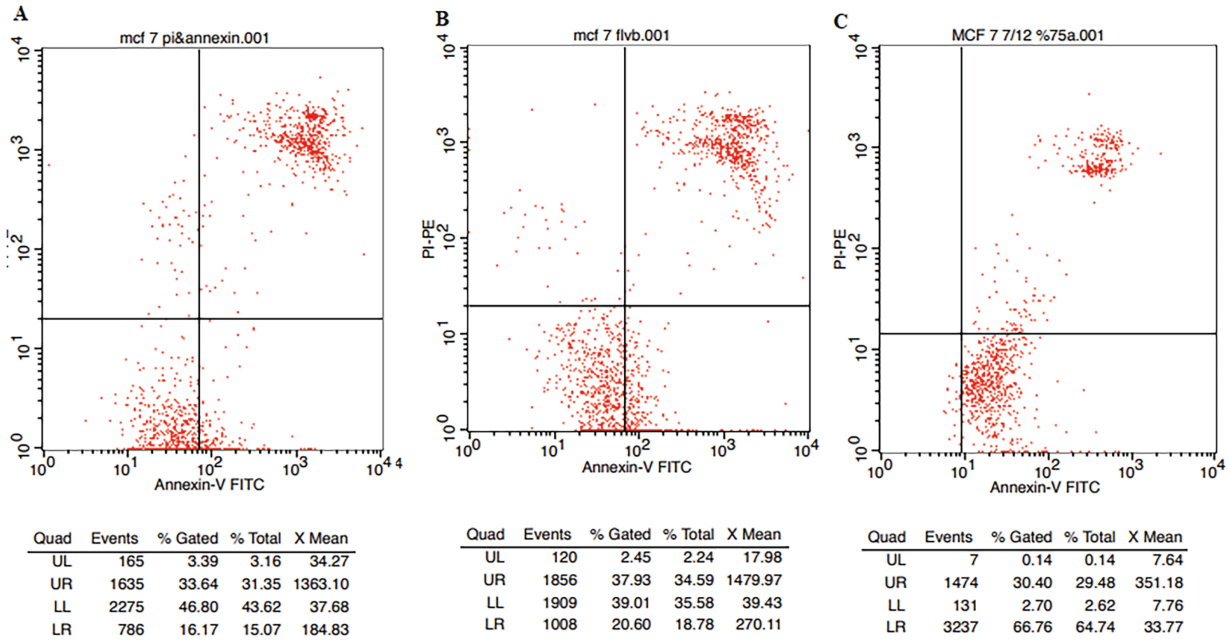
3.2 Annexin V-PI Analysis

MCF-7 and MCF-10A cell lines were exposed to 35% and 75% concentrations of the polyphenolic mixture. Following the treatment of MCF-7 cells with the 35% concentrations of the polyphenolic mixture, 38% of total cells were late-apoptotic and 21% were early-apoptotic and 39% of were alive. Necrotic cells were nearly absent (2% of total cells). In contrast, following the exposure to the same concentration, 83% of MCF-10A cells were living and the percentage of total apoptotic cells was only 16%. When we focused on the treatment with 75% concentration of polyphenolic mixture for both cell lines, almost 97% of MCF cells were apoptotic (30% early, 67% late). On the other hand, 85% of MCF-10A cells remained alive, with only 12% being apoptotic (Figures 3 and 4).

3.3 Microarray Analysis

For the whole genome expression assay, both cell lines were treated with the 35% and 75% concentrations of

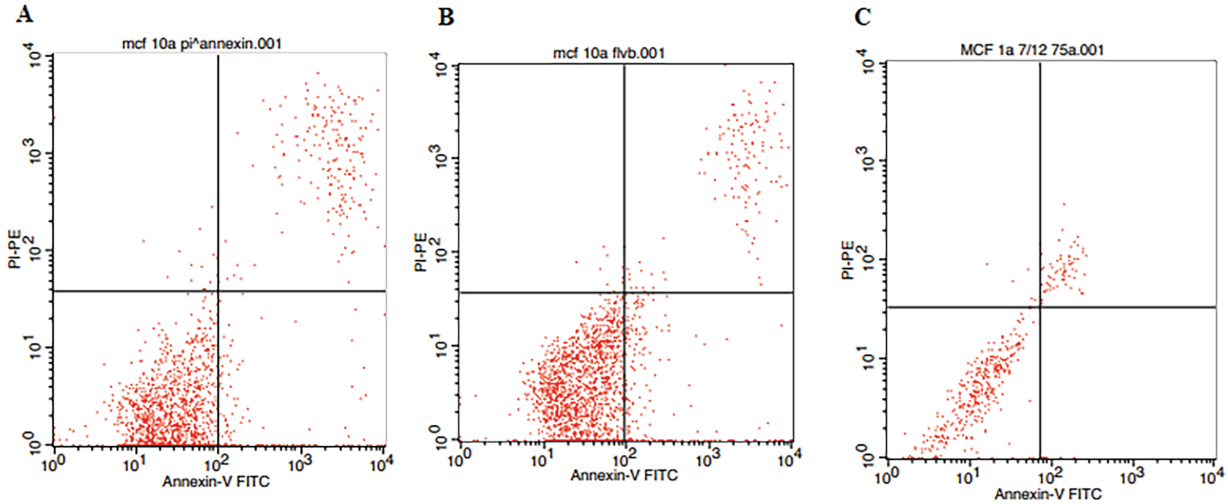


Quad	Events	% Gated	% Total	X Mean
UL	165	3.39	3.16	34.27
UR	1635	33.64	31.35	1363.10
LL	2275	46.80	43.62	37.68
LR	786	16.17	15.07	184.83

Quad	Events	% Gated	% Total	X Mean
UL	120	2.45	2.24	17.98
UR	1856	37.93	34.59	1479.97
LL	1909	39.01	35.58	39.43
LR	1008	20.60	18.78	270.11

Quad	Events	% Gated	% Total	X Mean
UL	7	0.14	0.14	7.64
UR	1474	30.40	29.48	351.18
LL	131	2.70	2.62	7.76
LR	3237	66.76	64.74	33.77

Figure 3. Annexin V-PI assay results for MCF-7 cells (A: Untreated control, B: %35 concentration of PFK-V1, C: %75 concentration of PFK-V1)



Quad	Events	% Gated	% Total	X Mean
UL	29	0.52	0.45	55.51
UR	583	10.36	9.04	2849.57
LL	4645	82.50	72.00	30.16
LR	373	6.63	5.78	1361.99

Quad	Events	% Gated	% Total	X Mean
UL	28	0.49	0.44	62.59
UR	417	7.30	6.61	2872.80
LL	4736	82.88	75.08	35.33
LR	533	9.33	8.45	860.02

Quad	Events	% Gated	% Total	X Mean
UL	35	1.65	1.13	52.35
UR	249	11.75	8.06	149.14
LL	1822	85.94	58.96	15.77
LR	14	0.66	0.45	150.33

Figure 4. Annexin V-PI assay results for MCF-10A cells (A: Untreated control, B: %35 concentration of PFK-V1, C: %75 concentration of PFK-V1)

Table 3. Number of genes changed by PFK-V1 in MCF-7 and MCF-10A cell lines

		P < 0.05			P < 0.01		
		Up-regulated	Down-regulated	Total	Up-regulated	Down-regulated	Total
MCF-10A	0–35%	221	5	226	221	5	226
MCF-10A	0–75%	98	156	254	42	82	124
MCF-10A	35%–75%	10	14	24	10	14	24
MCF-7	0–35%	79	15	94	46	5	51
MCF-7	0–75%	169	313	482	33	40	73
MCF-7	35%–75%	43	214	257	43	214	257

Table 4. Microarray results (Genes that are associated with cell division)

Cell Cycle			Histones			Centromere/Kinetochore		
Name	P value	FC	Name	P value	FC	Name	P value	FC
CCNB2	0,000107	−4,0419	*HIST1H4L*	0,004551	−3,3544	*CENPA*	0,000236	−4,5915
CDCA3	0,000147	−4,4821	*HIST2H3D*	0,005950	−4,6650	*NDC80*	0,000426	−4,3104
CDC45	0,000239	−3,5055	*HIST1H4C*	0,006074	−3,4439	*BUB1*	0,000861	−3,2456
CDCA2	0,000549	−3,3862	*HIST1H1B*	0,008074	−5,1135	*CENPE*	0,000956	−4,4195
CDKN3	0,000810	−3,7351	*HIST1H3F*	0,008159	−3,7929	*CEP152*	0,001552	−3,1382
CCNB1	0,001139	−3,1699	*HIST2H3A*	0,008185	−4,8329	*CENPF*	0,001798	−3,4969
CDC25C	0,002123	−3,8471	*HIST1H3J*	0,009949	−4,4337	*CEP55*	0,003136	−3,0515
CCNO	0,002155	−2,6905	*HIST1H3B*	0,019352	−4,9809	*BUB1B*	0,004462	−3,9830
CDK1	0,003318	−4,0556	*H2AFX*	0,020125	−3,3721	*TPX2*	0,005359	−3,5696
CDCA5	0,004052	−2,8366	*HIST1H3H*	0,023894	−3,4250	*SPC24*	0,005527	−4,9010
E2F1	0,006214	−2,6725	*HIST1H3C*	0,030678	−1,7828	*BORA*	0,006613	−3,4004
CDCA8	0,006475	−3,2423	*HIST1H3G*	0,031133	−3,0526	*KNTC1*	0,008427	−2,8655
CDC7	0,007551	−2,1990	*HIST1H3E*	0,032407	−4,2909	*SPC25*	0,009205	−6,0388
CDKN2A	0,010112	−2,1238	*HIST1H2BF*	0,046733	−1,9787	*CENPU*	0,009331	−3,6017
GTSE1	0,013668	−4,5548				*KNSTRN*	0,010971	−2,4665
DBF4	0,015622	−1,9852				*DSN1*	0,011094	−2,3225
CDC20	0,020758	−3,2376				*UBE3B*	0,012717	−2,0290
RBL1	0,023132	−1,8084				*CENPI*	0,016813	−4,5526
CDKN2C	0,025754	−3,7023				*CEP78*	0,018548	−1,9242
MCM7	0,026007	−2,4796				*NUF2*	0,024563	−3,789
						AURKA	0,025635	−2,9371
						CENPN	0,025753	−2,7957
						MAD2L1	0,02924	−2,9886
						CENPW	0,030967	−3,2065
						AURKB	0,03603	−4,4123

the polyphenolic mixture for 48 hours. The effects of the polyphenolic mixture were given in Table 2. In MCF-7 cells, when we compared 35% concentration with untreated control cells (shown as "0" in Table 3), 79 genes were up-regulated and only 15 genes were down-regulated. On the other hand, when we compared the 75% concentration with untreated control cells, 169 genes were up-regulated, 313 genes down-regulated. In contrast, when we examined the data of MCF-10A, 221 up-regulated and only 5 down-regulated genes were detected when compared to the 35% concentration with untreated control cells. When we compared 75% concentration with untreated control cells, 42 genes were up-regulated and 82 genes were down-regulated (Table 3). Afterwards, pathway analysis was performed via and GeneSpring software and results were given in Table 4. Then, log fold change (logFC) values of altered genes were admitted to Bioinformatics website platform in order to perform GO pathway enrichment analysis. According to the GO pathway enrichment analysis, 35% concentration changed the gene expression statistically in the pathways such as arachidonic acid and metabolic process, epoxygenase P450 pathway, positive regulation of vascular endothelial growth factor production, secondary metabolic process and toxin metabolic process in MCF-7 cells (Figure 5). Furthermore, the 75% concentration altered expressions in the pathways such as chromosome segregation, mitotic nuclear division,

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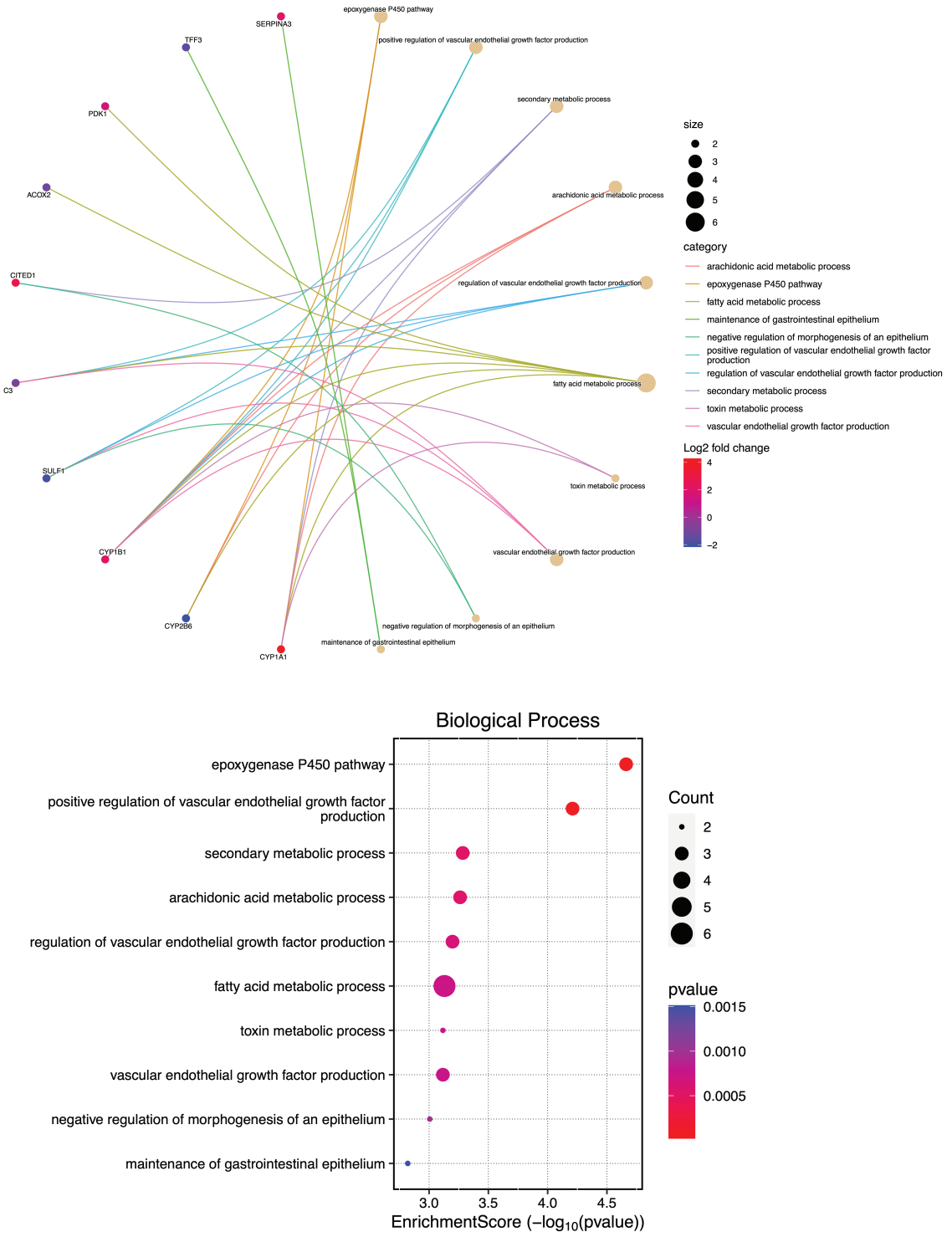

Figure 5. Functional enrichment analysis of 35% concentration of polyphenolic mixture on MCF-7 cell line. Upper figure: The cnetplot for the biological process data in GO analysis. Below figure: The significantly enriched Biological process in GO annotations

and mitotic sister chromatid segregation and organelle fission in MCF-7 cells (Figure 6). On the other hand, in MCF-10-A cells, the 35% concentration of affected pathways such as carbohydrate derivative catabolic process, negative regulation of axonogenesis, response to iron ion (Figures 7 and 8) and, the 75% concentration expressions in pathways including negative regulation of peptidase activity, neutrophil activation involved in immune response, negative regulation of endopeptidase activity, negative regulation of proteolysis.

3.4 Validation of Microarray Results via Q-PCR

CCBB2, CDKN3, CDK1, BIRC5, E2F1, and CDC25C were selected for the validation of the microarray data. The

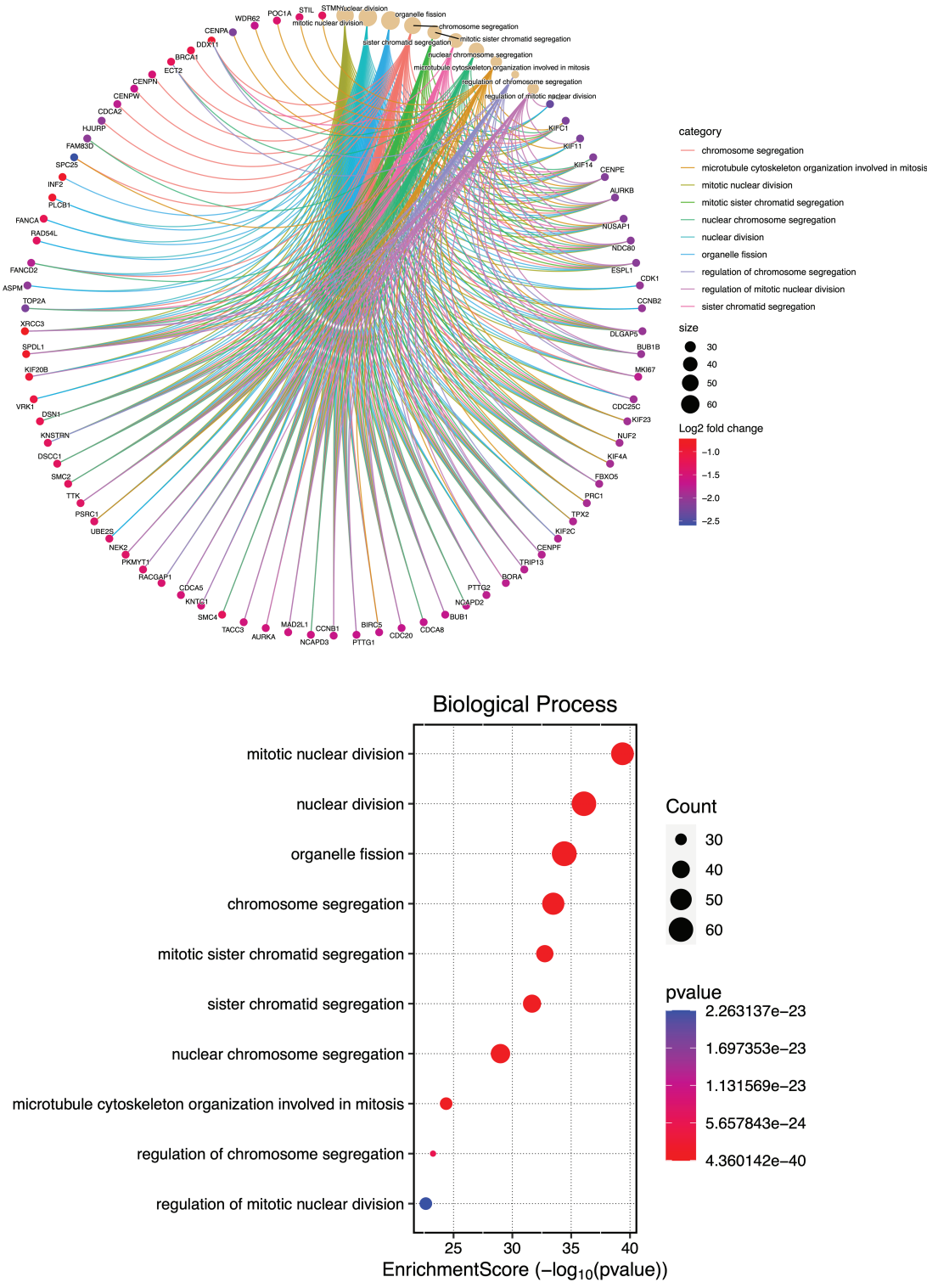

Figure 6. Functional enrichment analysis of 75% concentration of polyphenolic mixture on MCF-7 cell line. Upper figure: The cnetplot for the biological process data in GO analysis. Below figure: The significantly enriched Biological process in GO annotations

selected genes were found down-regulated in microarray findings with the treatment of 75% versus the untreated control. Q-PCR findings showed that the selected genes were also down-regulated (Figure 9). These findings validate our microarray data.

Breast cancer is a multifactorial condition that develops due to the alterations of various genes associated with oncogenic activation, inhibition of tumor suppressor genes, and defect in crucial signaling pathways such as cell survival, growth, differentiation and apoptosis (Shubbar et al., 2013). In recent years, there has been a focus on developments

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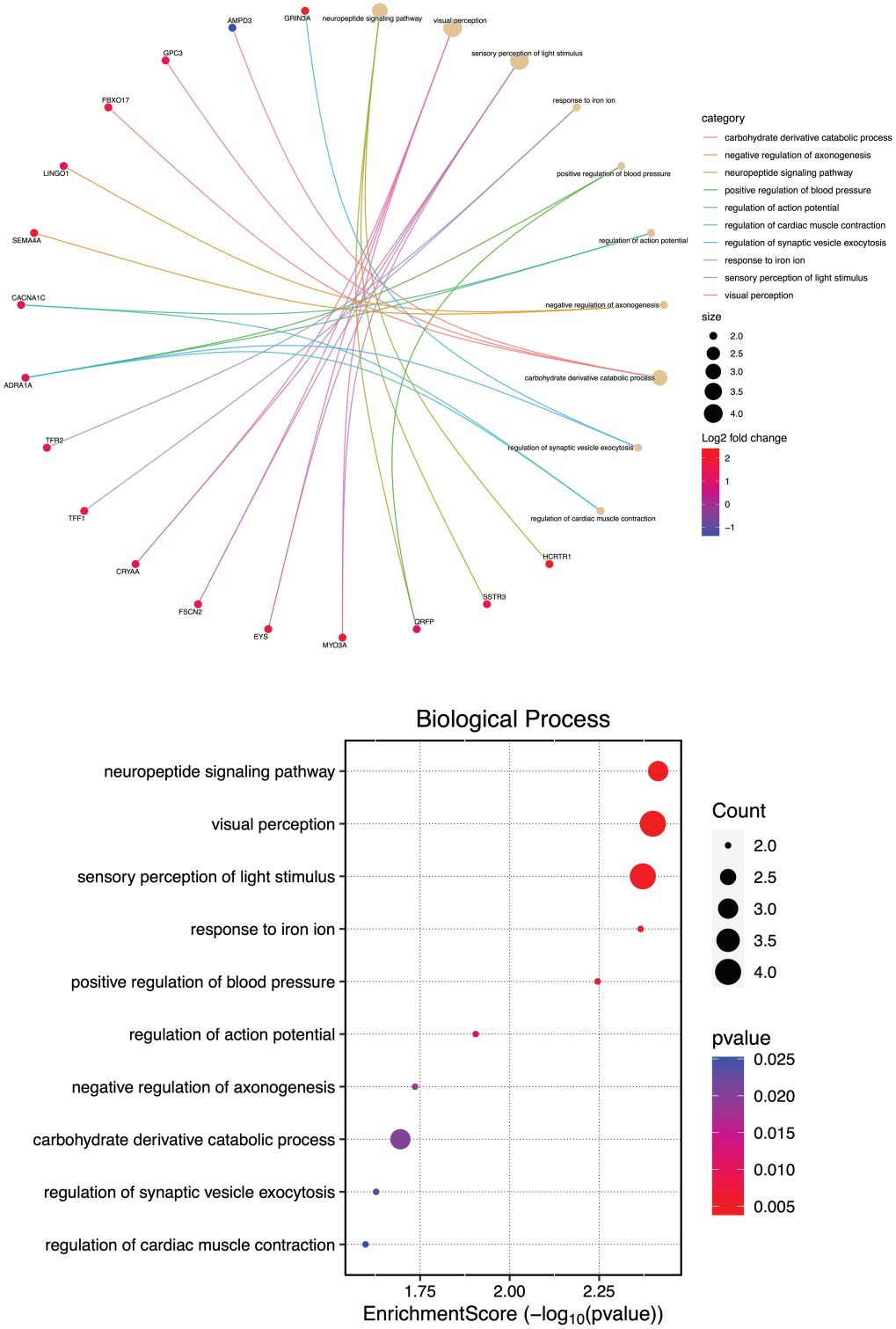

Figure 7. Functional enrichment analysis of 35% concentration of polyphenolic mixture on MCF-10A cell line. Upper figure: The cnetplot for the biological process data in GO analysis. Below figure: The significantly enriched Biological process in GO annotations

that could be effective in the diagnosis and treatment of breast cancer by revealing the cellular and molecular characterization of the disease (Khleif et al., 2010; Perou et al., 2000). There are currently several treatment options available, including adjuvant therapy combined with surgery, which are used to enhance quality of life and long-term survival rates. Breast cancer is still the second leading cause of death for women, though, despite improvements in treatment. Therefore, the development of new treatments for breast cancer prevention has focused on targeted therapies (Shubbar et al., 2013). Various studies have demonstrated that flavonoids prevent cancer especially by activating the

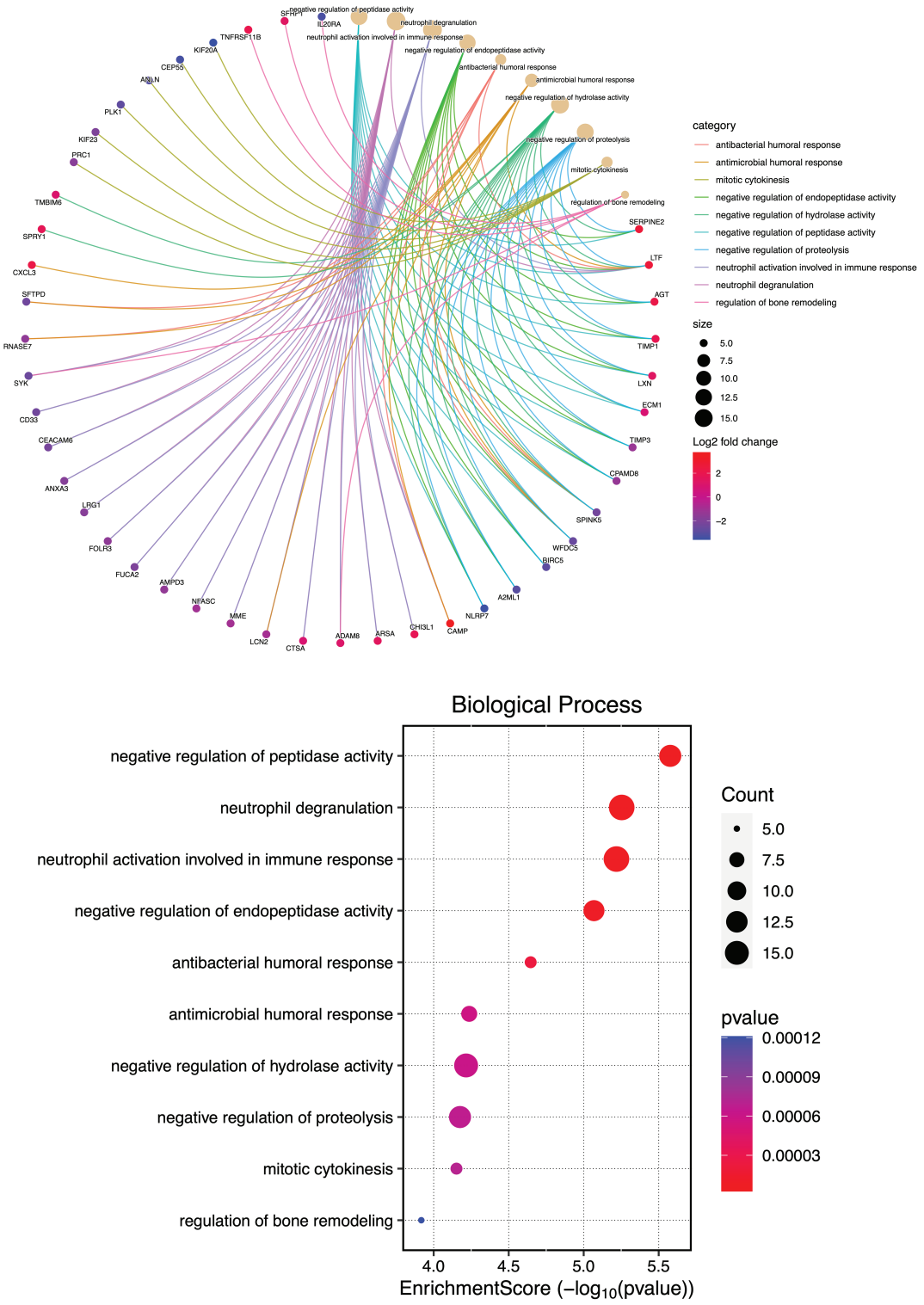

Figure 8. Functional enrichment analysis of 75% concentration of polyphenolic mixture on MCF-10A cell line. Upper figure: The cnetplot for the biological process data in GO analysis. Below figure: The significantly enriched Biological process in GO annotations

scavenging of reactive oxygen species (ROS) and stimulating cell death such as apoptosis and autophagy, which may be acting as positive modulators of pro-inflammatory cytokine signaling pathways (Vladu et al., 2022; Oh et al., 2019). Recently, among this kind of studies also anticarcinogenic features of polyphenols have shined out.

In the present study, the 75% concentration the polyphenolic mixture has shown the most statistically significant results regarding to whole genome expression data. In consequence, we detected statistically significant down-regulated genes associated with especially cell division process such as cell cycle, mitosis, histones, centromere, kinetochore and

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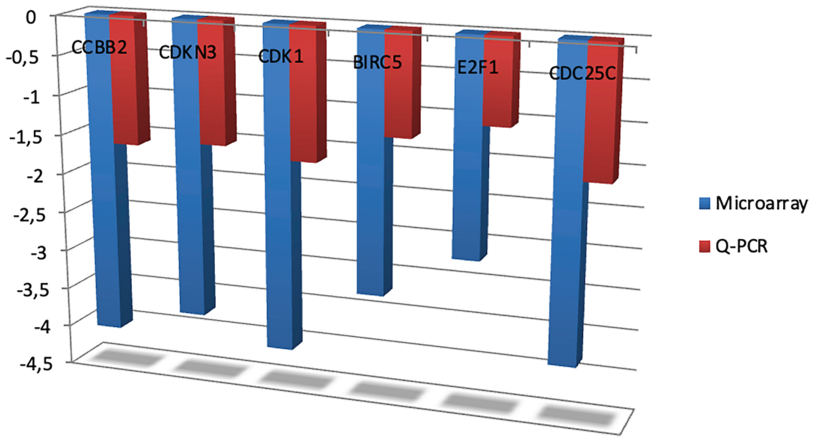

Figure 9. Validation of microarray data with selected genes (untreated vs treated for concentration of %75 polyphenolic mixture)

cytokinesis (Table 4). Following DNA replication, the cell cycle concludes cell division as a result of a series of processes. Various mechanisms contribute to the proper and correct progression and control of the cell cycle (Sanhaji et al., 2011) such as G1/S and G2/M checkpoints. According to our findings, down regulation of CDKN2C and CDKN2A genes, which inhibit cyclin dependent kinases like 4 and 6 that phosphorilate retinonblastoma (RB), was observed. Additionally, retinoblastoma ligand (RBL) and E2F1 gene expressions were down regulated, interacting with RB, indicating G1 arrest. In addition, cyclin B1 gene which controls G2/M checkpoint by interacting with cyclin independent kinase 1 (CDK1), was also down regulated by the polyphenolic mixture. Furthermore, down regulation was detected in CDC45L and MCM7 gene expressions, which control DNA replication. Furthermore, we have reported similar results that polyphenol-rich propolis inhibited cell division remarkably in SK-BR-3 cells (Seyhan et al., 2024).

Kinetochore is a complex molecular structure containing more than 100 proteins, which attaches to the spindle fibers during cell division to pull sister chromatids apart (Cheeseman & Desai, 2008). The key centromere-mediated element is the Histone H3 variant CENPA, which is found only at the centromere and is associated with nucleosome formation (Palmer et al., 1991). There are two arms that serve to connect the kinetochore to DNA. The first arm contains CENPC, which binds directly to the CENPA nucleosome (Carroll et al., 2010; Kato et al., 2013) and interacts with the Mis12 complex with its four subunits (Screpanti et al., 2011). This Mis12 complex, in turn, interacts with KNL1 and the four-subunit NDC80 complex, microtubule-binding proteins on the kinetochore (Petrovic et al., 2010). The second arm contains the DNA-binding CENP-T-W-S-X complex. In addition to binding with DNA, CENP-T interacts directly with the NDC80 complex (Nishino et al., 2012). The most important regulatory elements controlling kinetochore function are serine protein kinases that control kinetochore-microtubule attachments. These kinases include Aurora B (Lampson & Cheeseman, 2011), Polo-like Kinase 1 (Plk 1) (Liu et al., 2012), Mps 1 (Liu & Winey, 2012), Bub1 (Elowe, 2011) and CDK (Chen et al., 2008; Gascoigne & Cheeseman, 2013). Aurora B kinase is a key element for the accuracy of this mechanism (Tanaka et al., 2002). We have detected that the 75% concentration induced a down regulation in CENPA, CENPW, NDC80, Aurora B and Bub1 genes that have a role at the kinetochore complex. The down-regulation of these critical genes, and their relationship with the kinetochore structure explained above, shows how important the prepared mixture is. This mixture led to a down-regulation genes coding for important proteins functioning in important check points of the cell cycle, ones that control histone proteins and related to the centrosome and kinetochore structures, which play a key role in ensuring proper cell division. This shows that the prepared polyphenolic mixture prevents cell proliferation in targeting different aspects of cell division.

Moreover, genes of kinesins family, metallothioneins, and DNA repair mechanism were also altered statistically significantly as response to the %75 concentration (Table 5). Kinesins serve as molecular motors for the transport of materials and cargo along microtubules in eukaryotic cells. In addition, they provide the energy for various transport processes required during cell cycle progression, including mitosis and meiosis (Miki et al., 2005). The kinesin-13 family plays an essential role by depolymerizing microtubules (Zhu et al., 2005). Especially, KIF2C or mitotic centromere-associated kinesin (MCAK) has an important role (Schlisio et al., 2008), KIF2C/MCAK is localized at the centromere and regulates microtubule turnover at the kinetochore (Zhu et al., 2005). MCAK is also involved in the repair of microtubule misattachments at the kinetochore before anaphase. Therefore, accurate control of MCAK activity and localization is crucial for maintaining genetic integrity during mitosis. MCAK has been shown to be phosphorylated by Aurora B, which strongly inhibits the microtubule destabilizing ability of MCAK. Thus, inhibition of Aurora B activity prevents MCAK accumulation at the centromere (Schlisio et al., 2008). These findings suggest that Aurora B phosphorylation positively regulates MCAK activity by increasing its localization at the centromere and negatively regulates its microtubule destabilizing activity by controlling

Table 5. Other genes that are found statistically significant in microarray experiment

Gene Name	Entrez Gene ID	Description	p Value	Fold Change
		Kinesins		
KIF14	9928	Kinesin family member 14	0,000276	−4,443109
KIF4A	24137	Kinesin family member 4A	0,020402	−3,769752
KIF11	3832	Kinesin family member 11	0,018591	−4,593041
KIF23	9493	Kinesin family member 23	0,000560	−3,829111
KIF20A	10112	Kinesin family member 20A	0,000088	−7,348681
KIF15	56992	Kinesin family member 15	0,000266	−4,15647
KIF2C	11004	Kinesin family member 2C	0,008136	−3,502179
KIFC1	3833	Kinesin family member C1	0,005000	−4,628552
KIF20B	9585	Kinesin family member 20A	0,020313	−1,997791
		Metallothioneins		
MT-1E	4493	Metallothionein 1E	0,000560	8,69085
MT-1F	4494	Metallothionein 1F	0,000566	5,562584
MT-1X	4501	Metallothionein 1X	0,028654	2,014739
MT-1A	4489	Metallothionein 1A	0,038402	1,717077
MT-1M	4499	Metallothionein 1M	0,002379	22,59839
		DNA Repair Genes		
BRCA1	672	Breast cancer 1, early onset	0,033121	−2,2187383
ANP32E	81611	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	0,025245	−3,006694
ECT2	1894	Epithelial cell transforming 2	0,021960	−2,37945
EXO1	9156	Exonuclease 1	0,047356	−3,3593988
KPNA2	3838	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0,016003	−2,2493408
RRM1	6240	Ribonucleotide reductase M1	0,018728	−2,2708619
XRCC3	7517	X-ray repair complementing defective repair in Chinese hamster cells 3	0,0234125	−1,649334
POLQ	10721	Polymerase (DNA directed), theta	0,003969	−2,825743
ASPM	259266	Asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	0,002540	−4,1950955
KIAA0101	9768	KIAA0101	0,036727	−4,072048
MELK	9833	Maternal embryonic leucine zipper kinase	0,002085	−4,260255
DOK7	285489	Docking protein 7	0,000381	−4,44688
DNA2	1763	DNA replication helicase/nuclease 2	0,005332	−2,2664468
BLM	641	Bloom syndrome, RecQ helicase-like	0,004467	−2,865799
FANCA	2175	Fanconi anemia, complementation group A	0,025951	−2,3251495
FANCD2	2177	Fanconi anemia, complementation group D2	0,001086	−3,491924
FANCG	2189	Fanconi anemia, complementation group G	0,03114	−2,009618
FANCI	55215	Fanconi anemia, complementation group I	0,000381	−3,177208
FANCL	55120	Fanconi anemia, complementation group L	0,003199	−2,739362

it. Aurora A, a member of the Aurora kinase family, has been found to be associated with coactivators such as BORA and TPX2 in cell division (Lan et al., 2004). Selective inhibition of Aurora A causes the formation of abnormal spindle fibers and chromosome segregation problems (Hutterer et al., 2006). Therefore, down-regulation in genes encoding MCAK activity and related proteins such as Aurora A, Aurora B, CDK1, BORA and TPX2 further emphasizes the importance of the polyphenolic mixture.

Metallothionein (MT) binds to trace elements such as Zinc and Copper, and to date, MTs have been found overexpressed in cancers such as breast, prostate, bladder and colon (Hoar et al., 2007). In addition, MTs are involved in processes such as cell cycle regulation (Lai et al., 2011). MT-1E, 1F, 1X and 2A have been found to be significantly up-regulated in breast cancer cell lines and tissues (Tai et al., 2003). Moreover, MT-1E has been observed to be overexpressed in ER-negative invasive ductal cancers, leading to speculation that this MT isoform may functionally compensate for the ER deficiency in this neoplasia (Jin et al., 2000). While MT-1F has been associated with tumor differentiation, the role of MT-1X in breast cancer progression is not fully known (Jin et al., 2001). Based on our microarray findings on polyphenolic mixture treated MCF-7 cells, a statistically significant up-regulation was detected in the expression of Metallothionein 1E, 1F, 1M, 1X, 1A genes. The upregulation the expression of

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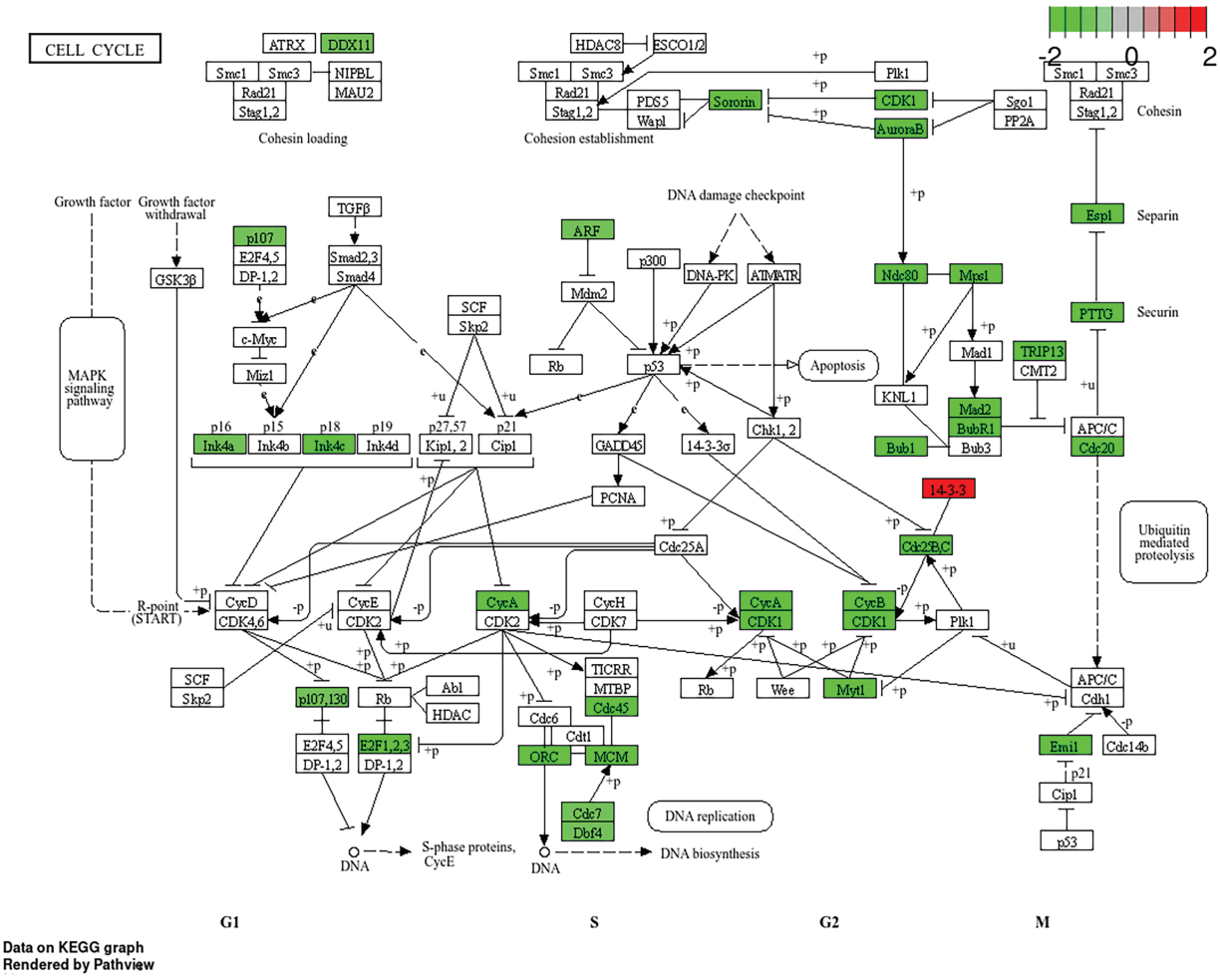

Figure 10. The effect of 75% concentration of polyphenolic mixture (hsa04110.pathview) in cell cycle on MCF-7 cell line

Metallothionein 1E, 1F, 1M, 1X, 1A genes was found similarly with literature which has been focused on breast cancer and metallothionein expression and this result showed that polyphenolic mixture did not change the metallothionein expression in breast cancer.

DNA repair mechanism is highly crucial in breast cancer carcinogenesis and mutations in DNA repair pathways can lead to genomic instability. Various mutations have been observed in DNA repair pathways breast cancer (Tufail, 2023). The % 75 c oncentration i nduced a d own regulation in several genes that associated with DNA repair mechanism similarly with the literature including BRCA1, ANP32E (Gursoy-Yuzugullu et al., 2015), ECT2 (Srougi & Burridge, 2011), POLQ (Brandalize et al., 2014), ASPM (Higgins et al., 2010), KIAA0101 (Simpson et al., 2006), DNA2 (Zhou et al., 2015). It was also reported that EXO1 (Kretschmer et al., 2011), RRM1 (Metro et al., 2010), XRCC3 (Martinez-Marignac et al., 2011) and DOK7 (Heyn et al., 2013) genes were found to be upregulated in breast cancer cases. However, the %75 concentration of the polyphenolic mixture down regulated the expressions of these genes in MCF-7 cells (Table 5).

In MCF-7 cells, which are caspase and Beclin-1 mutant, down-regulations were observed in the Caspase recruitment domain family, member 9, CARD9, BIRC5 (Survivin) genes. Since we have shown that apoptosis induced in these cells, apoptosis must be induced in these alternative pathways to caspase-3. Cell cycle arrest seems to be the most prominent outcome of this treatment. Nevertheless, this polyphenolic mixture obviously shows promising anti-carcinogenic effects. On the contrary of the whole genome expression data of MCF-7, in terms of treated cells, more up-regulated genes were found statistically significant in the MCF-10-A cell line. However, they were either unnamed genes or cell structure related genes. Therefore, they are regarded unrelated with cancer, which is a desirable finding regarding the direction of this study. No mutually altered pathways were observed between MCF-7 and MCF-10-A. In MCF-10-A, mainly affected pathways including carbohydrate derivative catabolic process, negative regulation of axonogenesis and response to iron ion, negative regulation of peptidase activity, neutrophil activation involved in immune response, negative regulation of endopeptidase activity, and negative regulation of proteolysis. In our fight against cancer, conventional chemotherapy approaches are highly toxic and lead to severe even fatal adverse effects, which turned researchers to agents effective on and selective to cancer cells. Here, we see that in

MCF-10 cells modeling fibrocystic breast epithelium, instead of cell cycle arrest or apoptosis, pathways related to immune response, negative regulation of proteolysis were altered. This shows us that it is triggering immune system in non-carcinogenic cells rather than showing cytotoxicity, which makes this mixture promising.

In summary, expression levels of genes related to several checkpoints of cell cycle and genes coding histone and centromere proteins in cell division process were down-regulated in MCF-7 cells treated with this distinctively prepared polyphenolic mixture. Furthermore, genes in the kinetochore complex, contributing to a smooth cell division (Table 4, Figure 10) indicate that the polyphenolic mixture inhibited the cell division process at distinct, important checkpoints. Down regulation of these kinesins family genes, increase this effect. Moreover, down-regulation of DNA repair genes in breast cancer cells exposed to the polyphenolic mixture may imply DNA damage repair, leading to apoptosis. Statistically significant down-regulation of various genes related to the development of breast cancer and other cancers explains the broad efficacy of the polyphenolic mixture. In conclusion, our findings suggest that polyphenols may be used as a complementary approach to current breast cancer therapy after rigorous *in vivo* and clinical studies and in the future, polyphenols may find a place amongst more efficient novel natural agents with minimal side effects.

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Author Contributions

Mehmet Fatih Seyhan: Methodology, Investigation, Conceptualization, Data curation, Formal analysis, Visualization, Writing—original draft. Allison Pinar Eronat: Conceptualization, Writing—original draft. Ayca Diren: Methodology, Investigation. Mete Bora Tuzuner: Formal analysis, Visualization. Ayse Begum Ceviz: Methodology, Investigation. Hulya Yilmaz Aydogan: Supervision, Project administration, Writing—review and editing. Tulin Oztürk: Supervision, Project administration. Oguz Ozturk: Methodology, Conceptualization, Supervision, Resources, Project administration, Writing—review and editing.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they have no competing financial interests.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products.

ORCID

Mehmet Fatih Seyhan: 0000-0002-6218-0049
Allison Pinar Eronat: 0000-0002-8095-1759
Ayca Diren: 0000-0003-2033-3577
Mete Bora Tuzuner: 0000-0001-8924-4850
Ayşe Begüm Ceviz: 0000-0002-3635-8421
Hulya Yilmaz-Aydogan: 0000-0002-8837-6664
Tulin Ozturk: 0000-0001-6910-1131
Oguz Ozturk: 0000-0002-2439-9269

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