



Investigation of The Synergistic Effects of Trastuzumab And Gambogic Acid in Her-2 Positive Breast Cancer Cell Line

Her-2 Pozitif Meme Kanseri Hücre Hattında Trastuzumab ve Gambojik Asit Sinerjik Etkilerinin İncelenmesi

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ABSTRACT

HER2 positive breast cancer is one of the biggest health problems in the world, causing millions of deaths every year. Drug combination modeling studies are extensively evaluated in treating many diseases. Pharmacological studies over the last half-century have shown that gambogic acid has potent anti-tumor activity against many types of cancer, including breast cancer. In this study, we examined the synergistic anticancer effect of gambogic acid and trastuzumab in HER2 positive breast cancer cell line (MDA-MB-453). In-vitro synergistic and antiproliferative effects of trastuzumab plus gambogic acid studies were determined with XTT method and the combination index (CI) values of the trastuzumab and gambogic acid combination were calculated by CompuSyn software. To determine molecular mechanisms of the trastuzumab and gambogic acid combination in MDA-MB-453 cells, the differences of gene and protein expression levels of HER2, caspase-9 and Bax were analyzed with using RT-qPCR and ELISA techniques. The combination of 50 µg/ml trastuzumab and 5 µM gambogic acid showed the best synergistic effect at 24 h incubation in MDA-MB-453 cells according to the in-vitro cell proliferation, RT-qPCR and ELISA test. Gambogic acid effects on HER2 positive breast cancer cell line shows its potential as natural compound to inhibit breast cancer cell proliferation in combination with trastuzumab.

Key Words

Trastuzumab, gambogic acid, breast cancer, HER-2.

Öz

HER2 pozitif meme kanseri, her yıl milyonlarca ölüme neden olan dünyanın en büyük sağlık sorunlarından biridir. İlaç kombinasyonu modelleme çalışmaları birçok hastalığın tedavisinde kapsamlı bir şekilde değerlendirilmektedir. Son yarım yüzyıl boyunca yapılan farmakolojik çalışmalar, gambojik asidin meme kanseri de dahil olmak üzere birçok kanser türüne karşı güçlü bir anti-tümör aktiviteye sahip olduğunu göstermiştir. Bu çalışmada HER2 pozitif meme kanseri hücre hattında (MDA-MB-453) gambojik asit ve trastuzumabın sinerjistik antikanser etkisini inceledik. Trastuzumab artı gambojik asit çalışmalarının in vitro sinerjistik ve antiproliferatif etkileri XTT yöntemi ile belirlenmiş ve trastuzumab ve gambojik asit kombinasyonunun kombinasyon indeksi (CI) değerleri CompuSyn yazılımı ile hesaplanmıştır. MDA-MB-453 hücrelerinde, trastuzumab ve gambojik asit kombinasyonlarının moleküler mekanizmalarını belirlemek ve HER2, kaspaz-9 ve Bax'ın protein ve gen ekspresyonu seviyelerindeki farklılıklar RT-PCR (qRT-PCR) ve ELISA teknikleri kullanılarak analiz edildi. RT-qPCR ve ELISA testine göre; 50 µg / ml trastuzumab ve 5 µM gambojik asit kombinasyonu, MDA-MB-453 hücre hattında 24 saatlik inkübasyonda en iyi sinerjistik etkiyi gösterdi. Gambojik asitin HER2 pozitif meme kanseri hücre hattı üzerine etkisi, , trastuzumab ile kombinasyonunun meme kanseri hücre proliferasyonunu inhibe eden doğal bileşik potansiyeline sahip olduğunu gösterir.

Anahtar Kelimeler

Trastuzumab, gambojik asit, meme kanseri, HER-2.

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INTRODUCTION

Breast cancer is the most diagnosed cancer type in women worldwide, with approximately 2 million new cases diagnosed and 627,000 patients died in 2018 [1]. Breast cancer is mainly categorized into three types: hormone receptor-positive (estrogen receptor and progesterone receptor), human epidermal growth factor receptor-2 positive (HER2-positive, HER2+) and triple-negative breast cancer. Amplification of HER2 gene or overexpression of HER2 protein is called HER2-positive and these processes cause occurrence and progression of tumorigenesis in normal breast cells. HER2 is a transmembrane tyrosine kinase receptor which is over-expressed in about 10-20 percent of breast cancers. There are several treatment strategies available for HER2-positive breast cancer, depending on the type and stage [2,3].

Chemotherapy is one of the most important steps in treatment of HER2-positive breast cancer and especially, development of HER2 targeted drugs has been significant therapeutic strategy in breast cancer. Trastuzumab (Herceptin®) is a recombinant DNA-derived humanized monoclonal antibody which selectively inhibits HER2-positive breast cancer tumorigenesis alone or in combination with other chemotherapeutics and natural products. HER-2 receptor consists of three conserved domains: extracellular ligand-binding domain, a transmembrane region, and an intracellular (cytoplasmic tyrosine kinase) domain. Trastuzumab binds to the extracellular domain of HER2 receptor with high affinity and prevents cleavage of this domain, resulting in interruption of cancer cell survival [4,5]. In order to increase therapeutic efficiency, administration of trastuzumab in combination with FDA approved drugs and natural products have been investigated in pre-clinical and clinical studies in cancer. In early and advanced HER2-positive breast cancer patients, trastuzumab is used in combination with paclitaxel, docetaxel, carboplatin [6-8]. Overexpression of HER2 is associated with resistance to hormonal therapy (particularly tamoxifen) in breast cancer. Therefore, combining hormonal agents, tamoxifen and aromatase inhibitors, with trastuzumab may be potential therapeutic aspect as hormonal therapy in breast cancer [9].

Gambogic acid is a natural product which is originally isolated from *Garcinia hanburyi* tree grown in Southeast Asia. Traditionally, gambogic acid has been used in

treatment of many different diseases for a long time. Numerous studies reported that, gambogic acid possesses diverse biological properties such as anti-cancer, anti-microbial, anti-oxidant and anti-inflammatory [10,11]. In recent decades, biological activities of gambogic acid have been investigated in almost all steps of tumorigenesis, and it inhibits the proliferation of various human cancer cells. According to *in-vitro* and *in-vivo* studies, gambogic acid induces apoptosis, inhibits angiogenesis and overcomes drug resistance in human cancer cells via different signaling pathways [10-13]. Gambogic acid has been approved for phase II clinical trial for solid tumor therapy by the Chinese Food and Drug Administration [14]. However, the anticancer mechanisms of the gambogic acid are not fully understood yet. Therefore, further molecular studies are needed to understand the biological effect of the gambogic acid in cancer.

In this study, we examined the combined effect of trastuzumab and gambogic acid as well as its mechanism of action was determined by using RT-PCR and ELISA techniques on HER2-positive human breast cancer cell line, MDA-MB-453. Obtained results indicated that gambogic acid plus trastuzumab directly decreased cell proliferation and induced inhibition of HER-2 signaling pathway. Moreover, this combination affects apoptosis via caspase-9 and Bax pathways. Combined therapy of trastuzumab and gambogic acid may be promising medicinal compound to treat HER-2 positive breast cancer.

MATERIALS and METHODS

Materials

MDA-MB-453 cell line was from ATCC (American Type Culture Collection, USA). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum, trypsin-EDTA, phosphate buffer saline (PBS), L-glutamine, penicillin-streptomycin and XTT ((2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell proliferation kit was obtained from Biological Industries Ltd. Gambogic acid was purchased from Abcam. Trastuzumab was supplied from Roche. RNA isolation and cDNA synthesis kits were purchased from Thermo Scientific. Human Bax, HER-2 and caspase-9 ELISA kits were from Sinogeneclon Co., Ltd. All other chemical reagents were purchased from Merck and Sigma Aldrich.

Table 1. Definition of CI index values.

CI>1	Antagonism	CI=1.1-1.2	Slight antagonism
		CI=1.2-1.45	Moderate antagonism
		CI=1.45-1.33	Antagonism
		CI=3.3-10	Strong antagonism
		CI>10	Very strong antagonism
CI=1	Additive	CI=1	Additive
CI<1	Synergism	CI=0.85-0.9	Slight synergism
		CI=0.7-0.85	Moderate synergism
		CI=0.3-0.7	Synergism
		CI=0.1-0.3	Strong synergism
		CI<0.1	Very strong synergism

Cell line and culture

For *in-vitro* experiments, MDA-MB-453 (HER2-positive human breast cancer) cell line was cultured in DMEM (high glucose) medium with 10% fetal bovine serum, 1% l-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin. Cells were cultivated in a humidified incubator at 37°C within an atmosphere containing 5% CO₂.

Cell proliferation assay

The XTT test was used to quantify the number of viable cells in each of the well in different concentrations [15,16]. Initially, the cancer cells were seeded in sterile 96-well culture plate (10x10⁴ cells in each well), and the different concentrations of gambogic acid (10-5-2.5-1.25-0.625-0.3125 µM), trastuzumab (100-50-25-12.5-6.25-3.125 µg/ml) and gambogic acid+trastuzumab were incubated with cells for 12-72 hours at 37 °C in a humidified incubator within an atmosphere containing 5% CO₂. At the end of the incubation times, 50 µl XTT reagents were added to each well for determination of living cells. After 4h, the absorbance was measured using micro plate reader at 450 nm, and then the percentage of cell viability was calculated.

Combined effect analysis

The interactions of the gambogic acid and trastuzumab were determined with using the combination index (CI) method (median-effect principle). CI values of gambogic acid and trastuzumab were calculated using CompuSyn free software. To enhance therapeutic efficiency and minimize resistance of drugs, combination chemotherapy is widely used in treatment of various diseases such as cancer. The Chou-Talalay method based on the median-effect equation was developed for analyzing drug combinations quantitatively. Further, this method

encompasses the Michaelis-Menten, Henderson-Hasselbalch, Scatchard, and Hill equations. In this context, CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) is analyzed drug combinations using median-effect principle, and calculated CI values to determine combination types (CI<1, CI=1 and CI>1 was accepted as synergism, addition and antagonism, respectively (Table-1) [17].

RT-qPCR Analysis

To determine the alteration of HER2, Bax, caspase-9 and GAPDH gene expressions with gambogic acid (5 µM), trastuzumab (50 µg/ml) and gambogic acid+trastuzumab (5 µM+50 µg/ml) at the end of the 24h incubation, qPCR experiments were carried out on MDA-MB-453 cells. Total RNA was extracted and first strand cDNA synthesis made according to the manufacturer's protocols. Primers were designed using Primer 3.0 program and synthesized by Macrogen Inc., with the following sequences (5' to 3') and qPCR conditions: HER-2 forward: TTGTGGCCTTCTTTGAGTTCGGTG and reverse: GGTGCCGGTTCAGGTACTCAGTCA at 95°C (10 min) followed by 45 cycles of 95°C (30 sec), 59°C (30 sec) and 72°C (45 sec), caspase-9 forward: CGA-ACTAACAGGCAAGCAGC and reverse: ACCTACCAA-ATCCTCCAGAAC at 95°C (10 min) followed by 45 cycles of 95°C (15 sec), 60°C (1 min) and 72°C (45 sec), Bax forward: CCTGTGCACCAAGGTGCCGGAAGT and reverse: CCACCCTGGTCTTGGATCCAGCCC at 95°C (10 min) followed by 45 cycles of 95°C (30 sec), 59°C (30 sec) and 72°C (30 sec), GAPDH forward: CGAACTAACAGGCAAGCAGC and reverse: TCGCCCCACTTGATTTTGG at 95°C (10 min) followed by 45 cycles of 95°C (15 sec), 64°C (30 sec) and 72°C (15 sec). qPCR was carried out using a SYBR Green Master Mix (Qiagen-330500) with a reacti-

on mixture containing 1 µl cDNA, 2 µl each primer and 12,5 µl SYBR Green Master Mix (total volume 25 µl). All assays were run in triplicate (Qiagen Rotor Gene). C_T values were assessed and relative expression of target genes was determined using the $2^{-\Delta\Delta C_T}$ method.

ELISA Tests

To investigate protein expression levels of the HER2, Bax and caspase-9 with gambogic acid (5 µM), trastuzumab (50 µg/ml) and gambogic acid+trastuzumab (5 µM+50 µg/ml) at the end of the 24h incubation, commercial sandwich ELISA kits were used on MDA-MB-453 cells according to the manufacturers' instructions. Expression levels of the proteins were measured spectrophotometrically.

Statistical Analysis

Differences in the mean values of measured activities were evaluated statistically using the SPSS 17.0 program (Univariate Variance Analyses and Pearson Correlation). Probability values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Cell Proliferation Assay

The cytotoxic activities of trastuzumab and gambogic acid were evaluated on MDA-MB-453 cell line by XTT assay individually and in combination. CI values of trastuzumab-gambogic acid combination were determined with CompuSyn software and Table-2 shows mean CI values of combinations at different incubation times. Gambogic acid was dissolved in DMSO and diluted in DMEM before cell proliferation assay. The control cells were treated with DMEM containing 0.1% DMSO to determine cytotoxicity of the gambogic acid. We treated MDA-MB-453 cells with different concentrations of trastuzumab 100-50-25-12.5-6.25-3.125 µg/ml, 10-5-2.5-1.25-0.625-0.3125 µM gambogic acid and the combination of trastuzumab and gambogic acid during 12, 24 and 48h.

Significant anti-proliferative activity did not observe when the treatment was applied for 12h. Treatment with gambogic acid alone as well as the combination of trastuzumab significantly inhibited cell proliferation after 24h and 48h. trastuzumab did not serve essential anti-proliferative activity from 12h to 48h in 100-3.125 µg/ml concentrations range. However, trastuzumab inhibited cell proliferation in combination with gambogic acid for certain concentrations for 24 and 48h (Figure-1-3). Trastuzumab is routinely used in the first-line treatment of patients with advanced breast cancers that express HER2. Nevertheless, initial and eventual resistance to HER2-based therapy with trastuzumab is frequently observed in significant number of patients with HER2-positive breast cancer. To overcome trastuzumab-induced resistance mechanisms in HER-2 positive breast cancer, incorporation of new compounds in combinational therapy with trastuzumab are being extensively studied in pre-clinical and clinical studies [18,19].

100 µg/ml trastuzumab +10 µM gambogic acid, 50 µg/ml trastuzumab +5 µM gambogic acid, and 25 µg/ml trastuzumab +2,5 µM gambogic acid significantly reduced cell viability when the treatment was applied for 48h, and 50% reduction in cell viability was achieved with these combinations. Moreover, 50 µg/ml trastuzumab+5 µM gambogic acid and 25 µg/ml trastuzumab+2,5 µM gambogic acid exhibited anti-proliferative activity during 24h against MDA-MB-453 cells. Briefly, 50 µg/ml trastuzumab+5 µM gambogic acid, and 25 µg/ml trastuzumab+2,5 µM gambogic acid showed similar combinational effect to inhibit survival of MDA-MB-453 cells in 24 and 48h. According to the CI values of the trastuzumab-gambogic acid combinations (Table-2), 50 µg/ml trastuzumab+5 µM gambogic acid exhibited the lowest CI values for 24h and therefore this combination was accepted as optimum for HER-2 positive breast cancer cells.

Table 2. CI values of the trastuzumab-gambogic acid combinations.

	12 HOURS	24 HOURS	48 HOURS
	CI		
100 µg/ml trastuzumab + 10 µM gambogic acid	0.98 ± 0.01	0.91 ± 0.02	1.64 ± 0.04
50 µg/ml trastuzumab + 5 µM gambogic acid	0.62 ± 0.02	0.50 ± 0.03	0.86 ± 0.05
25 µg/ml trastuzumab + 2.5 µM gambogic acid	0.78 ± 0.04	0.59 ± 0.03	0.78 ± 0.02

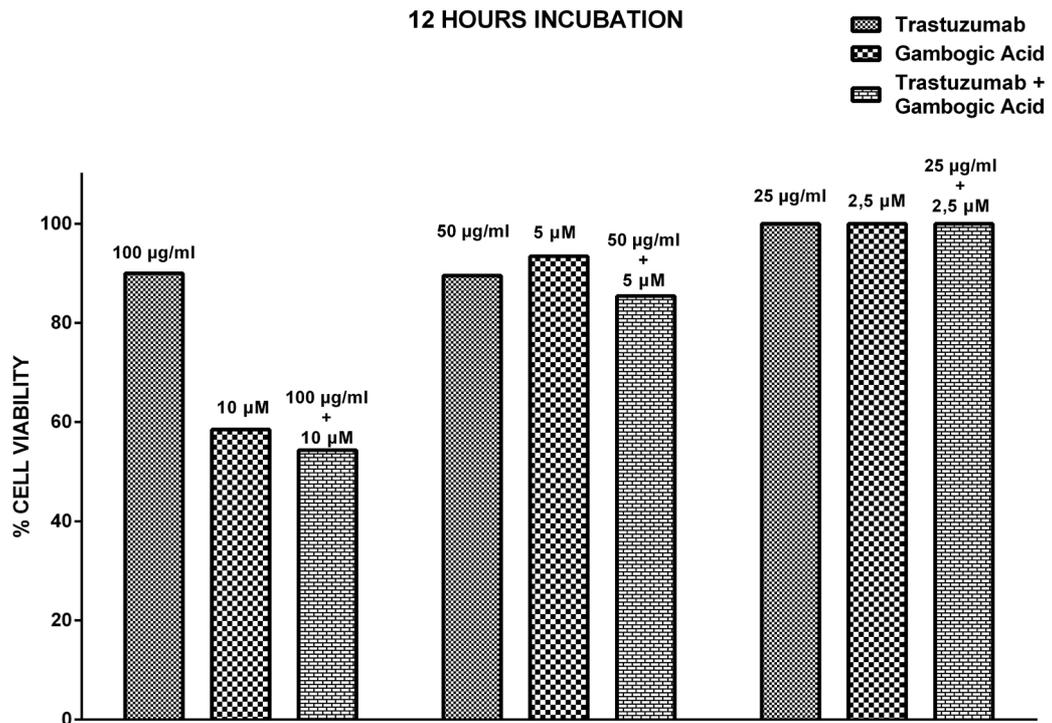


Figure 1. Antitumor activity of trastuzumab, gambogic acid and trastuzumab+gambogic acid for MDA-MB-453 cell line at 12h.

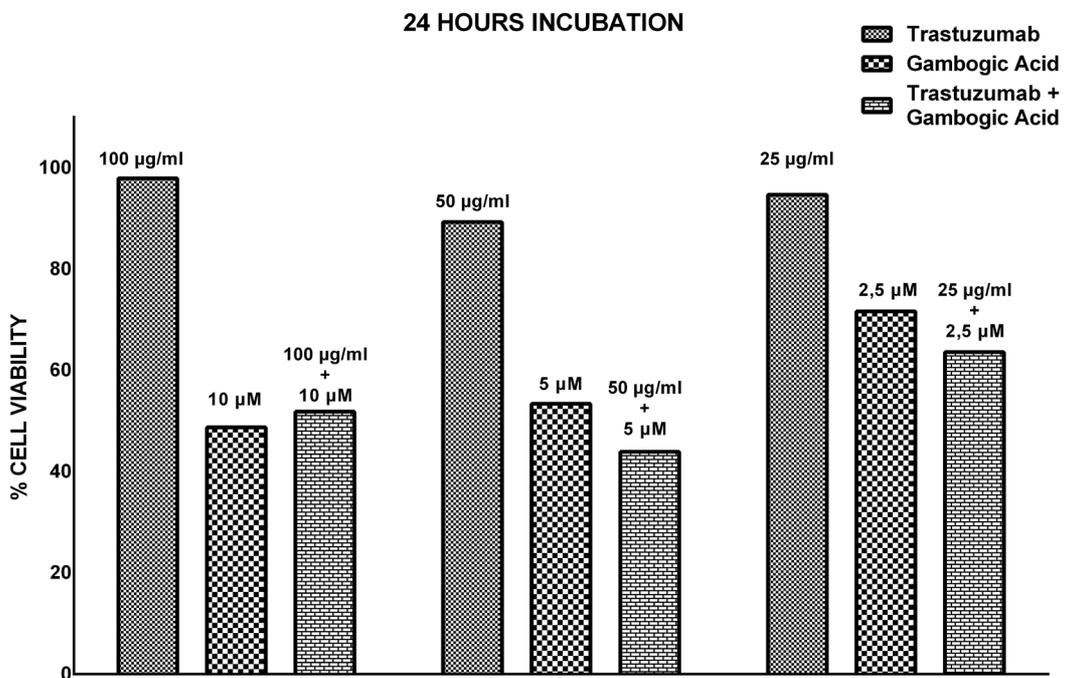


Figure 2. Antitumor activity of trastuzumab, gambogic acid and trastuzumab+gambogic acid for MDA-MB-453 cell line at 24h.

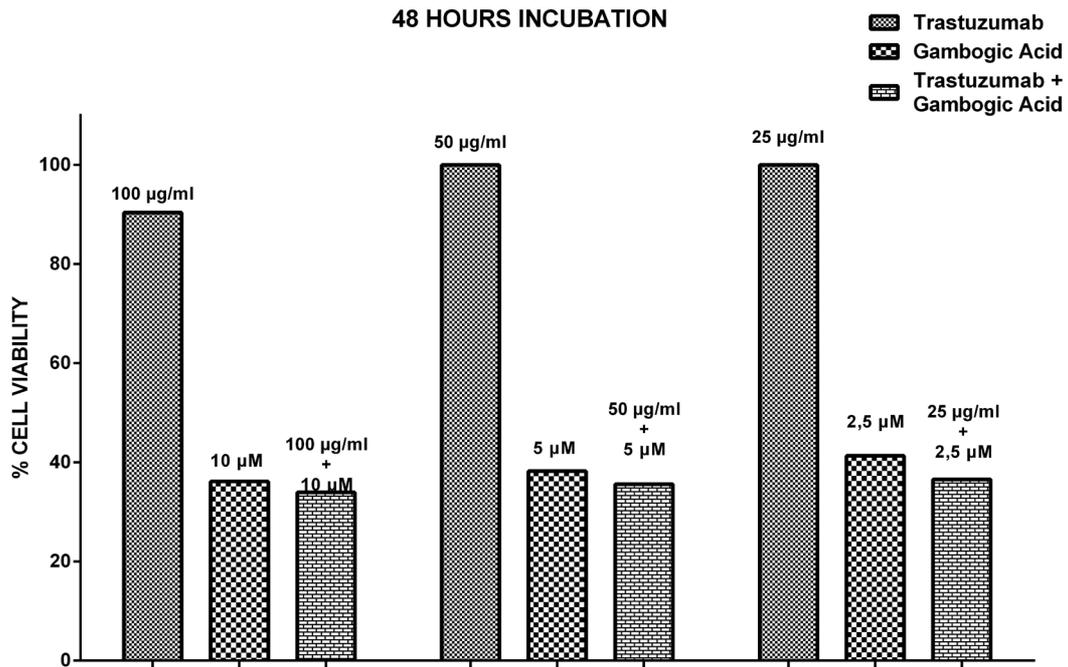


Figure 3. Antitumor activity of trastuzumab, gambogic acid and trastuzumab+gambogic acid for MDA-MB-453 cell line at 48h.

RT-qPCR Analysis and ELISA Tests

Gene expression analysis was performed 50 µg/ml TRS and 5 µM gambogic acid combinations at 24h with the best agonistic effect calculated as CI 0.50 ± 0.03 . Almost all cancer patients, upregulation of HER2 expression at the gene and protein level have been correlated with poor prognosis in breast cancer [20]. In this study, it was shown that treatment with combination of trastuzumab and gambogic acid suppressed HER2 expression levels better than individual treatment (Figure-4). In cells treated with a combination of 50 µg/ml trastuzumab and 5 µM gambogic acid, the ELISA findings of HER2 protein decreased by 60.17% (36.4 IU/L) compared to control, correlating with decreased gene expression levels (Table-3). Gambogic acid increasing anti-proliferative activity of trastuzumab, suggests that the use of gambogic acid with trastuzumab as an adjunct therapy in the treatment of HER2 positive breast cancer may be more effective.

The intrinsic apoptotic pathway begins with the release of cytochrome-C (Cyt-C) into the cytosol, causing a change on the permeability of mitochondrial membrane by Bax (B-cell lymphoma-2-associated X) protein. Apoptotic Protease Activating Factor-1 (Apaf-1) after release of Cyt-C, polymerizes in an dATP or ATP dependent mechanism [21,22]. Apaf-1 enabling the assembly of the apoptosome, activates caspase-9 (cas-9) and in

this manner initiating the caspase cascade [20]. According to the findings trastuzumab and gambogic acid induced to intrinsic apoptotic pathway individually. The gene expression level of cas-9 was found to increase approximately 6 and 5 times (Figure-5) in trastuzumab and gambogic acid treatment, respectively. On the other hand, in cells treated with a combination of 50 µg / ml trastuzumab and 5 µM gambogic acid, ELISA results of cas-9 protein level was determined as lowest (21 ng/ml) and correlating with gene expression levels (Table-3). This maybe suggests that treated with a combination of trastuzumab and gambogic acid induces cell death by a pathway other than intrinsic apoptosis. In order to explain this situation to be precise, it is necessary to investigate gene expression levels of some genes such as Bcl2, Apaf-1 and protein levels by western-blot in addition to flow-cytometry. According to the results of gene expression analysis, when trastuzumab and gambogic acid were applied separately, it was found to down-regulate Bax gene (0.5 and 1.5 fold change, respectively). When ELISA results are examined, it is seen that there is a similar change in the amount of protein supporting these results (Table-3). Conversely, trastuzumab-gambogic acid combination treatment (50 µg/ml trastuzumab and 5 µM gambogic acid) increased Bax gene expression (Figure-6) and amount of protein. In this case it may be considered an indication of the formation of trastuzumab resistance.

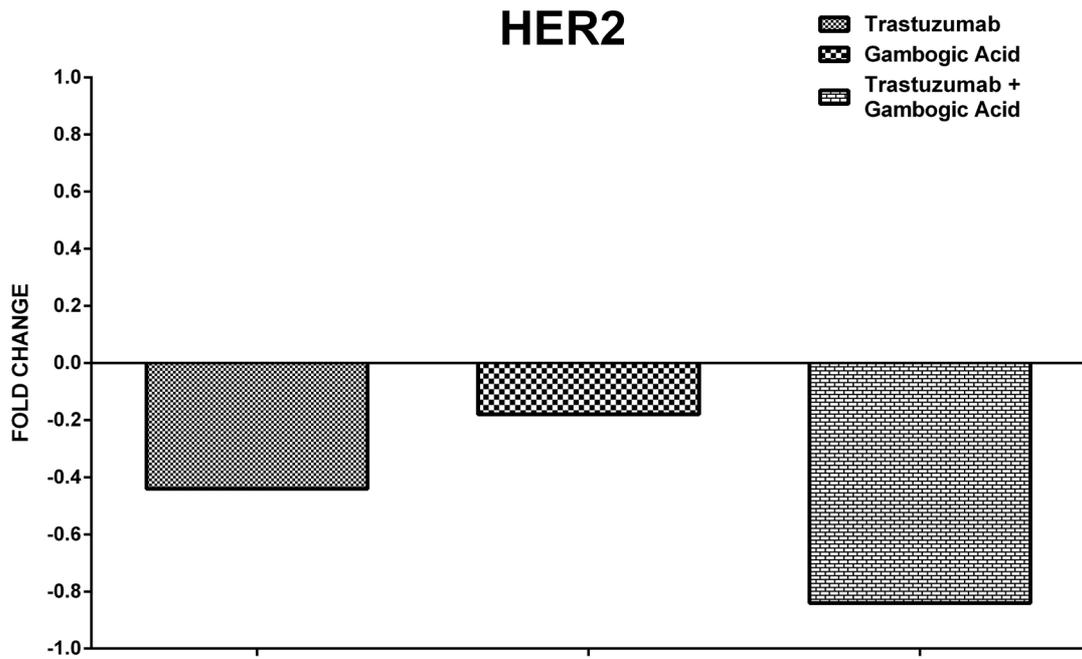


Figure 4. Relative fold change for HER2 gene in trastuzumab, gambogic acid and trastuzumab+gambogic acid by RT-qPCR.

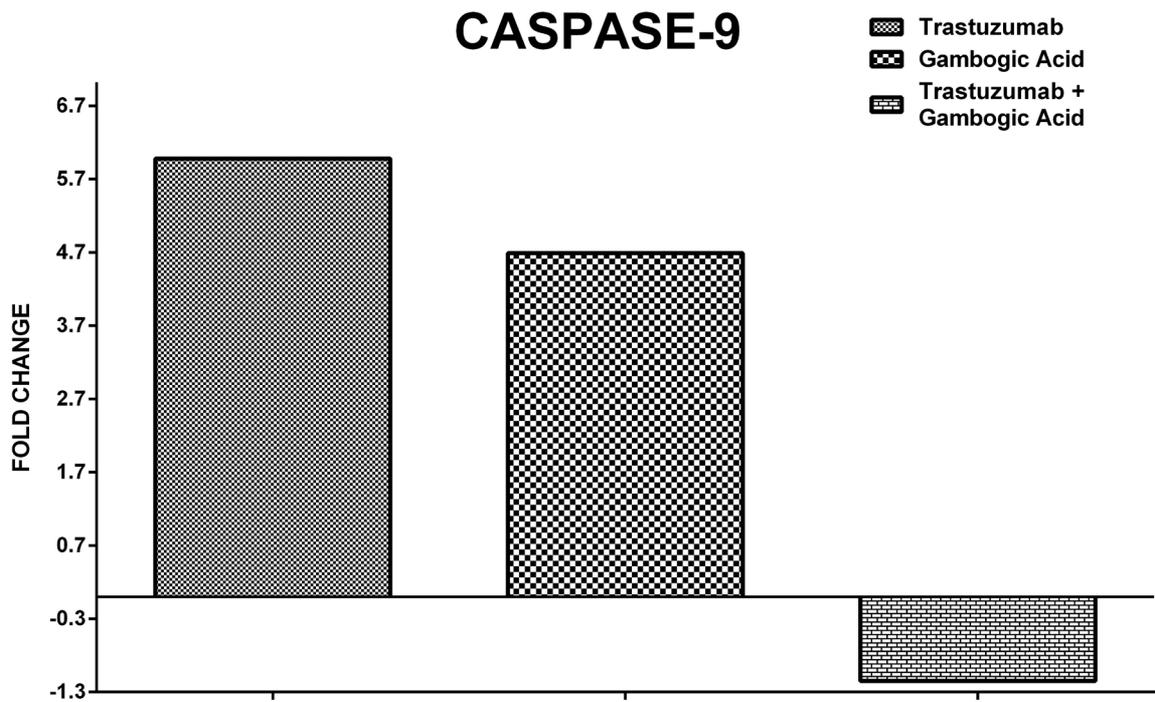


Figure 5. Relative fold change for caspase-9 gene in trastuzumab, gambogic acid and trastuzumab+gambogic acid by RT-qPCR.

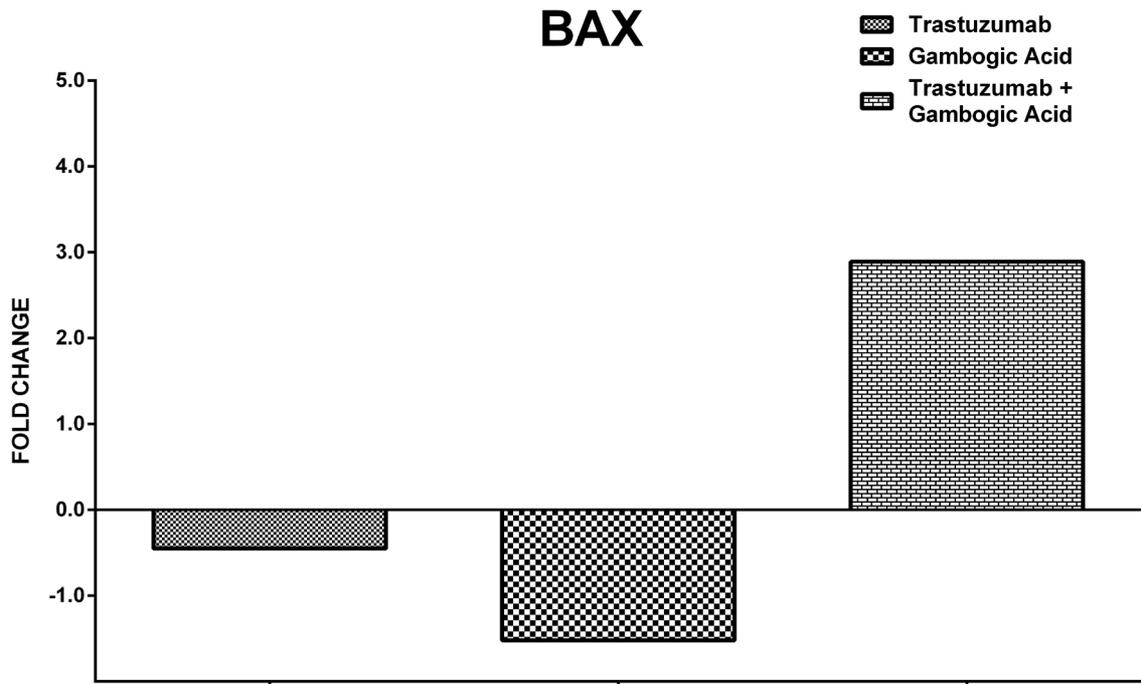


Figure 6. Antitumor activity of trastuzumab, gambogic acid and trastuzumab+gambogic acid for MDA-MB-453 cell line at 48h.

Table 3. ELISA Tests results of trastuzumab, gambogic acid and trastuzumab+gambogic acid.

	Bax (ng/ml)	Caspase 9 (ng/ml)	HER2 (IU/L)
Control	10.27	28.50	91.40
Trastuzumab (50µg/ml)	9.90	46.50	80.30
Gambogic Acid (5µM)	9.59	37.08	51.33
Trastuzumab (50µg/ml) + Gambogic Acid (5µM)	12.05	21.00	36.40

Trastuzumab is the standard treatment for HER2 positive breast cancer and significantly improved clinical outcomes. Nevertheless, approximately 50% of HER2 positive breast cancer patients can not heal with this drug [23]. Compared to monotherapy, agonistic growth inhibition and anti-proliferative effect was achieved by the combination treatment of gambogic acid and trastuzumab. Findings showed significant agonistic cytotoxic effect in MDA-MB-453 cells at 24th hour 50 µg / ml trastuzumab and 5 µM gambogic acid according to the cell proliferation, RT-qPCR and ELISA studies.

Conclusion

Trastuzumab is clinically used target specific drug to treat either early-stage or advanced-stage/metastatic HER-2 positive breast cancer. Nevertheless, resistance to trastuzumab is often observed in significant number of patients with HER-2 positive breast cancer. To increa-

se therapeutic efficiency and minimize drug resistance of trastuzumab is important research topics in HER2-positive breast cancer. Therefore, many natural and synthetic compounds have been utilized in combinational therapy with trastuzumab. In this study, gambogic acid has increased anti-proliferative activity of the trastuzumab according to the cytotoxicity, RT-qPCR and ELISA tests. Especially, gambogic acid+trastuzumab was dramatically decreased expression level of the HER-2 at gene and protein level. Further, this combination may stimulate cell death by a pathway other than intrinsic apoptosis according to the expression of Bax and caspase-9 in MDA-MB-453 cells. In conclusion, gambogic acid-trastuzumab combination can be suggested as a potent candidate for treatment of HER-2 positive breast cancer.

Conflict of Interest

Authors declare that he has no conflict of interest.

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