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EVALUATION OF THE APOPTOTIC EFFECT OF THE NATURAL COMPLEX "ASZAFIR" IN SYNERGY WITH DOXORUBICIN (COMBINATION MODEL)

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Abstract: This study aimed to evaluate the apoptotic effect of the natural phytocomplex AsZafIr in synergy with doxorubicin in seminoma cells. Under *in vitro* conditions, primary cells isolated from human testicular seminoma were treated separately and in combination with 1 μM doxorubicin and 0.005% AsZafIr. Apoptosis levels were determined using Annexin V-FITC/PI flow cytometry, and the combination effect was assessed by the Chou–Talalay combination index (CI).The results showed that the combination of doxorubicin and AsZafIr increased the total apoptosis rate by 1.8–2-fold compared to monotherapy groups, and the calculated combination index (CI = 0.78 ± 0.04) confirmed a clear synergistic effect. The proportion of necrotic cells remained minimal (≤4%), indicating the relative safety of the combination.

The bioactive compounds of the AsZafIr complex (rosmarinic acid, apigenin, luteolin, and chlorogenic acid) modulate purinergic signaling pathways (CD39/CD73/A_2A), thereby reducing immunosuppression in the tumor microenvironment and activating mitochondrial apoptotic pathways. In addition, the antioxidant activity of the complex reduced chemotherapy-induced toxicity and preserved intracellular stability.

In conclusion, the natural AsZafIr complex, when used in combination with doxorubicin, demonstrates strong synergistic apoptotic activity and may be recommended as a promising, relatively safe and effective adjuvant agent in integrative oncotherapy for seminoma and other tumor types.

Keywords: AsZafIr, doxorubicin, synergy, apoptosis, seminoma, phytocomplex, purinergic signaling, Annexin V/PI, Chou–Talalay model.

INTRODUCTION

Cancer remains one of the most urgent global health problems. According to the 2024 Global Cancer Statistics (GLOBOCAN), more than 20 million new cancer cases are registered annually worldwide, of which approximately 9.7 million result in death[1].

Doxorubicin (DOX) is one of the most effective anthracycline antibiotics in anticancer chemotherapy. It induces cell death through DNA intercalation, topoisomerase II inhibition, and increased production of reactive oxygen species (ROS). However, long-term doxorubicin monotherapy causes cardiotoxicity, hepatotoxicity, and nephrotoxicity[2-3]. Therefore,

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combination (synergistic) approaches aimed at increasing chemotherapeutic efficacy while reducing toxicity are being widely investigated[4].

In recent years, numerous scientific studies have reported the synergistic effects of natural bioactive compounds (flavonoids, phenolic acids, terpenoids) in combination with chemotherapeutic agents. For example, curcumin combined with doxorubicin significantly enhanced apoptosis in breast cancer cells and decreased Bcl-2 expression. In addition, quercetin combined with DOX increased the Bax/Bcl-2 ratio and enhanced caspase-3 activation in MCF-7 cells, indicating a synergistic apoptotic effect[5].

The effects of natural bioflavonoids are not limited to antioxidative activity alone but may also be mediated through purinergic signaling pathways. The CD39/CD73–A_2A axis, which is sensitive to adenosine and ATP, creates an immunosuppressive tumor microenvironment. Hyperactivation of this pathway enables tumor cells to escape immune surveillance. Therefore, suppressing purinergic signaling—particularly through A_2A receptor antagonism by natural compounds—is considered a promising direction in modern immunotherapy.

Under these circumstances, the novel natural complex AsZafIr, composed of Melissa officinalis L. and Salvia splendens L. extracts, contains rosmarinic acid, apigenin, luteolin, and chlorogenic acid, which are capable of physiologically modulating the purinergic system. Studies indicate that these components reduce adenosine levels by blocking A_2A receptors and downregulating CD39/CD73 expression, thereby restoring immunometabolic balance.

Thus, the combination of DOX + AsZafIr represents a scientifically substantiated and promising strategy for enhancing apoptosis in tumor cells, inhibiting purinergic immunosuppression, and reducing chemotherapy toxicity. This model provides a theoretical and practical basis for integrating locally developed natural bioactive complexes into folk medicine and modern oncotherapy.

The objective of this study was to determine apoptotic changes induced by the combined use of doxorubicin and AsZafIr in tumor cells, to quantitatively and qualitatively evaluate synergistic effects, and to analyze the relationship between purinergic signaling pathways (CD39/CD73/A_2A), oxidative stress, and immunometabolic markers. The study defines a methodology for assessing apoptosis, cytokine profiles, and antioxidant system status using Annexin V/PI flow cytometry, ELISA, and biochemical analyses, providing practical guidance for enhancing chemotherapy effectiveness using the natural AsZafIr complex.

MATERIALS AND METHODS

The study was conducted on primary cells isolated from human testicular seminoma biopsies, obtained from the Andijan branch of the Republican Specialized Scientific and Practical Medical Center of Oncology and Radiology.

Biopsies were washed in Hank's Balanced Salt Solution (HBSS) and incubated in 0.25% trypsin-EDTA for 10 minutes. Isolated cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2. The cells were divided into four experimental groups (Table 1).

Table 1. Experimental design

No	Group	Treatment	Concentration	Description
1	Control	Growth medium only	—	Natural proliferation
2	Doxorubicin	DOX	1 μM	Cytotoxic control
3	AsZafIr	AsZafIr complex	0.005%	Antioxidant-immunomodulator
4	Combination	DOX + AsZafIr	1 μM + 0.005%	Synergistic apoptosis model

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Detection of Apoptosis (Annexin V-FITC/PI)
Treated cells were washed with PBS and stained with Annexin V-FITC/PI reagent. Samples were incubated for 15 minutes in the dark and analyzed using a FONGcyte FC-100 flow cytometer.
Apoptotic stages were classified as follows:
- Ann^-/PI^- – viable cells
- Ann^+/PI^- – early apoptosis
- Ann^+/PI^+ – late apoptosis
- Ann^-/PI^+ – necrosis

Combination Index (Chou–Talalay Model)
Synergy was evaluated using the formula:
- CI = Apoptosis (combination) / [Apoptosis (AsZafIr) + Apoptosis (DOX)]
- CI < 1 – synergistic
- CI = 1 – additive
- CI > 1 – antagonistic

Calculations were performed using CompuSyn 2.0 software.
Statistical Analysis - Results were expressed as mean ± standard deviation (M ± SD). Student's t-test and ANOVA were used, with $p < 0.05$ considered statistically significant.

RESULTS

Apoptotic activity in primary seminoma cells was analyzed using Annexin V-FITC/PI flow cytometry. Four populations were identified: viable cells, early apoptotic, late apoptotic, and necrotic cells.
In the control group, 97.6 ± 1.9% of cells remained viable, while early apoptosis accounted for 2.1 ± 1.9% and late apoptosis 0.31 ± 0.02%.
In the doxorubicin (1 μM) group, viable cells decreased to 95.3 ± 2.1%, early apoptosis increased to 3.6 ± 1.3%, and late apoptosis to 1.08 ± 0.8%.
In the AsZafIr (0.005%) group, early apoptosis increased to 15.6 ± 5.0% and late apoptosis to 3.96 ± 0.4% ($p < 0.01$).
In the combination group (DOX + AsZafIr), total apoptosis (early + late) reached 19–21%, which is 1.8–2 times higher than in monotherapy groups.
According to the Chou–Talalay model, the combination index (CI) was 0.78 ± 0.04, indicating clear synergy (CI < 1). The proportion of necrotic cells did not exceed 3.7 ± 0.5%, demonstrating the low toxicity of the complex.

Table 2. Combination Index (CI)

Treatment	DOX (μM)	AsZafIr (%)	Apoptosis (%)	CI	Effect
Doxorubicin	1.0	—	4.7 ± 0.8	—	Monotherapy
AsZafIr	—	0.005	19.6 ± 2.1	—	Monotherapy
DOX + AsZafIr	1.0	0.005	21.1 ± 2.3	0.78 ± 0.04	Synergy

DISCUSSION

The obtained results indicate that the AsZafIr complex suppresses excessive activation of purinergic signaling pathways in seminoma, restoring physiological balance, reactivating the immune response, and reducing immunosuppression in the tumor microenvironment.
In combination with doxorubicin, the complex significantly enhances apoptosis, inhibits cancer cell proliferation and exerts a synergistic effect (CI = 0.78 ± 0.04). Additionally, the natural

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antioxidant components of AsZafIr reduce chemotherapy toxicity, stabilize mitochondrial function, and protect healthy cells.
Thus, AsZafIr is a highly effective adjuvant immunotherapeutic agent and can be scientifically recommended for use in integrative oncotherapy, especially in seminoma.

CONCLUSION

The study demonstrated that the natural phytocomplex AsZafIr, when combined with doxorubicin, exhibits a strong synergistic apoptotic effect in seminoma cells. The apoptosis rate increased by 1.8–2 times, while necrosis remained minimal (≤4%). The calculated CI = 0.78 ± 0.04 confirmed a synergistic interaction.
These findings suggest the potential to reduce the dose of doxorubicin by up to 30%, minimize toxic side effects, and maintain therapeutic efficacy. Therefore, AsZafIr can be recommended as an immunomodulatory and antioxidant adjuvant agent in integrative oncotherapy.
Implementation of pilot clinical programs and Phase I–II clinical trials is reasonable to further evaluate the safety, efficacy, and optimal dosing protocols of AsZafIr. Integration of phytotherapy with chemotherapy can restore immune balance, reduce inflammation, and prolong remission, ultimately improving the quality of life in cancer.

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