

Fullpaper Oral Presentations

► Fullpaper Oral Presentation 2

Synergistic Anticancer Effects of *Cannabis sativa* L. (Narlı variety) Seed Oil Combined with Doxorubicin through Modulation of Apoptosis in SH-SY5Y Neuroblastoma Cells

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ABSTRACT

This study aimed to investigate the synergistic and additive anticancer effects of *Cannabis sativa* L. (Narlı variety) seed oil (CSSO) in combination with doxorubicin (DOX) on SH-SY5Y human neuroblastoma cells, with a particular emphasis on apoptosis.

Cell viability was evaluated using the MTT assay to determine the IC₅₀ values of CSSO and DOX. Drug combination effects were analyzed using the SynergyFinder platform, and the most favorable synergistic combination was evaluated using reverse transcription-quantitative PCR (RT-qPCR) to assess the expression of apoptosis-related genes.

SH-SY5Y cells were treated with CSSO (1–800 µg/mL) and DOX (1–50 µg/mL) for 24 h. The IC₅₀ values were determined to be 96.84 µg/mL for CSSO and 1.84 µg/mL for DOX, respectively. SynergyFinder analysis revealed synergistic interactions at various concentrations. Among all tested combinations, 0.92 µg/mL DOX in combination with 48.42 µg/mL CSSO showed the most favorable synergistic interaction (CI = 0.88) and was selected for further analyses. At this concentration, the corresponding ZIP synergy score was 5.1, with a 54% growth inhibition. Furthermore, this combination significantly increased the mRNA expression of Caspase-

3 and Bax to 1.28 ± 0.05 -fold and 1.19 ± 0.11 -fold of the DOX group, respectively, corresponding to 28% and 19% increases, respectively, while reducing Bcl-2 mRNA expression to 0.68 ± 0.07 -fold of the DOX group (32% reduction) ($p < 0.05$).

These findings suggest that CSSO may enhance the anticancer activity of DOX by inducing apoptosis, indicating its potential as an experimental adjuvant strategy for combination therapy in neuroblastoma.

Keywords: *Cannabis sativa* L. Seed oil, Doxorubicin, SH-SY5Y, Apoptosis

INTRODUCTION

Neuroblastoma is a common malignancy in infancy and childhood and is a lethal extracranial solid tumor, accounting for approximately 8–10% of childhood cancers and nearly 15% of cancer-related deaths among children. Current treatment combines surgery, chemotherapy, radiotherapy, stem cell transplantation, and immunotherapy [1]. Doxorubicin (C₂₇H₂₉NO₁₁; DOX) is a broad-spectrum anthracycline chemotherapeutic agent whose anticancer activity is primarily mediated by DNA intercalation and topoisomerase II inhibition, leading to DNA damage and apoptosis [2]. It is widely used to treat various malignancies, including neuroblastoma [3]. However, its clinical use is limited by serious adverse effects, particularly dose-limiting cardiotoxicity and the development of multidrug resistance [4, 5]. Apoptosis eliminates damaged cells, with Bax and Bcl-2 serving as key pro- and anti-apoptotic regulators, respectively, and caspase-3 mediating apoptotic cell death. Because dysregulated apoptosis promotes cancer cell survival and proliferation, its induction is an effective anticancer strategy [6].

Cannabis sativa L. seed oil (CSSO) is rich in polyunsaturated fatty acids, linoleic and α -linolenic acids, tocopherols, phytosterols, and phenolic compounds [7]. These components have antioxidant, anti-inflammatory, and antiproliferative properties, supporting the application of CSSO in cancer research [8], with antiproliferative/cytotoxic effects reported in A549 and colon cancer cell lines [9, 10]. Combination therapy can enhance efficacy while reducing drug resistance and adverse effects [11]. Although DOX effectively treats neuroblastoma, its use is limited by cardiotoxicity and drug resistance [12]. Natural compounds can be used in combination with chemotherapeutic agents [4, 11]. This study aimed to evaluate the potential synergistic anticancer

effects of CSSO in combination with DOX in SH-SY5Y human neuroblastoma cells.

METHODS

Chemicals, Reagents, and Cell Culture

Cannabis sativa seed oil (CSSO; ‘NARLI’ variety), obtained by cold pressing and supplied by the Ondokuz Mayıs University Hemp Research Institute (Samsun, Türkiye), was used as described previously [7]. Doxorubicin (DOX; purity $\geq 98\%$) and MTT were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS at 37 °C and 5% CO₂. Cells were seeded at 2×10^4 cells/well in 96-well plates for viability assays and 2×10^5 cells/well in 6-well plates for RT-qPCR analysis.

Cell Viability and Synergy Analysis

Cell viability was evaluated using the MTT assay after 24 h of treatment with CSSO (1–800 $\mu\text{g/mL}$) or DOX (1–50 $\mu\text{g/mL}$). Cells were incubated with MTT (5 mg/mL) for 4 h, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm, and IC₅₀ values were determined using GraphPad Prism 9. For combination analysis, 0, 1/4 IC₅₀, 1/2 IC₅₀, and IC₅₀ concentrations of CSSO and DOX were evaluated

using SynergyFinder Plus [13] based on the Zero Interaction Potency (ZIP) model and Combination Index (CI).

Experimental Design and RT-qPCR Analysis

Based on the synergy analysis, CSSO (48.42 $\mu\text{g/mL}$) and DOX (0.92 $\mu\text{g/mL}$) were selected for the RT-qPCR analysis. The cells were divided into control, DMSO (0.05%), DOX, CSSO, and CSSO + DOX groups and treated for 24 h. Total RNA was extracted using the EcoPURE Total RNA Kit (EcoTech, Türkiye) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Caspase-3, Bax, and Bcl-2 expression was quantified using SYBR Green qPCR Master Mix on a StepOnePlus Real-Time PCR System, with β -actin as the reference gene [14]. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method, and the Bcl-2/Bax ratio was determined from normalized expression values.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 9). Normality was assessed using the Shapiro–Wilk test. Normally distributed data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test. Statistical significance was set at $p < 0.05$.

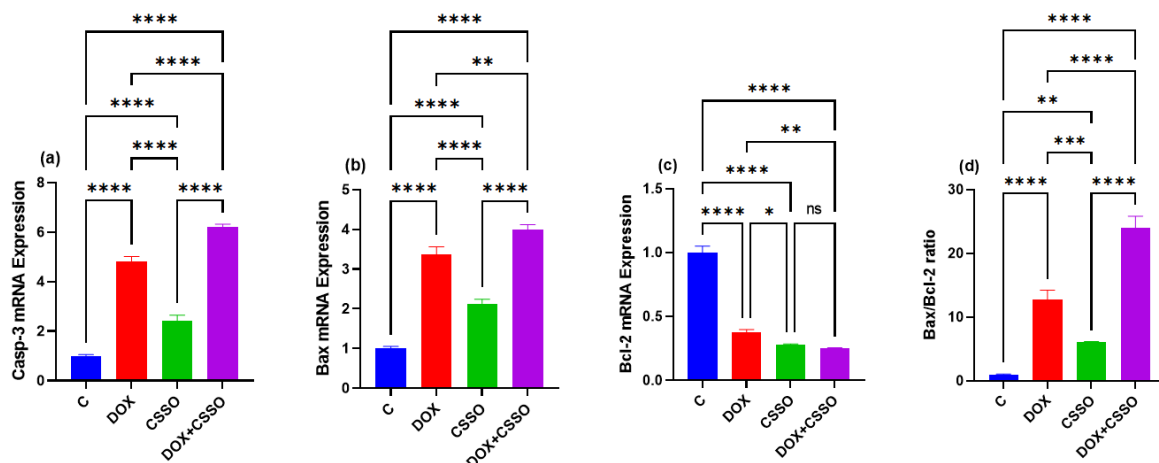


Figure 1. The mRNA expression levels of (a) caspase-3, (b) Bax, and (c) Bcl-2, as well as (d) the Bax/Bcl-2 ratio, were determined following treatment with CSSO, DOX, or their combination. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey’s honestly significant difference (HSD) post hoc test. Data are expressed as the mean $\pm$ SD of three independent biological replicates. Statistical significance between the indicated groups is shown by connecting brackets: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. **Abbreviations:** C, control; DOX, doxorubicin; CSSO, *Cannabis sativa* L. seed oil.

RESULTS

Cell Viability and Synergy Analysis

The IC₅₀ values of CSSO and DOX were 96.84 and 1.84 µg/mL, respectively. Their combination at 1/2 IC₅₀ concentrations (48.42 µg/mL CSSO + 0.92 µg/mL DOX) showed synergism (ZIP = 5.1; CI = 0.88) and was selected for subsequent RT-qPCR analysis.

Effects of CSSO and DOX on Apoptosis-Related Gene Expression

As shown in Figure 1a–d, DOX and CSSO alone significantly increased caspase-3 (4.83 ± 0.18 - and 2.43 ± 0.21 -fold) and Bax (3.36 ± 0.20 - and 2.13 ± 0.11 -fold) expression and decreased Bcl-2 expression (0.37 ± 0.03 - and 0.28 ± 0.01 -fold) compared with the control (all $p < 0.0001$). The Bax/Bcl-2 ratio was also increased by DOX (12.76 ± 1.43 -fold; $p < 0.0001$) and CSSO (6.09 ± 0.09 -fold; $p < 0.01$). Compared with DOX alone, the combination of DOX + CSSO further increased caspase-3 (1.28 ± 0.05 -fold; $p < 0.0001$) and Bax (1.19 ± 0.11 -fold; $p < 0.01$) expression and Bax/Bcl-2 ratio (1.91 ± 0.24 -fold; $p < 0.0001$), while further decreasing Bcl-2 expression (0.68 ± 0.07 -fold; $p < 0.01$).

DISCUSSION

To our knowledge, this is the first study to investigate the antiproliferative and apoptosis-related effects of CSSO alone and in combination with DOX in SH-SY5Y cells. CSSO reduced cell viability in a concentration-dependent manner, and its combination with lower DOX concentrations enhanced the anticancer response. Combined CSSO + DOX treatment increased the mRNA expression of caspase-3 and Bax, decreased that of Bcl-2, and increased the Bax/Bcl-2 ratio, indicating a pro-apoptotic shift. These findings suggest that CSSO may enhance DOX activity in SH-SY5Y cells by modulating apoptosis-related responses.

DOX is an effective chemotherapeutic agent used in neuroblastoma; however, its clinical utility is limited by dose-dependent toxicities, particularly cardiotoxicity, and by acquired or multidrug resistance [6, 15]. These limitations have encouraged the development of combination strategies aimed at enhancing anticancer efficacy while reducing DOX exposure [4, 5]. Therefore, natural bioactive compounds have gained attention as potential adjuvants or chemosensitizing agents for their effects on cancer cell survival, proliferation, and apoptosis [5, 11].

CSSO alone exhibited a concentration-dependent antiproliferative effect on SH-SY5Y cells, with an IC₅₀ of 96.84 µg/mL after 24 h. This finding is consistent with previous reports on the antiproliferative effects of hemp seed oil and related *Cannabis sativa*-derived preparations in cancer cell models [9, 10]. CSSO also increased caspase-3 and Bax mRNA expression, decreased Bcl-2 expression, and increased the Bax/Bcl-2 ratio [9, 16], indicating a shift toward a pro-apoptotic profile.

Previous studies have shown that agents such as napabucasin, barakol, and betulinic acid enhance the anticancer effects of DOX in neuroblastoma cells [4-6]. These effects are associated with increased apoptotic activity and modulation of survival and chemoresistance-related pathways [6]. Consistent with these observations, our findings suggest that CSSO enhances the anticancer response of SH-SY5Y cells to DOX.

A key finding was the favorable interaction between CSSO and DOX at half of their respective IC₅₀ concentrations (48.42 and 0.92 µg/mL, respectively). This combination yielded a CI of 0.88 and a ZIP score of 5.1. According to the predefined criteria, CI < 1 indicates synergy, whereas a ZIP score between -10 and +10 indicates additivity [9, 17]. Thus, the interaction was model-dependent, with CI indicating synergy and ZIP indicating additivity; importantly, neither model indicated an antagonism. The weaker effects observed with other dose combinations further suggest that the CSSO–DOX interaction is concentration- and ratio-dependent. Further studies with broader concentration matrices are required to determine the optimal combination ratio and its reproducibility across neuroblastoma models.

The findings on apoptosis-related gene expression may provide a molecular basis for the enhanced antiproliferative effect of this combination. The balance between pro-apoptotic Bax and anti-apoptotic Bcl-2 is an important regulator of mitochondrial apoptosis [18], whereas caspase-3 contributes to the execution of apoptotic cell death [19, 20]. DOX alone increased Bax and caspase-3 expression and decreased Bcl-2 expression, consistent with its reported pro-apoptotic effects in neuroblastoma cells [3, 6, 11]. More importantly, CSSO + DOX further increased Bax and caspase-3 expression and reduced Bcl-2 expression compared with DOX alone, resulting in an increased Bax/Bcl-2 ratio. These findings suggest that enhanced apoptosis-related signaling may contribute to the greater anticancer response of the combination, consistent with previous studies on DOX combinations [4-6].

However, because only mRNA expression was assessed, these changes indicate a pro-apoptotic molecular profile rather than directly confirming apoptotic cell death in the cells.

The main limitations of this study include the use of a single cell line and the assessment of apoptosis only at the mRNA level. Further studies using additional models, direct apoptosis and protein analyses, and in vivo approaches are required to confirm these findings and clarify the relevance of the CSSO + DOX combination. In conclusion, CSSO enhanced the anticancer effects of DOX in SH-SY5Y cells and promoted a pro-apoptotic gene expression profile in these cells. The interaction was concentration-dependent, with CI indicating synergy and ZIP indicating an additive effect. These findings support further investigation of CSSO as a potential combination agent for use with DOX.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

Disclosure

The authors have reported no conflicts of interest in preparing and publishing this article.

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