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Anticancer Effects of Fenugreek and Cinnamon Extracts in Combination with Doxorubicin and Cisplatin on U87 Glioblastoma Cell Line

Çemen Otu ve Tarçın Ekstraktlarının Doksorubisin ve Sisplatin ile Kombinasyon Halinde U87 Glioblastoma Hücre Hattı Üzerinde Antikanser Etkileri

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ABSTRACT

Objective: Glioblastoma multiforme is one of the most aggressive brain tumors, with a low survival rate and limited treatment options. Moreover, resistance to chemotherapy complicates the treatment. Plant extracts are commonly used to overcome drug resistance and support currently used chemotherapies. This study aimed to determine the anticancer effect of fenugreek and cinnamon extracts in combination with doxorubicin and cisplatin on U87 glioblastoma cells.

Methods: Fenugreek and cinnamon extracts were donated by Indus Biotech (India). The 50% viability of fenugreek, cinnamon, doxorubicin, and cisplatin was determined by MTT assay. The combination of plant extracts and drugs was also applied to U87 cells. Apoptosis and cell cycle analysis were performed by flow cytometry.

Results: IC50 concentrations for 24h were found to be 224.8 µg/mL for fenugreek, 3269 µg/mL for cinnamon, 3.2 µM for doxorubicin, and 770 µM for cisplatin, respectively. Fenugreek, doxorubicin, and fenugreek-cisplatin groups were found to be more cytotoxic than cinnamon-doxorubicin and cinnamon-cisplatin groups. The treatment of plant extracts and drugs seems to shift the cell cycle distribution, particularly increasing the G0/G1 phase.

Conclusion: These results are thought to be meaningful in understanding the potential effectiveness of the combination of fenugreek and cinnamon with traditional chemotherapeutic agents in cancer treatment.

Keywords: Fenugreek, cinnamon, cytotoxicity, glioblastoma, anti-cancer

ÖZ

Amaç: Glioblastoma multiforme, düşük hayatta kalma oranı ve sınırlı tedavi seçenekleriyle en agresif beyin tümörlerinden biridir. Ayrıca kemoterapiye direnç tedaviyi zorlaştırmaktadır. İlaç direncinin aşılması ve halihazırda kullanılan kemoterapilerin desteklenmesi amacıyla bitki ekstraktları oldukça yaygın olarak kullanılmaktadır. Bu çalışmada, çemen otu ve tarçın ekstraktlarının doksorubisin ve sisplatin ile kombinasyon halinde U87 glioblastoma hücreleri üzerindeki antikanser etkilerinin belirlenmesi amaçlandı.

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Yöntem: Çemen otu ve tarçın ekstraktları Indus Biotech (Hindistan) tarafından bağışlanmıştır. Çemen otu, tarçın, doksorubisin ve sisplatinin %50 canlılık oranı MTT yöntemi ile belirlendi. Bitki ekstraktları ve ilaç kombinasyonu da U87 hücrelerine uygulandı. Apoptoz ve hücre döngüsü analizi akış sitometrisi ile yapıldı.

Bulgular: Çemen otu, tarçın, doksorubisin ve sisplatinin 24 saat için sırasıyla 224,8 µg/mL, 3269 µg/mL, 3,2 µM ve 770 µM konsantrasyonları IC50 olarak bulunmuştur. Çemen otu, doksorubisin ve çemen otu-sisplatin gruplarının sırasıyla tarçın-doksorubisin ve tarçın-sisplatin gruplarına göre daha sitotoksik olduğu gözlenmiştir. Uygulanan bitki ekstaktları ve ilaçların hücre döngüsü dağılımını değiştirdiği, özellikle G0/G1 fazını artırdığı belirlendi.

Sonuç: Bu sonuçların, çemen otu ve tarçının geleneksel kemoterapötik ajanlarla kombinasyonunun kanser tedavisindeki potansiyel etkinliğini anlamak açısından anlamlı olabileceği düşünülmektedir.

Anahtar Kelimeler: Fenugreek, cinnamon, sitotoksisite, glioblastoma, anti-kanser

INTRODUCTION

Glioblastomas are among the most aggressive forms of brain cancer, and because of their high permeability and molecular heterogeneity, patient survival rates are quite low.[1] Chemotherapy and radiotherapy are among the most popular treatment options. It is important to find new treatment agents due to the side effects and damage to healthy cells caused by existing treatments. Recently, research has been shifting to medicinal plants and phytochemicals consisting of natural ingredients.[2]

Herbal sources present effects on cancer pathways due to their antitumor potential and are evaluated as safe therapeutic helpers.[3] It has been stated that phenolics (including apigenin, curcumin, and resveratrol), terpenoids (betulinic acid, cucurbitacin), and steroids (diosgenin, ergosterol) from plants may be useful in the treatment and prevention of cancer.[4]

Trigonella foenum graecum, also known as fenugreek, is a beneficial and traditional plant used as an adjuvant for cancer patients. Fenugreek extract was found to target metastasis, angiogenesis, invasion, inflammation, and proliferation of the cancer cells.[5,6] Diosgenin is a steroid that is present in fenugreek and potatoes, among other plants. In glioblastoma, it increases deoxyribonucleic acid damage, reactive oxygen species (ROS) production, and apoptosis.[7] Moreover, it inhibits the cell cycle at the G0/G1 phase.[8] However, genistein, a type of flavonoid present in fenugreek, has been shown to have anticancer properties against glioblastomas.[9]

Cinnamon is a unique herbal medicine with several biological properties that attract scientists working on diabetes, inflammatory diseases, microbial diseases, and oxidative stress.[10] Moreover, anti-carcinogenic effects of cinnamon have been shown in several types of cancers.[11-13] The anti-proliferative activity of cinnamon was shown in hematological cancer cell lines with a cytotoxic dose between 0.05 and 0.2 mg/mL.[14] In another study, a concentration of 80 µg/mL cinnamon decreased the growth of cervical cancer cells.[15] Cinnamon's in different cell lines are believed to be due to the distinct structures of the cells.

The main goal of this study was to find out how fenugreek and cinnamon extracts, along with anticancer drugs, doxorubicin, and cisplatin, affected glioblastoma cells by causing apoptosis and cell cycle arrest. In particular, we wanted to find out if these herbal extracts could improve the therapeutic effects of these chemotherapeutic agents by changing important cellular processes controlling the cell cycle and inducing apoptosis in tumor cells.

METHODS

Chemicals and Drugs

Fenugreek and cinnamon extract were kindly requested from Indus Biotechnology (India). Extracts were dissolved in Dulbecco's modified Eagle medium (DMEM) prior to use. We purchased doxorubicin and cisplatin from Cayman Chemical Company (USA) and dissolved them in dimethyl sulfoxide following the instructions provided.

Cell Culture

Experiments were performed on the glioblastoma U87 cell line from the American Type Culture Collection (ATCC® HTB-14). Cells were cultured in DMEM (Gibco, Waltham, MA, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), 100 U/mL penicillin, and 100 g/mL streptomycin (Sigma-Aldrich), and 10% heat-inactivated fetal bovine serum (Serox GmbH, Mannheim, Germany). Cells were incubated in 5% CO_2 atmosphere at 37 °C. Cells were passaged during the experiments at 80% confluency.

Assessment of Cell Viability

U87 cells were seeded at a density of 6 x 104 cells/well in 96-well plates by using trypan blue cell counting method on Neubauer counting chamber and incubated for 24 h. Cinnamon and fenugreek extracts were prepared at six different concentrations, such as 31.25, 62.5, 125, 250, 500, 1000 µg/mL for cinnamon and 312.5, 625, 1250, 2500, 5000, 7500 µg/mL for fenugreek respectively. Doxorubicin and cisplatin were prepared at different concentrations: 1.25,

2.5, 5, 10, 20 µM for doxorubicin, 1.25, 2.5, 5, 10, 20 µM 37.5, 75, 150, 300, 600, 1200 µM for cisplatin, respectively. The cells were exposed to different concentrations of fenugreek, cinnamon, doxorubicin, and cisplatin for 24, 48, and 72 h. 50% cell viability (IC50) was determined by 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) (Glentham Life Sciences, UK), and optic densities were measured at 570 nm using a multiplate reader (ThermoScientific Multiscan Go, USA).

To calculate the IC50 values, We expressed the data as the percentage of cell viability relative to untreated controls. A dose-response curve was constructed by plotting the concentration of each compound on the x-axis and the percentage of cell viability on the y-axis. The IC50 values were determined using nonlinear regression analysis (e.g., GraphPad Prism software or similar), where the concentration of the drug or extract that reduced cell viability by 50% was calculated. Each experiment was performed in triplicate, and the results were presented as the mean ± standard deviation.

The Combinational Effect of Fenugreek and Cinnamon with Doxorubicin and Cisplatin on Cell Viability

The IC50 values of fenugreek, cinnamon, doxorubicin, and cisplatin were determined using the MTT method at 24, 48, and 72 h. To determine the combined effect of the plant extracts and drugs, 24-hour IC50 levels were applied onto cells. Briefly, 6 x 104 cells/well were seeded in 96-well plates, and after 24 h incubation: IC50 doses of fenugreek, cinnamon, doxorubicin, cisplatin, fenugreek + doxorubicin, fenugreek + cisplatin, cinnamon + doxorubicin, and cinnamon + cisplatin were applied onto cells for 24 h. MTT assay was performed as explained above.

Annexin V-Fluorescein Isothiocyanate/Propidium Iodide Staining Assay

To determine apoptosis in U87 cells after plant extract and drug treatment, flow cytometric analysis was performed using annexin V, fluorescein isothiocyanate (FITC)/ propidium iodide (PI) dual staining. In brief, U87 cells were seeded at a density of 1.8 × 10^6 cells per well in a 6-well plate and incubated for 24 hours. Then, the cells are exposed to plant extracts and drugs at their IC50 concentrations at 24 hours. The combinations of plant extracts and drugs were also applied to the cells. The cells were harvested after 24 h and washed with PBS three times. 5µL of Annexin V/FITC (Elabscience) and 5µL of PI (Invitrogen) were added onto cells and incubated for 15-20 minutes at room temperature. Finally, the Beckman Coulter DxFLEX was used to detect apoptotic cell percentage.[16]

Cell Cycle Assay

Modifications in the phases of the cell cycle were assessed by flow cytometry. The cells were treated with the plant extracts and drugs, as previously described. Afterwards, cells were harvested and washed with PBS three times. PI in powder form was diluted with deionized water to 1 mg/mL (1.5 mM). Dilute solution was prepared from the resulting stock solution. To prepare the dilution solution, the stock solution was diluted 1:500 with staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet P-40). Cold ethanol was added to cells and incubated for 15 minutes at -20 °C. The cells were centrifuged, and the supernatant was discarded. 5 mL PBS was added onto cells and incubated for 15 minutes. After centrifugation, staining buffer was added onto cells and the cells were analyzed.

Statistical Analysis

The experiments were run in triplicate. One-way analysis of variance was used to test the data, and the samples were compared to their reference controls (GraphPad Prism Software v. 9.01). At $p<0.05$, the results were deemed statistically significant.

RESULTS

Determination of Cell Viability Using 3-(4.5-Dimethylthiazol-2-yl)-2.5-Diphenyltetrazolium Bromide Assay

Fenugreek, cinnamon, doxorubicin, cisplatin were applied to the U87 cells for 24, 48, and 72 hours, cell viability was determined using the MTT method. The concentrations found as IC50 for 24 hours were 224.8 µg/mL for fenugreek, 3269 µg/mL for cinnamon, 3.2 µM for doxorubicin, and 770 µM for cisplatin, respectively (Figure 1).

Combinational Effect of Fenugreek, Cinnamon with Doxorubicin and Cisplatin were Determined

Combinatorial effects of fenugreek and cinnamon with doxorubicin and cisplatin were detected for 24 hours. Concentrations that result in 50% cell viability were applied to cells (3269 µg/mL fenugreek, 3269 µg/mL fenugreek and 3.2 µM doxorubicin, 3269 µg/mL fenugreek and 770 µM cisplatin, 224.8 µg/mL cinnamon, 224.8 µg/mL cinnamon and 3.2 µM doxorubicin, 224.8 µg/mL cinnamon and 770 µM cisplatin). When we compared the combination groups including doxorubicin, we detected that fenugreek-doxorubicin was more cytotoxic than cinnamon-doxorubicin (mean % cell viability was 18.7 and 29.8, respectively). Moreover, fenugreek-cisplatin combination was evaluated more cytotoxic than cinnamon-cisplatin group (36.4% and 38,633%, respectively) (Figure 2).

Flow Cytometric Analysis for Biochemical Feature of Apoptosis

The biochemical features of apoptosis at IC50 doses were examined by flow cytometry. 10,000 cells were analyzed for each group, and the results were compared with the control group. The Q1-LL part shows the percentage of living cells, Q1-LR shows the percentage of cells in early apoptosis, Q1-UR shows the percentage of cells in late apoptosis, and Q1-UL shows the percentage of necrotic cells. Each group showed an increase in apoptotic cells compared to the control group (Figure 3). In the fenugreek-treated group, 9% of the cells were in early apoptosis and 4% were in late apoptosis, for a total of 13%. In the cinnamon-treated group, 10% of the cells were early apoptosis and 28% were late apoptosis; 38% in total. In the cisplatin group, 48% of the cells undergo early apoptosis, 4% undergo late apoptosis, and 52% undergo apoptosis in total. 2% are late apoptosis, 97% are necrotic in the doxorubicin group, and no cells were detected in the early apoptosis group. When we evaluated the combination groups, the most apoptotic group was identified as the fenugreek-cisplatin group (45% in total). The fenugreek-doxorubicin group had 28% late apoptotic cells and 70% necrotic cells, while the cinnamon-doxorubicin group had 12% late apoptotic cells and 71% necrotic cells. In total, fenugreek-doxorubicin groups had 28% apoptotic cells, while cinnamon-doxorubicin groups had 12%. Cinnamon-cisplatin combination seemed to mainly bring cells to necrosis (64%), while 5% of necrotic cells were detected in the fenugreek-cisplatin group. Interestingly, fenugreek-cisplatin combination mainly brings cells to early apoptosis (34%); cinnamon-cisplatin leads to late apoptosis (19%). Statistical differences between the control and drug-treated groups were presented in Supplementary Table 1. According to the results, live cell percentages decreased statistically significantly in all groups compared to the control group. cinnamon groups were statistically significant in early apoptosis, late apoptosis, and necrosis cell percentages in the fenugreek-cisplatin and cinnamon groups compared to the control group.

Cell Cycle Analysis by Using Propidium Iodide

The effect of fenugreek, cinnamon, doxorubicin, and cisplatin on the cell cycle was detected after PI staining by flow cytometry. Ten thousand cells were evaluated for each

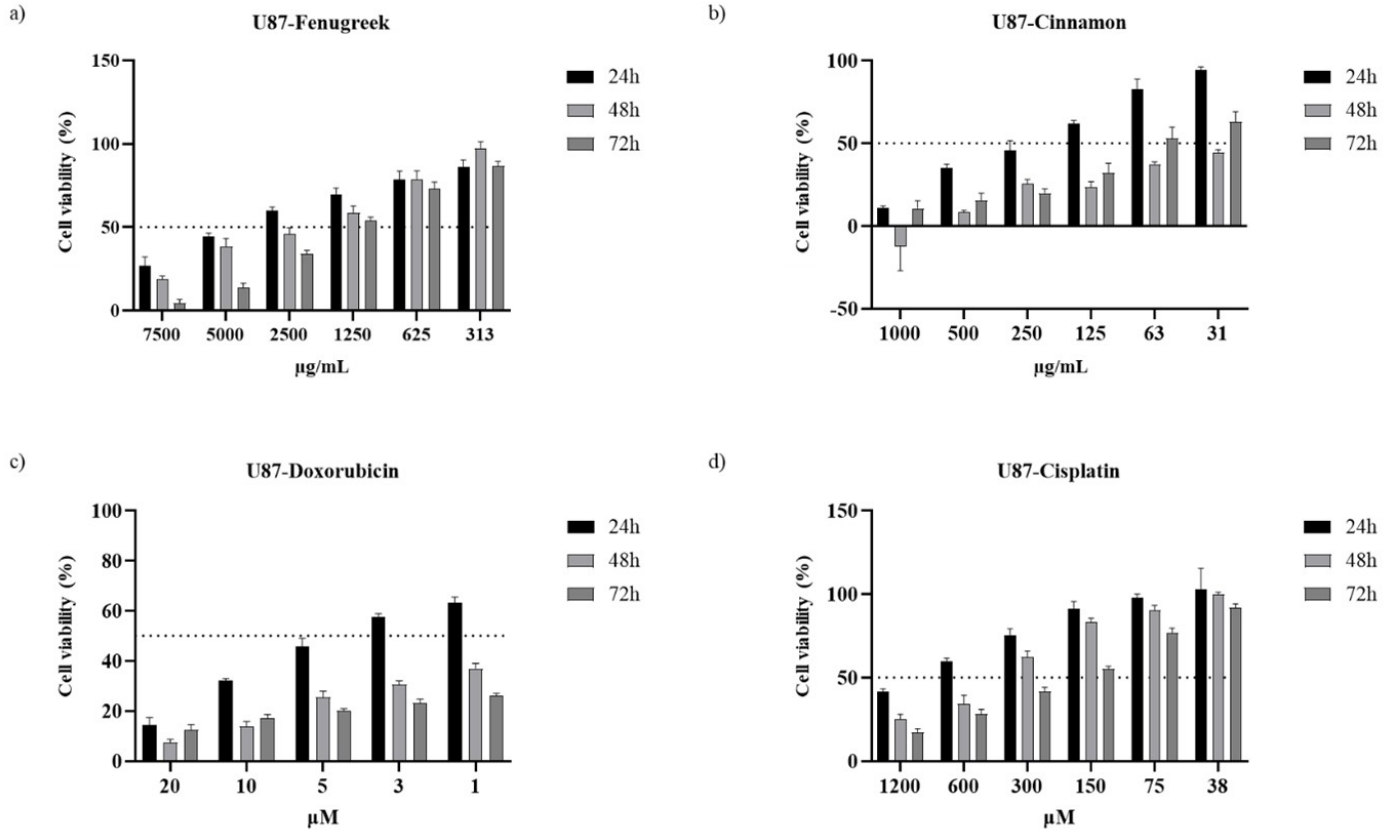

Figure 1. Cell viability results of the a) fenugreek b) cinnamon c) doxorubicin d) cisplatin due to 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide assay results

treatment group. Cell rates in sub-G1, G0/G1, S and G2-M phases were determined as percentage values (Figure 4).

Fenugreek was evaluated to have a significant portion in G0/G1 (36%) and G2 (61%). Similar to fenugreek, cinnamon was higher in the G0/G1 phase (56%), and lower in the G2 phase (40%). Doxorubicin and cisplatin 51% and 62%, respectively. The combinational experiments showed that fenugreek-doxorubicin arrests cell cycle in G0/G1 phase dominantly while cinnamon-doxorubicin still has higher cell percentages in G2 phase (46%). When we evaluated the effect of cisplatin, the G2 phase is prominent (39%) while cinnamon-cisplatin have more cells in the G0/G1 phase (57%).

DISCUSSION

The effects of fenugreek and cinnamon on glioblastoma cells have garnered attention due to their potential apoptotic and cell cycle-modulating properties. Varjas et al.[17] indicated that these natural compounds can significantly impact glioblastoma cell viability and induce apoptosis. Researchers have explored the protective effects of fenugreek (trigonella foenum-graecum) on glioblastoma cells through its bioactive compounds, which exhibit anticarcinogenic properties. Research has revealed that fenugreek can inhibit cell proliferation and modulate gene expression associated with tumorigenesis. Fenugreek contains compounds like protodioscin and diosgenin, which have shown potential in inhibiting the proliferation of cancer cells.[17] In studies involving mice, fenugreek consumption was associated with a protective effect against the expression of arachidonic acid metabolizing enzymes, which are linked to tumor growth. The inhibition of prostaglandin synthesis by fenugreek may contribute to its protective effects against tumorigenesis, suggesting a potential mechanism for its action against glioblastoma.[17] While specific studies on fenugreek's direct effects on glioblastoma cells are limited, its general antitumor properties indicate a promising avenue for further research. In contrast, other compounds have been more extensively studied for their direct antiproliferative effects on glioblastoma cells, highlighting the need for more focused research on fenugreek in this context.[18,19]

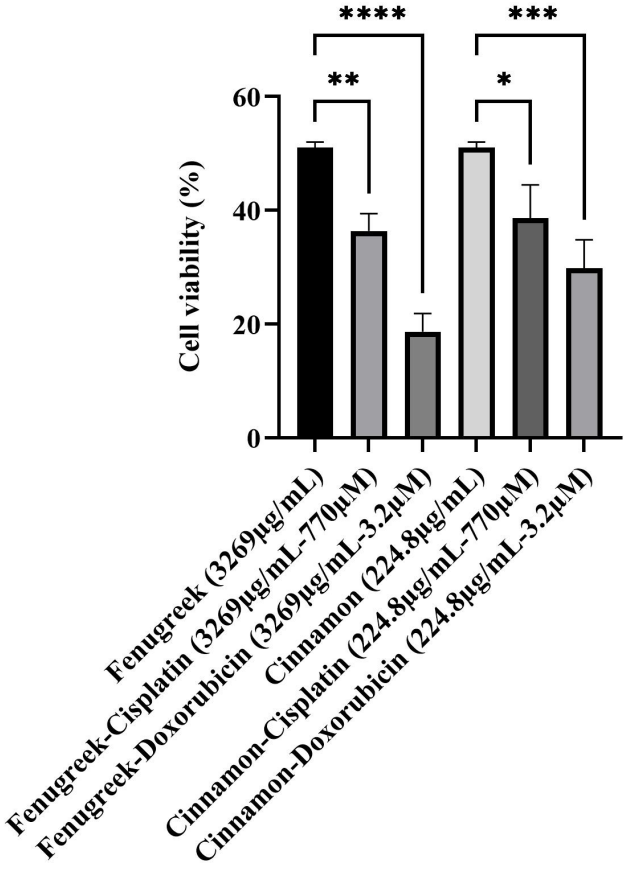

Figure 2. Combinational effect of fenugreek, cinnamon, doxorubicin and cisplatin on cell viability. *p<0.05, **p<0.005, ***p=0.0001, ****p<0.0001

Fenugreek has been shown to trigger programmed cell death in glioblastoma cells, potentially through the modulation of signaling pathways involved in cell survival.[20] When combined with standard chemotherapeutics like temozolomide, fenugreek may enhance the overall therapeutic effect, overcoming some resistance mechanisms.[21]

Cinnamon, specifically its active compound cinnamaldehyde, exhibits promising effects against glioblastoma multiforme (GBM) by inducing apoptosis and disrupting metabolic pathways. According to research, cinnamaldehyde raises the levels of ROS, and causes GBM cell lines like U87 and U251 to undergo cell death.[22] Cinnamon extracts have also shown strong anticancer properties, which lower the number of living cells and the level of protein expressed in U87 cells. Cinnamon phytochemicals' suggests their potential integration into existing GBM therapies, potentially enhancing efficacy with minimal side effects.[23] Cinnamon contains compounds that can reduce inflammation, a crucial factor in glioblastoma progression. This may help in mitigating tumor growth and enhancing patient outcomes.[24] Cinnamon has been reported to influence key signaling pathways that are often deregulated in glioblastoma, potentially restoring normal cellular functions and inhibiting tumor invasion.[25]

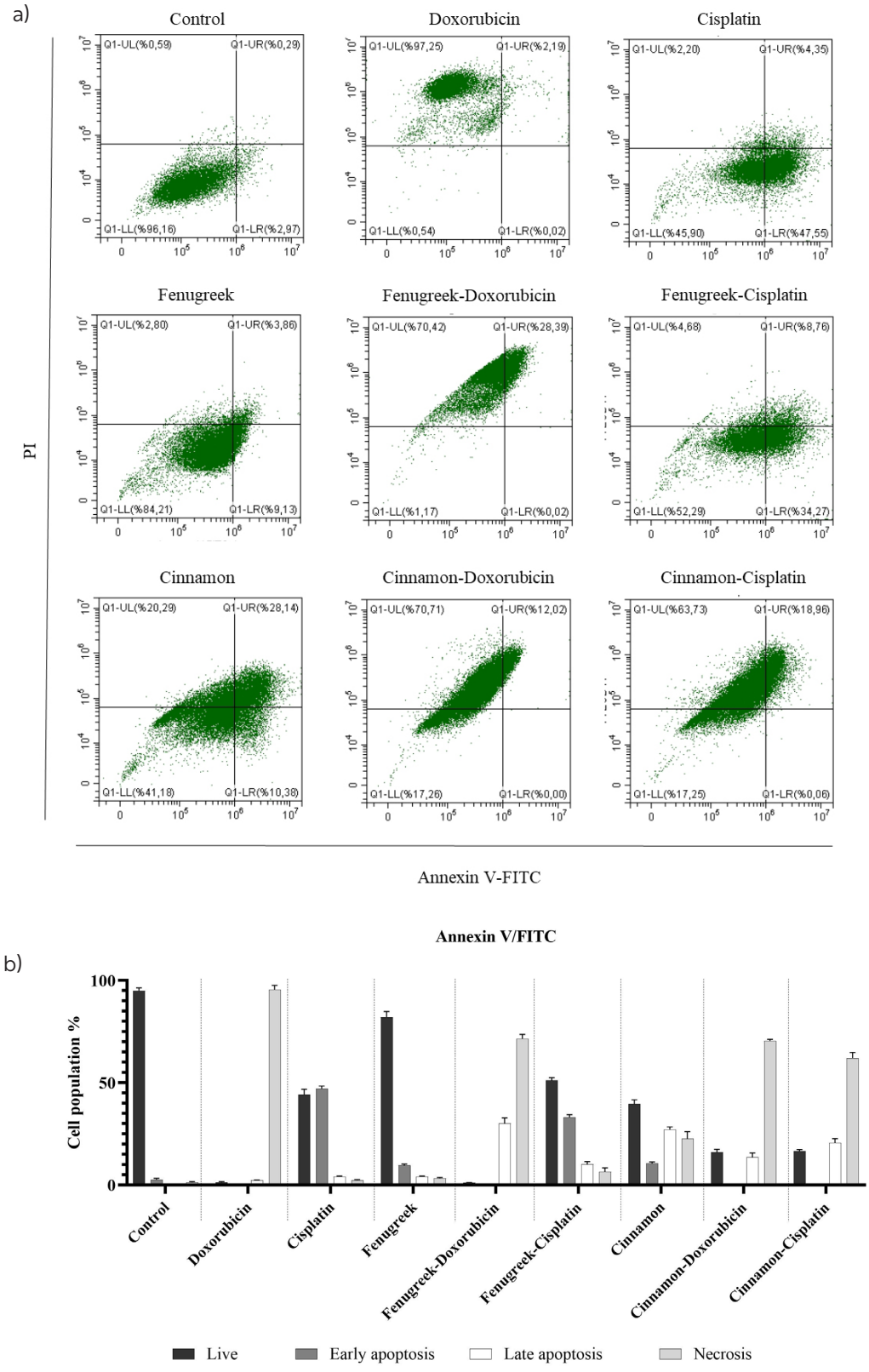

Figure 3. a) Flow cytometry plots of Annexin V-fluorescein isothiocyanate/propidium iodide staining. b) Bar graph showing percentages of live cells (both Annexin-V and propidium iodide negative), early apoptotic cells (Annexin-V positive, propidium iodide negative), late-apoptotic cells (Annexin-V positive, propidium iodide positive) and necrotic cells (Annexin V negative, propidium iodide positive)

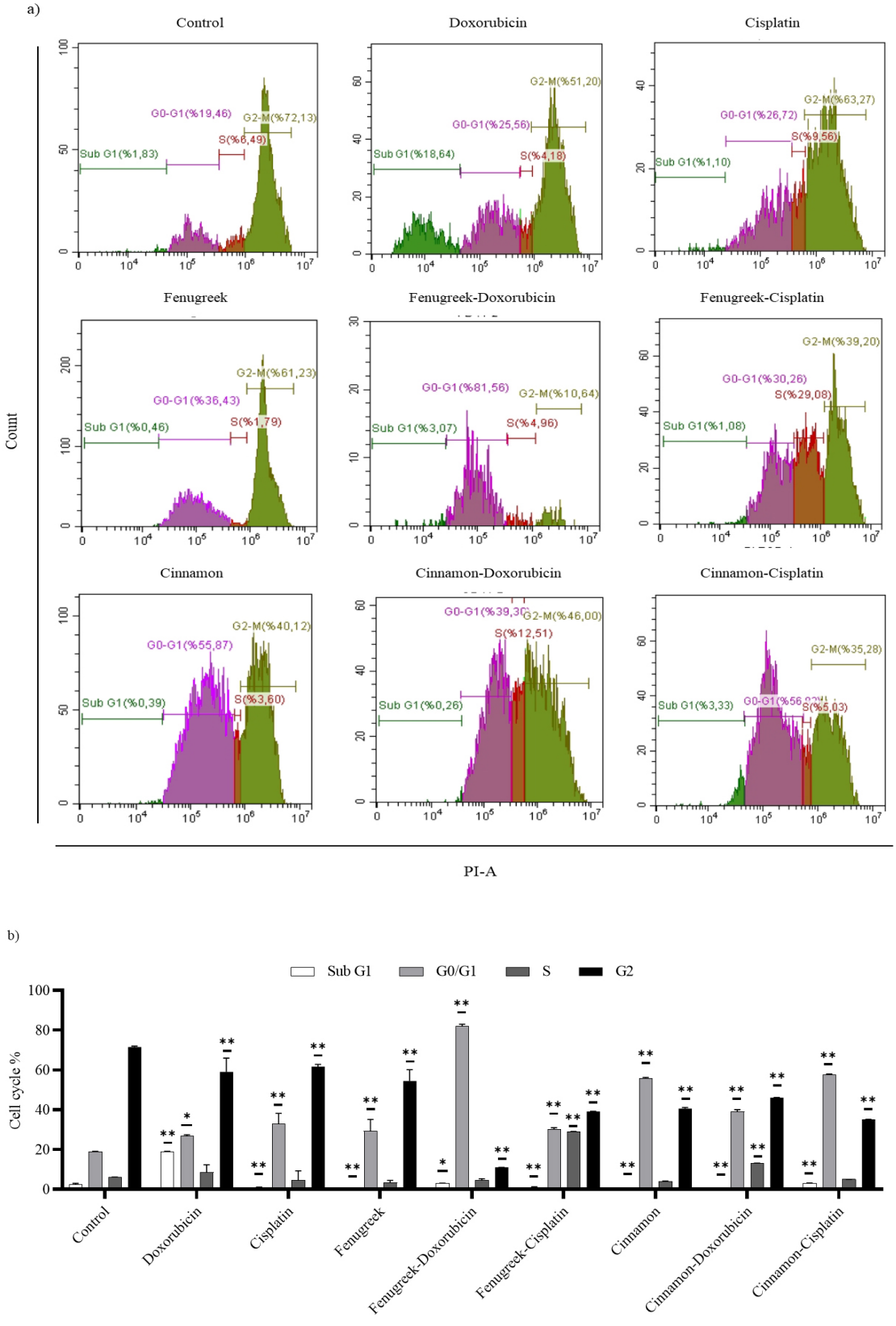

Figure 4. Cell cycle analysis of U87 cells after treatment with fenugreek, cinnamon, doxorubicin, cisplatin and combinations for 24 h. a) Plots of cell cycle phases. b) Data are presented as the means ± standard error of triplicate experiments and compared to the control. *p<0.05, and **p<0.01

In this study, it was observed that the fenugreek-treated group exhibited less cytotoxicity compared to the cinnamon-treated group, which showed a significant decrease in cell viability. Conversely, both doxorubicin and the combination treatments (fenugreek-doxorubicin, and cinnamon-doxorubicin) resulted in very low cell viability. Doxorubicin demonstrated high cytotoxicity, primarily resulting in necrosis, while fenugreek appeared to have a protective effect in comparison to the others. Treatments combining cinnamon and cisplatin induced significant apoptosis, particularly with cisplatin. These results suggest that while some treatments effectively induce cell death (such as doxorubicin and cinnamon), others may offer protective benefits (like fenugreek). However, due to its cytotoxic effects, further analysis of fenugreek's impact on cancer pathways is necessary.

When we compared cell cycle arrest results of the extracts and the drugs fenugreek induces G0/G1 phase arrest and promotes apoptosis, while cinnamon causes G0/G1 phase arrest and limits cell proliferation. Cisplatin and doxorubicin both induce G2/M phase arrest and promote apoptosis. These effects highlight the potential of using these substances, either individually or in combination, as therapeutic strategies for managing cancer, including glioblastoma. Fenugreek and cinnamon may serve as complementary agents to conventional chemotherapies like cisplatin and doxorubicin, enhancing their effectiveness while potentially mitigating side effects.

Combining fenugreek and cinnamon with conventional chemotherapeutic agents (like doxorubicin and cisplatin) has shown promise in enhancing the overall therapeutic effects, suggesting a potential role as an adjunct treatment.[23,26,27] However, more research is needed to validate these findings and understand the mechanisms involved.

Study Limitations

Despite the conduct of apoptosis and cell cycle research, the fundamental molecular mechanisms of action for the plant extracts and drug combination remain ambiguous and necessitate additional inquiry.

CONCLUSION

Fenugreek and cinnamon are generally considered safe when consumed in food quantities. However, their effects at higher doses and in isolated extract forms require further investigation to assess potential toxicity.

Ethics

Ethics Committee Approval and Informed Consent: Since the study is a cell culture study, ethical approval and informed consent are not required.

Footnotes

Authorship Contributions

Concept: B.Ç.A., Design: B.Ç.A., Data Collection or Processing: B.Ç.A., T.K.A., Analysis or Interpretation: B.Ç.A., T.K.A., M.P., M.S., Literature Search: B.Ç.A., M.P., M.S., Writing: B.Ç.A., M.S., İ.P.

Conflict of Interest: No conflict of interest was declared by the authors.

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Supplementary Table 1. Statistical differences between control and drug treated groups		
	95.00% CI	**Adjusted p value**
Live		
Control vs. doxorubicin	89.39 to 98.11	<0.0001
Control vs. cisplatin	46.64 to 55.36	<0.0001
Control vs. fenugreek	8.640 to 17.36	<0.0001
Control vs. fenugreek-doxorubicin	89.54 to 98.26	<0.0001
Control vs. fenugreek-cisplatin	39.64 to 48.36	<0.0001
Control vs. cinnamon	51.14 to 59.86	<0.0001
Control vs. cinnamon-doxorubicin	74.64 to 83.36	<0.0001
Control vs. cinnamon-cisplatin	74.14 to 82.86	<0.0001
Early apoptosis		
Control vs. doxorubicin	-1,810 to 6,910	0.4609
Control vs. cisplatin	-48.81 to -40.09	<0.0001
Control vs. fenugreek	-11.31 to -2,590	0.0006
Control vs. fenugreek-doxorubicin	-1,810 to 6,910	0.4609
Control vs. fenugreek-cisplatin	-34.81 to -26.09	<0.0001
Control vs. cinnamon	-12.31 to -3,590	<0.0001
Control vs. cinnamon-doxorubicin	-1,810 to 6,910	0.4609
Control vs. cinnamon-cisplatin	-1,810 to 6,910	0.4609
Late apoptosis		
Control vs. doxorubicin	-6,560 to 2,160	0.6167
Control vs. cisplatin	-8,510 to 0.2099	0.068
Control vs. fenugreek	-8,510 to 0.2099	0.068
Control vs. fenugreek-doxorubicin	-34.36 to -25.64	<0.0001
Control vs. fenugreek-cisplatin	-14.36 to -5,640	<0.0001
Control vs. cinnamon	-31.36 to -22.64	<0.0001
Control vs. cinnamon-doxorubicin	-17.86 to -9,140	<0.0001
Control vs. cinnamon-cisplatin	-24.86 to -16.14	<0.0001
Necrosis		
Control vs. doxorubicin	-98.61 to -89.89	<0.0001
Control vs. cisplatin	-5,360 to 3,360	0.9891
Control vs. fenugreek	-6,360 to 2,360	0.7082
Control vs. fenugreek-doxorubicin	-74.61 to -65.89	<0.0001
Control vs. fenugreek-cisplatin	-9,510 to -0.7901	0.0144
Control vs. cinnamon	-25.61 to -16.89	<0.0001
Control vs. cinnamon-doxorubicin	-73.61 to -64.89	<0.0001
Control vs. cinnamon-cisplatin	-65.11 to -56.39	<0.0001
CI: Confidence interval		