


Tetrazolium-Based Cytotoxicity Tests May Not Always Reflect Accurate Results

Tetrazolium Bazlı Sitotoksosite Testleri Her Zaman Doğru Sonuçları Yansıtmayabilir

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Abstract

Background: Cell viability and/or cytotoxicity analysis is one of the most important tools used for biological evaluation in vitro studies. The selection of the right cytotoxicity tests is critical to form the basis for in vivo and preclinical studies, specifically for cancer research. In the present study, we aimed to investigate the cytotoxic effects of bromelain, a widely-used phytochemical product in the medical field, and idarubicin, an anthracycline antibiotic used in the treatment of cancer, in normal lymphocytes and a promyelocytic leukemia cell line (HL-60) with MTT, WST-1, and luminescent ATP assays and to compare the results of these tests.

Materials and Methods: We obtained peripheral blood lymphocytes from healthy, young, non-smoker male volunteers and obtained the HL-60 cell line from the American Type Culture Collection (ATCC). Bromelain and idarubicin were added in increasing concentrations to both cell lines. Cells were incubated at 37°C in a carbon dioxide incubator for 24 h. After incubation, cytotoxicity levels were determined by MTT, WST-1, and ATP assays, and morphological evaluations were performed by fluorescent staining.

Results: The MTT and WST-1 assays demonstrated that cell viability/formazan formation increased with bromelain concentration; however, the luminescent ATP assay demonstrated that cell viability decreased with increasing concentrations of bromelain. Whereas fluorescent staining methods confirmed the ATP assay results, the MTT and WST-1 assays contradicted the ATP assay results. The cytotoxic effects of idarubicin were similar in the two cell lines according to the three different measurement methods and were positively correlated with the results of the fluorescent staining methods.

Conclusion: The detection of cell viability and cytotoxicity by bromelain with the MTT and WST-1 assays in lymphocytes and HL-60 cells is limited. To obtain accurate and reliable results from cytotoxicity studies, a measurement method should be carefully selected by considering that the phytochemicals to be tested could interfere with the results, and the results should be verified by other methods.

Key words: Bromelain, Idarubicin, Cytotoxicity tests, Interference, Tetrazolium salts

Öz.

Amaç: Hücre canlılığı ve/veya sitotoksosite analizleri in vitro çalışmalarda biyolojik değerlendirme için kullanılan en önemli göstergelerdendir. Özellikle kanser araştırmalarında, in vivo ve prelinik çalışmalara kaynaklık etmesi bakımından sitotoksosite testlerinin seçimi kritik öneme sahiptir. Bu çalışmada medikal kullanımı yaygın fitokimyasal bir ürün olan bromelain ve kanser tedavisinde kullanılan antrasiklin antibiyotik bir ilaç olan idarubisin'in MTT, WST-1 ve Luminesan ATP yöntemleriyle sitotoksik etkilerinin, normal lenfosit hücre kültürü ve HL-60 promyelositik lösemi hücre hattında araştırılması ve testler arasındaki ilişkinin incelenmesi amaçlandı.

Materyal ve Metod: Periferik kan lenfositleri sigara içmeyen, sağlıklı, gönüllü, genç erkek bireyden, HL-60 promyelositik lösemi hücre hattı ise ATCC firmasından sağlandı. Bromelain ve idarubisinin artan konsantrasyonları her iki hücre hattına eklendi. Hücreler, 24 saat 37 °C'de karbondioksit inkübatöründe inkübe edildi. İnkübasyon sonrası MTT, WST-1 ve ATP analizleriyle sitotoksosite düzeyleri araştırılırken, floresan boyama yöntemleriyle de morfolojik incelemeler yapıldı.

Bulgular: MTT ve WST-1 analizlerinde, bromelain konsantrasyon artışına paralel olarak hücre canlılığının/formazan oluşumunun arttığı, ATP testinde ise konsantrasyon artışıyla hücre canlılığının azaldığı bulundu. Floresan boyama yöntemleri ile ATP analiz sonuçları doğrulanırken, MTT ve WST-1 negatif ilişkiliydi. İdarubisin'in sitotoksik etkisinin her iki hücre hattında 3 ölçüm yöntemi ile benzer olduğu, floresan boyama yöntemleri ile de pozitif ilişkili olduğu bulundu

Sonuç: Bromelain'in lenfosit ve HL-60 hücre dizileri üzerinde MTT ve WST-1 analiz yöntemleri ile hücre canlılığı/sitotoksosite tespitinde bazı ölçüm sınırlamalarına neden olduğu bulunmuştur. Sitotoksosite çalışmalarında doğru ve güvenilir sonuçlar elde etmek için analiz edilecek fitokimyasalların interferanslara sebep olabileceği göz önüne alınarak yöntem seçimi dikkatlice yapılmalı ve başka metodlarla sonuçlar doğrulanmalıdır.

Anahtar kelimeler: Bromelain, İdarubisin, Sitotoksosite testleri, İnterferans, Tetrazolyum

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Introduction

Focused on the development of targeted drugs, the pharmaceutical industry frequently conducts treatment studies for cancer in both in vitro cell lines and in vivo models (1). The characteristics of an agent, which likely cause unknown therapeutic and toxic effects, are investigated by cytotoxic analyses, and the findings of these analyses are the preliminary data for future cancer models and clinical experiments. Therefore, the accuracy and reliability of data derived from cytotoxicity studies are extremely valuable (2). When cells are exposed to a cytotoxic agent, they can die due to apoptosis, autophagy, and necrosis, or they can lose their proliferative features due to cytostasis (3). Cell-based cytotoxicity studies provide general information about the cytostatic and cytotoxic effects of a tested agent (4). There are various tests that use staining, colorimetric, luminometric, and enzymatic methods to determine cell viability and proliferation (5-7). The basic principle of colorimetric methods is based on the spectrophotometric measurement of water-insoluble formazan crystals (purple) formed from the increased mitochondrial dehydrogenase activity of proliferated cells and tetrazolium (4). The tetrazolium ring can be cleaved by mitochondrial dehydrogenase and reductase, and thus, a color reaction can only be induced by living cells (8). No tinctumutation occurs in cells that have lost their viability (9). Tetrazolium salt-based assays (e.g., MTT, MTS, XTT, and WST-1), are the most commonly used tests in cancer research to evaluate cell proliferation and viability (10). The first generation of MTT compounds, which were developed by Mossman, can easily diffuse through the eukaryotic cell membrane and are reduced to formazan crystals. The amount of formazan, which is directly proportionate to the number of living cells, can be measured by optic density at an absorbance of 570 nm with a multi-plate reader. WST-1 and other test reagents are negatively charged and, thus, cannot diffuse through the cell membrane. These assays typically use an intermediate electrophile, which can transfer electrons from the cytoplasm or plasma membrane, to catalyze the reduction of tetrazolium to the colored formazan product (8, 11). When cytotoxicity analyses are performed using the MTT and WST-1 assays, the parameters (i.e., the medium pH, medium glucose, cell type, NAD⁺/NADH+H rate, presence of non-mitochondrial oxidoreductase and chemical structure of the substance to be investigated) affecting sensitivity and accuracy should be considered (10, 12). Phytochemicals, which contain phenolic compounds with high antioxidative and antimutagenic properties, continue to be relevant due to their potential to protect against life-threatening diseases (13, 14). Our aim in this study was to investigate the effects of bromelain and idarubicin on the cell viability of HL-60 cells and normal lymphocytes with the MTT, WST-1, and luminometric ATP assays and to discuss the conditions that cause interference by comparing these

tests.

Materials and Methods

The study was approved by the ethical committee of clinical research of the University of Gaziantep, and it was conducted in accordance with the Declaration of Helsinki. (Ethics Committee Decision Number: 2017/13, 25.01.2017). This study was conducted in the Department of Biochemistry, Medical Faculty, University of Gaziantep, Turkey between October 2016 and August 2018.

The Chemicals, Reagents and Test Samples Preparation

All chemical used for these experiments were of analytical grade. Idarubicin HCl (4-demethoxydaunorubicin) was purchased from Selleckchem (Houston, TX, USA). Bromelain and unless mentioned otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Titer-Glo™ Luminescent cell viability assay kit was purchased from Promega (Madison, WI, USA). WST-1 Cell Proliferation Assay Kit was purchased from INtRON Biotechnology, Inc. (Burlington, MA, USA). Primarily, bromelain was dissolved in phosphate buffer solution (PBS) to prepare stock solution (100 mg/mL). Bromelain final concentrations were 20, 10, 5, 2.5, 1, 0.5, 0.1 and 0.01 mg/mL. Idarubicin test solutions of different concentrations were prepared according to our previous experiments. Final concentrations of idarubicin solubilized in DMSO were 20, 10, 5, 2.5, 1, 0.5 and 0.1 µM. All of the samples diluted with RPMI 1640 medium. The final concentration of DMSO in the diluted samples was made kept <1%. Cell suspension without test solution was accepted as the control group.

Cell Culture and Experimental Design

The human peripheral blood mononuclear cells (PBMCs) was obtained from a young (33 years old), healthy, non-smoking volunteer. Healthy volunteers provided informed, written consent before participating. PBMCs separation processes and live cell count experiments were carried out as described previously (15). The HL-60 leukemia cancer cell line (ATCC® CCL-240™) was provided by ATCC (Manassas, VA, USA). Cultures with cell viability greater than 90-95% were included in the study (Using the trypan blue exclusion method). The HL-60 and PBMCs were placed separately on 96-well plates (~1.5-2.5X10⁴ cells in each well). After incubation for 24 h at 37 °C in an incubator containing 5% CO₂, 95% air in a humid atmosphere, the RPMI-1640 were discarded and the different concentrations of the test samples were incubated for 24 h. All experiments were replicated three times.

The MTT Assay

The cytotoxicity of test solutions was determined according to Mosmann's specifications (8) for the MTT assay with minor alterations (15). After 24 hours of exposure to the different concentrations of the idarubicin and bromelain, the medium was removed, and the cells were washed with

PBS. Following, MTT (100 µg/mL) was added to the wells and incubated at 37 °C for 4 hours. The insoluble formazan crystals that formed after the incubation were dissolved in DMSO, and to assess cell viability, absorbance at 570 nm were obtained using a multi-mode microplate reader (Bio-Tek® Synergy H1). The cell viability of the wells containing the test solutions at different concentrations was calculated by accepting 100% for cell viability according to the absorbance values of the wells containing only cell suspension.

The WST-1 Assay

The second cytotoxicity test, which determines the cytotoxic effects of test solutions, was evaluated with WST-1 kits (INiTRON Biotechnology, USA) according to the manufacturer's protocols. After 24 hours of exposure to the different concentrations of the idarubicin and bromelain, the medium was removed, and the cells were washed with PBS. WST-1 reagent was freshly prepared (100 µL Elektro Connecting Solution/1 mL WST-1 solution). WST-1 reagent of 10 µL was added to each well in the microplate and incubated for 3 hours. Following 3 h incubation, the measurements were taken on a plate reader (BioTek® Synergy H1) at wavelengths of 450 and 630 nm. Graphs were then created and the % cell viability of each test solutions was calculated.

The Luminescent Cell Viability Assay

The final assay to assess the cytotoxicity of test solutions in HL-60 and PBMCs was determined by The CellTiter-Glo Luminescent Cell Viability Assay. The basic principle of this method is to determine the number of living cells in the cell culture based on the amount of ATP that indicates the presence of metabolically active cells. In the presence of luciferase enzyme, ATP, Mg²⁺ and molecular oxygen, the density of luminescent signal that occurs during the oxidation of luciferin to oxyluciferin is parallel with the amount of ATP. After incubation for 24 h of exposure to the test solutions, microplate was kept at room temperature for 30 minutes. Following, the ATP reagent of 100 µL was added to all wells, and incubated on the orbital shaker for 2 minutes to induce cell lysis. The luminescence density was measured in the multiplate reader after 10 minutes at room temperature for stabilization of the luminescence signals. The cytotoxic effect of test solutions on the cells was calculated by comparison with the control group.

Morphological examination with fluorescent staining methods

Normal, apoptotic and necrotic cell morphologies were analyzed by Acridine orange / Ethidium bromide (AO/EB) and DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) fluorescence staining methods (16, 17). After the incubation period, nuclear morphology was assessed by fluorescence microscopy (Leica, DM IL LED, Wetzlar, Germany).

Statistical analysis

Statistical analyses and graph were performed using GraphPad Prism 8 software (San Diego, CA, USA) and

SPSS 16.0 statistic program (Chicago, IL, USA). The results are represented as a mean ± standard deviation. A *p* value <0.05 was considered statistically significant.

Results

In vitro cytotoxicity tests are often used to investigate the unknown toxic profile of agents. Although there are various cytotoxicity tests with different measurement methods and sensitivities used in the evaluation of mitochondrial activity, the most commonly used is the basic cytotoxicity test. In the present study, the cytotoxic effects of idarubicin and bromelain on HL-60 cells and PBMCs were investigated with the MTT, WST-1, and luminescence ATP assays.

The cytotoxic effects of different concentrations of idarubicin on HL-60 cell viability were the same for the three different cytotoxicity measurement methods. However, we did not record the same results for PBMCs. Instead, we determined that different concentrations of idarubicin have different cytotoxic potential on PBMCs viability. When the effect of bromelain was examined, two assays (i.e., the MTT and WST-1 assays) indicated that cell viability increased in both cell lines, especially in PBMCs, as the concentration increased. The observed PBMCs viability was higher from the MTT and WST-1 assays than from the ATP assay. The recorded cell viability from the ATP assay was inversely proportional to the bromelain concentration (Figure 1).

These contradictory results were also tested by fluorescent staining techniques (i.e., AO/EB and DAPI) (Figure 2a and 2b). Although the MTT and WST-1 assays indicated that cell viability increased as bromelain concentration increased, AO/EB and DAPI fluorescent staining results showed that 20 mg/mL bromelain, which was randomly selected, increased the rate of apoptosis and/or necrosis (i.e., the rate of cell death increased) (Figure 1). The results of the MTT and WST-1 assays, which were used to determine the cytotoxicity level of bromelain, negatively correlated with the results of AO/EB and DAPI fluorescent staining. In contrast, the results of the ATP assay, which was another cytotoxicity investigation method for bromelain, positively correlated with those of fluorescent staining. There was a linear relationship between AO/EB and DAPI and the results of the three assays investigating the cytotoxic effects of idarubicin.

The relationships between the MTT, WST-1, and ATP cytotoxicity assays were investigated by Spearman correlation (Figure 3), and a positive correlation was found among the three assays used to detect the cytotoxic effects of idarubicin (Figure 3a). In the cytotoxicity analysis of bromelain, the results of the MTT and WST-1 assays, which have similar measurement mechanisms, were positively correlated ($r=0.751$, $p<0.001$), which was consistent with the positive correlation between the results of the WST-1 and ATP assays ($r=0.026$, $p=0.862$); however, the results of the

ATP and MTT assays were negatively correlated ($r=-0.139, p=0.349$).

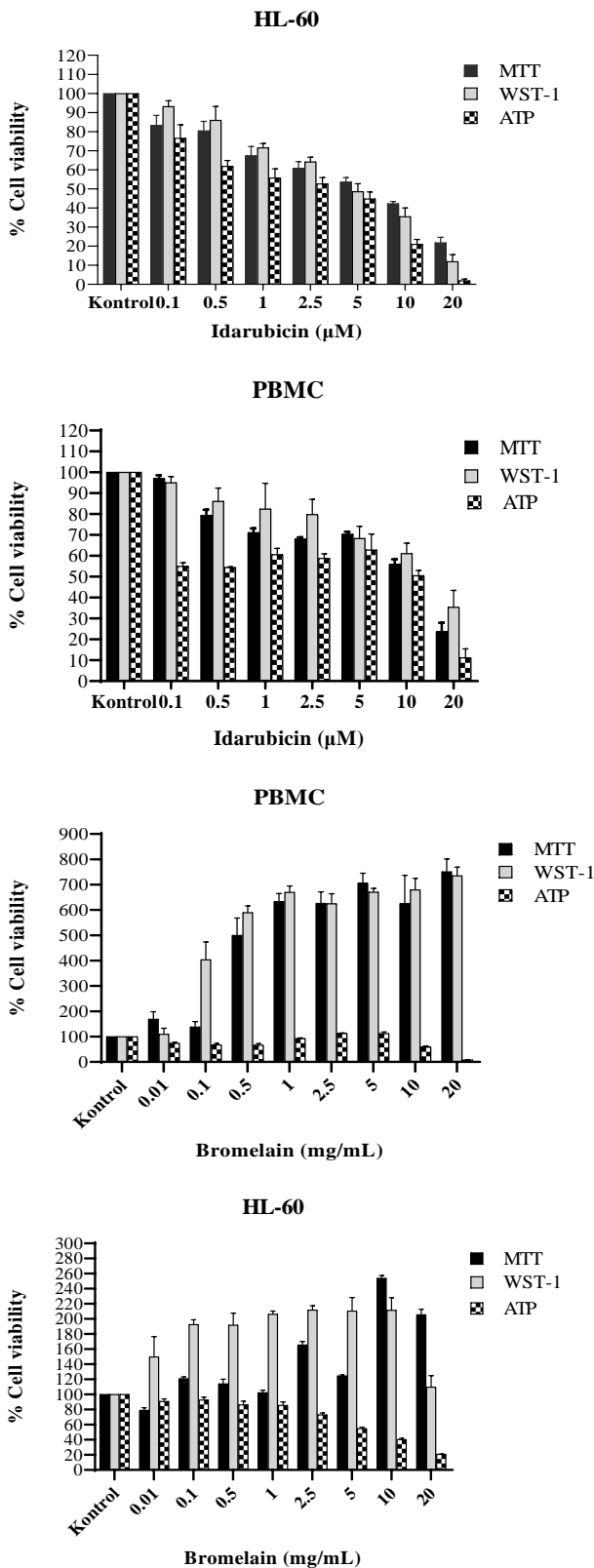


Figure 1. The effects of different concentrations of idarubicin and bromelain on cell viability in HL-60 cells and PBMCs as measured by the MTT, WST-1, and ATP cytotoxicity assays. Data represent three independent experiments.

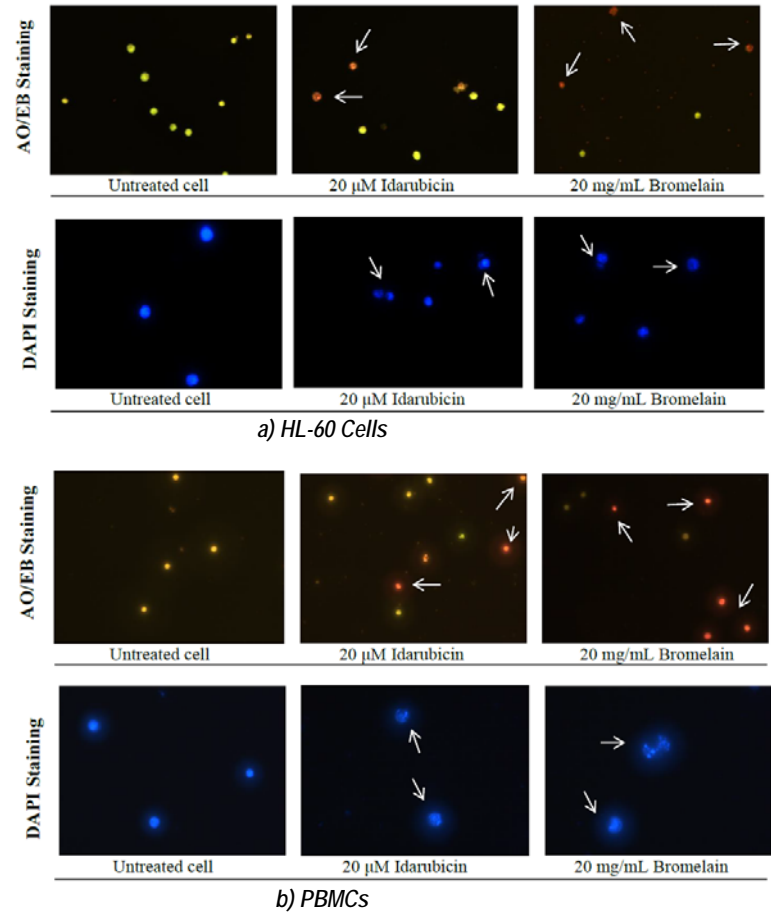


Figure 2. The view of normal, apoptotic, and necrotic HL-60 cells and PBMCs. AO/EB Staining: Untreated cells (normal cells) appear with evenly distributed circular nuclei at the center of the cell, while necrotic cells show irregular orange-red fluorescence and an opaque outline. DAPI staining: As a result of nuclear fragmentation and chromatin condensation, the fluorescence intensity of apoptotic cells was stronger than that of normal cells and not uniformly distributed. The magnification of these images was 40X.

Discussion

Cell viability is defined as the number of living cells in a biological sample, and it is a vital indicator for the understanding of the mechanisms of specific genes, proteins, and signal pathways and the determination of a cell's fate after exposure to a drug or a chemical agent. The effect of a chemical can be cytotoxic, which is defined as the inhibition of cell growth, toxicity, and cell death (18). *In vitro* cytotoxicity and/or cell viability analyses have emerged as an alternative for animal studies and have become more preferable due to their ease of application, speed, standardization, low cost, and compatibility with data from *in vivo* studies (19, 20). The basic principle of these analyses is based on various cellular functions, such as cell membrane permeability, enzyme activity, cell compatibility, ATP production, coenzyme production, and nucleotide uptake activity (21).

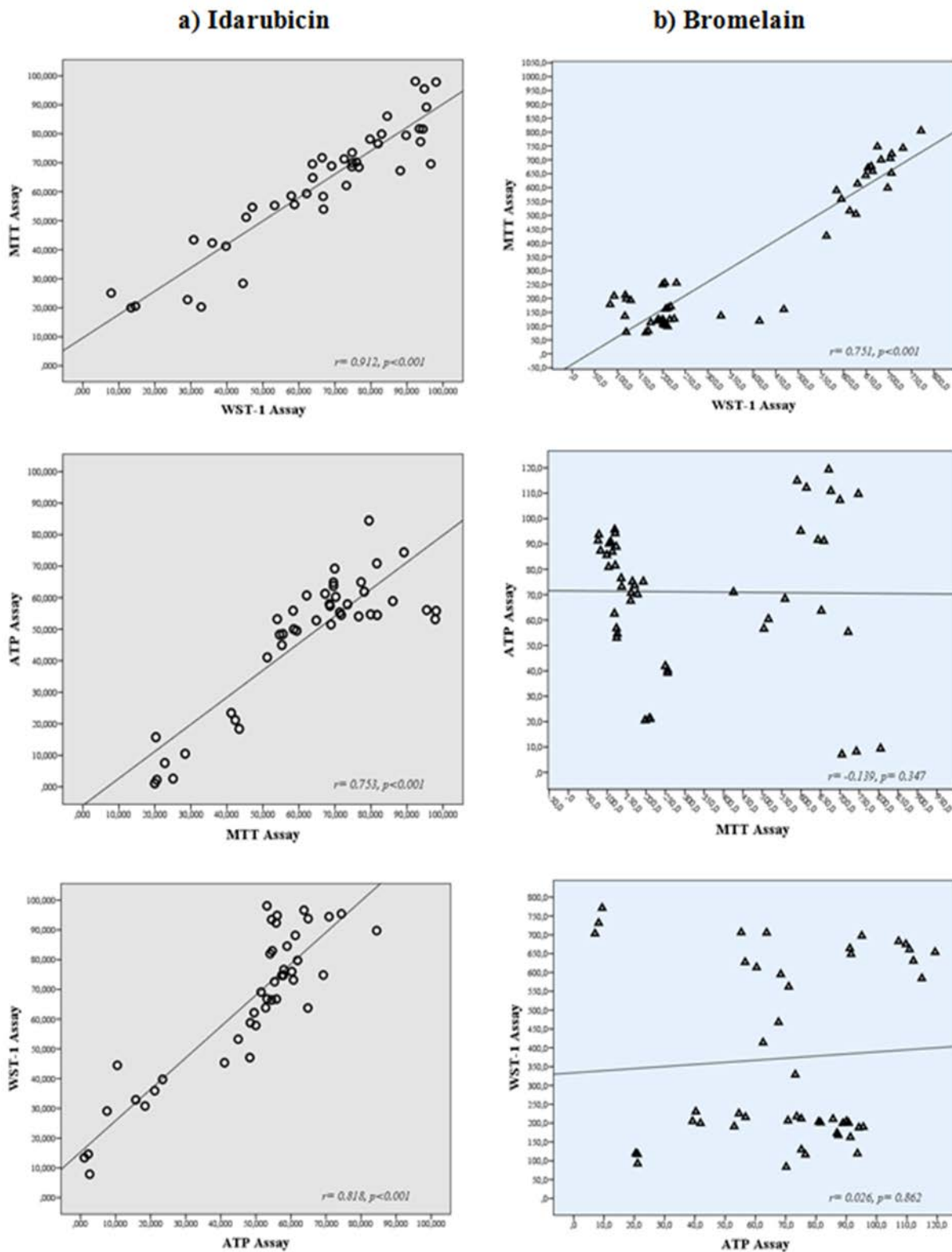


Figure 3. Correlation analyses among the MTT, WST-1, and ATP assays.

These analyses, which provide basic information about the behavior of an agent with unknown toxic effects, are widely used in the fields of toxicology and pharmacology, and their accuracy and reliability are extremely high and, therefore, provide reliable resources for future animal experiments and clinical trials (2, 22). Therefore, the selection of a suit

able method for an agent whose cytotoxicity will be analyzed is a critical stage for the success of studies (23, 24). Tetrazolium-based analyses (i.e., MTT, MTS, XTT, and WST-1 assays) that evaluate cytotoxicity via mitochondrial activity are widely used to measure cell proliferation, cell

viability, and drug cytotoxicity, especially in studies with cancer cells (10, 12). The MTT assay, which has been described as the gold standard for the detection of cell viability and proliferation since its development by Mossman in the 1980s, is the most commonly used among these tests (8, 25). However, interference, which causes false positive results, were detected in MTT assays, which have become popular due to their ease of application and low cost (25-28). The following main variations that cause interference may affect the absorbance readings at the end of the reaction: an interaction between the agents to be analyzed and tetrazolium dye (29), non-mitochondrial dehydrogenase activities (10), a reaction between phytochemicals and the MTT reagent even in the absence of cells (28), the property of cultured cells (cell adherence or suspension) (22), the pH of the reaction medium, and the cellular ion concentrations (30).

Having a net negative charge compared to MTT, the MTS, XTT, and WST-1 reagents are highly membrane-impermeable and, thus, require a main electron carrier (10). WST-1, which was developed as a new generation compound, is fast and ready to use compared to other equivalents and does not require an additional solution step for formazan crystals (31).

In cytotoxicity studies, the appropriate method should be selected by considering the mechanism of the agents to be tested to obtain accurate, reliable, and reproducible results (22, 23). Flavonoids and plant extracts, the cytotoxic effects of which have been especially investigated in cancer cell lines, have been shown to cause false positive results in tetrazolium-based tests (26, 28, 32, 33). In our study, bromelain, which is an extract of the pineapple plant, increased cell viability in a concentration-dependent manner in PBMCs and HL-60 cells according to the MTT and WST-1 assays. Interestingly, almost all bromelain concentrations increased cell viability in both cell lines compared to the untreated control groups. Conflicting bromelain results were derived between the results of the MTT and WST-1 assays and the results of the ATP assay, and cell viability decreased in a concentration-dependent manner in the ATP assay. The highest bromelain concentration-induced morphological changes stained by AO/EB and DAPI did not support the data from the MTT and WST-1 assays, but the results were compatible with the ATP assay results. In other words, although apoptotic and necrotic cell rates increased in both cell lines (i.e., the cell viability decreased) at the highest bromelain concentration according to the MTT and WST-1 assays, the rate of cell viability was highest. AO/EB and DAPI results confirmed the results of the ATP assay. When the results were combined, bromelain was considered to interfere with the MTT and WST-1 assays and, thus, led to false positive results. In addition, the agents to be analyzed and the metabolically inactive cells did not react with the tetrazolium salt and should not be

considered dead cells.

The luminescent ATP assay has been reported to be more sensitive than the MTT assay for the analysis of agents with cytotoxic effects (34, 35). However, although our study and recent studies detected interference that affected the results of the MTT and WST-1 assays, these tests remain reliable for some agents. A study performed by Ulukaya et al. indicated that the cytotoxic effects of doxorubicin, an analog of idarubicin, in lung cancer cells measured by MTT and ATP assays showed a significant positive correlation, (34) which is consistent with our results that showed a positive correlation between the cytotoxic effects of idarubicin obtained with the three different assays. When the relationships among the results of bromelain were examined, although the ATP and MTT assay results were negatively correlated, the ATP and WST-1 assay results trended towards a negative correlation. These tests, which should verify the results of each other, are required to be supported by another method.

Cytotoxic agents can damage and kill cells, in which case, a decrease in the reduction of MTT to formazan is expected (34). If these outcomes do not occur, the presence of interference should be considered. Whether a plant-derived agent, the cytotoxic effects of which were examined, reacted with tetrazolium salt was investigated in the medium without cells before the cell viability experiments, and that agent's reaction with tetrazolium indirectly affected the results of the experiment (36). The interference potential of new potential drugs or combinations should not be ignored before starting the evaluation of their cytotoxic effects in cell viability experiments, which will be used to evaluate cytotoxic effects.

To select the optimal cell viability analysis, the cost, speed, materials, cell type, culture conditions, and agents to be analyzed should be considered carefully. These data emphasize the need to use different methods to prevent misleading results in the determination of the effects of an agent with toxic potential on cell viability. We believe that the MTT and WST-1 assays can contribute to the experimental design of *in vitro* cell culture experiments to be performed in the future.

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Ethical approval:

The study was approved by the ethical committee of clinical research of the University of Gaziantep, and it was conducted in accordance with the Declaration of Helsinki. (Ethics Committee Decision Number: 2017/13, 25.01.2017).

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