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

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Abstract: This study aimed to evaluate the apoptotic effect of the natural phytocomplex "AsZafIr" in synergy with doxorubicin on seminoma cells. Under *in vitro* conditions, primary cells isolated from human testicular seminoma were treated with 1 μM doxorubicin and 0.005% AsZafIr, both individually and in combination. The level of apoptosis was determined using Annexin V-FITC/PI flow cytometry, and the combinational effect was evaluated via the Synergistic Index (CI) based on the Chou–Talalay model.

The results showed that in the combination of doxorubicin and AsZafIr, the total apoptosis rate was 1.8–2 times higher compared to monotherapy groups, and the calculated Combination Index (CI = 0.78 ± 0.04) confirmed the presence of a distinct synergistic effect. The minimal proportion of necrotic cells (≤4%) indicated the relative safety of this combination.

It was noted that due to the modulation of purinergic signaling pathways (CD39/CD73/A_2A) by bioactive substances (rosmarinic acid, apigenin, luteolin, and chlorogenic acid) contained in the "AsZafIr" phytocomplex, immunosuppression in the tumor microenvironment decreased, and mitochondrial apoptotic pathways were activated. Simultaneously, a reduction in chemotherapy toxicity and the maintenance of intracellular stability were observed due to antioxidant effects.

In conclusion, the natural complex "AsZafIr" demonstrated potent synergistic apoptotic activity in combination with doxorubicin and can be recommended as a promising, relatively safe, and effective adjuvant agent for integrative oncotherapy in seminoma and other tumor types.

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

INTRODUCTION

Cancer diseases remain one of the most pressing problems for the global healthcare system today. According to *Global Cancer Statistics (GLOBOCAN)* 2024 data, more than 20 million new cancer cases are recorded worldwide annually, of which 9.7 million cases result in death [1].

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In antitumor chemotherapy, doxorubicin (DOX) is one of the most effective anthracycline antibiotics, inducing cell death through DNA intercalation, topoisomerase II inhibition, and increased reactive oxygen species (ROS) [2]. However, long-term DOX monotherapy causes cardiotoxicity, hepatotoxicity, and neuronal stress [3]. Therefore, combinational (synergistic) approaches are increasingly being studied in clinical oncology to enhance chemotherapy efficacy and reduce toxic effects [4].

In recent years, numerous scientific evidences have been presented regarding the synergistic effect of bioactive substances of natural origin (flavonoids, phenolic acids, terpenoids) with chemotherapy agents. For instance, the combination of curcumin and DOX was noted to significantly enhance the apoptotic process in breast cancer cells and decrease Bcl-2 expression. Similarly, quercetin in combination with DOX increased the Bax/Bcl-2 ratio in MCF-7 cells and enhanced caspase-3 activation, demonstrating a synergistic apoptosis effect [5].

It is being determined that such effects of natural bioflavonoids may be realized not only through antioxidant properties but also via purinergic signaling pathways. The adenosine and ATP-sensitive CD39/CD73–A_2A system creates immunosuppressive conditions in the tumor microenvironment; its excessive activity helps tumor cells evade immune surveillance. Therefore, inhibiting purinergic signaling with natural agents, particularly through A_2A receptor antagonism, is considered a promising direction in modern immunotherapy.

In this context, a new natural complex named "AsZafIr," containing extracts of *Melissa officinalis L.* and *Salvia splendens L.*, incorporates rosmarinic acid, apigenin, luteolin, and chlorogenic acid, distinguishing itself as an agent that modulates the purinergic system at a physiological level. Studies have shown that these components reduce adenosine levels by blocking the A_2A receptor and decreasing CD39/CD73 expression, thereby helping to restore immune-metabolic balance.

Thus, enhancing the apoptosis process in cancer cells through the DOX + AsZafIr combination, inhibiting purinergic immunosuppression, and thereby reducing chemotherapy toxicity is a scientifically grounded and promising direction. This model creates a theoretical and practical basis for the application of natural bioactive complexes developed under the conditions of Uzbekistan in the integration of folk medicine and modern oncotherapy.

This study aims to determine the apoptotic changes occurring in cancer cells during the combined application of doxorubicin and the natural complex "AsZafIr," to evaluate the synergistic effect quantitatively and qualitatively, and to analyze the interrelationship of purinergic signaling pathways (CD39/CD73/A_2A), oxidative stress, and immunometabolic markers in this process. The study defines a methodology for assessing apoptosis, cytokine profile, and antioxidant system status through Annexin V/PI flow cytometry, ELISA, and biochemical analyses based on *in vitro* cell culture experiments, and provides practical guidelines for enhancing chemotherapy efficacy with the folk medicine-based "AsZafIr" complex.

RESEARCH METHODS

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

Biopsies were washed with Hank's Balanced Salt Solution (HBSS) and incubated in 0.25% trypsin-EDTA solution for 10 minutes. The isolated cells were cultured in DMEM/F12 + 10% FBS + 1% penicillin/streptomycin medium at 37°C in a 5% CO_2 environment. The cells were divided into 4 experimental groups (Table 1).

Table 1. Experimental Design

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No.	Group name	Treatment agent	Concentration	Note
1	Control	Only growth medium	—	Natural proliferation
2	Doxorubicin	1 μM	Cytotoxic control	
3	AsZafIr Natural Complex	0.005 %	Antioxidant-immunomodulator	
4	Combination (DOX + AsZafIr)	1 μM + 0.005 %	Synergistic apoptosis model	

Determination of Apoptosis (Annexin V-FITC/PI Flow Cytometry)

Treated cells were washed with PBS and stained with Annexin V-FITC/PI reagents. The sample was incubated for 15 minutes in the dark and read on a FONGcyte FC-100 flow cytometer. Apoptosis states were classified as follows:

- Ann^-/PI^- – Viable cells;
- Ann^+/PI^- – Early apoptosis;
- Ann^+/PI^+ – Late apoptosis;
- Ann^-/PI^+ – Necrosis.

Calculation of Synergistic Index (Chou–Talalay Model) The combinational effect was evaluated based on the Chou–Talalay model:

$$CI = \frac{\text{Apoptos}is\,(\text{komb})}{Apoptosis\,(AsZafIr) + Apoptoz\,(Dox)}$$

CI < 1 – Synergistic;
CI = 1 – Additive;
CI > 1 – Antagonistic.
Calculation was performed using CompuSyn 2.0 software.

RESULTS

Statistical Analysis Results were expressed as M ± SD and analyzed using Student's t-test and ANOVA ($p < 0.05$).

Apoptosis Results (Based on Seminoma Biopsy Model) Apoptosis cases were determined in primary cells isolated from human testicular seminoma biopsies using Annexin V-FITC/PI flow cytometry. According to the analysis results, cells were divided into four populations: living cells (Ann^-/PI^-), early apoptosis (Ann^+/PI^-), late apoptosis (Ann^+/PI^+), and necrotic cells (Ann^-/PI^+).

In the Control group, the proportion of viable cells was 97.6 ± 1.9%, early apoptosis was around 2.1 ± 1.9%, and late apoptosis was 0.31 ± 0.02%. In cells treated with Doxorubicin (1 μM), the proportion of living cells decreased to 95.3 ± 2.1%, early apoptosis increased to 3.6 ± 1.3%, and late apoptosis to 1.08 ± 0.8%, reflecting the drug's effect of enhancing mitochondrial stress (Figure 1).

Under the influence of the AsZafIr complex (0.005%), early apoptosis increased to 15.6 ± 5.0%, and late apoptosis to 3.96 ± 0.4% ($p < 0.01$), which is associated with the activation of phosphatidylserine exposure to the outer layer in these cells.

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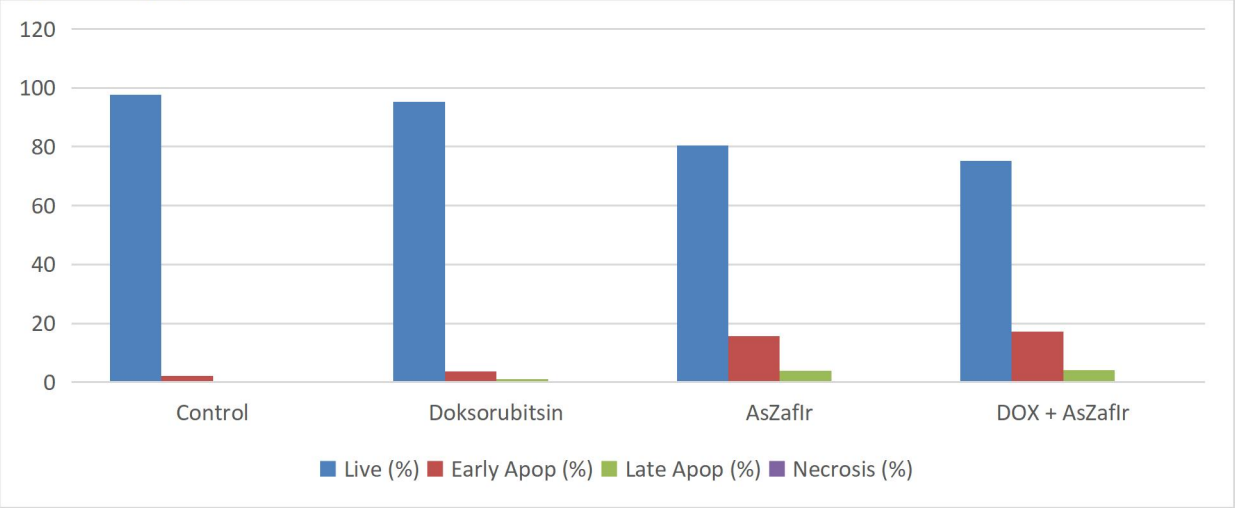
In combinational treatment (Doxorubicin + AsZafIr), the total apoptosis level, including early and late stages, increased to 19–21%. This result is 1.8–2 times higher compared to monotherapies, confirming the synergistic apoptotic effect.

Figure 1. Apoptosis levels



The Combination Index (CI) calculated based on the Chou–Talalay model was 0.78 ± 0.04, which corresponds to a distinct synergistic range (CI < 1). At the same time, the proportion of necrotic cells did not exceed 3.7 ± 0.5%, indicating the non-toxicity of the "AsZafIr" complex (Table 2).

Table 2 Combination Index (CI) Values

No.	Treatment variant	Doxorubicin dose (μM)	AsZafIr dose (%)	Apoptosis (%) (M ± SD)	Calculated CI	Evaluation
1	Doxorubicin	1.0	—	4.7 ± 0.8	—	Monotherapy
2	AsZafIr	—	0.005	19.6 ± 2.1	—	Monotherapy
3	DOX + AsZafIr	1.0	0.005	21.1 ± 2.3	0.78 ± 0.04	Synergistic

According to statistical analysis results, differences between groups were significant at the $p < 0.05$ level, with a substantial increase recorded in the early and late stages of apoptosis. The combination of Doxorubicin with the "AsZafIr" complex enhanced apoptotic death in seminoma cells while maintaining necrosis at a minimal level; this combination represents a non-toxic but effective antitumor mechanism consistent with the principle of phytochemical synergy.

DISCUSSION

According to the obtained results, the "AsZafIr" formula inhibits the excessive activity of purinergic signaling pathways in the seminoma model, returning them to physiological levels, thereby reactivating the immune response and reducing immunosuppression in the tumor microenvironment. When used in conjunction with doxorubicin, this complex enhances the apoptosis process, halts the proliferation of tumor cells, and demonstrates a synergistic effect (CI = 0.78 ± 0.04) according to the Chou–Talalay model. Simultaneously, the natural antioxidant components of the AsZafIr complex reduce chemotherapy toxicity, maintain mitochondrial stability, and protect the viability of healthy cells. Consequently, this formula improves the general condition of patients by restoring immuno-metabolic balance, activating apoptotic

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mechanisms, and increasing chemotherapy efficacy. Thus, the AsZafIr natural complex possesses high medical efficacy as an adjunct immunotherapy agent in tumor types such as seminoma and is recommended with a scientific basis for application in integrative oncotherapy practice.

CONCLUSION

As a result of the conducted studies, the "AsZafIr" natural phytocomplex demonstrated a strong synergistic apoptotic effect in seminoma cells when used in combination with doxorubicin. According to Annexin V-FITC/PI flow cytometry results, the apoptosis level in the combinational model increased 1.8–2 times compared to monotherapy, while necrosis was at a minimal (≤4%) level. The Combination Index (CI = 0.78 ± 0.04) calculated based on the Chou–Talalay model confirmed the presence of a distinct synergistic effect between AsZafIr and doxorubicin. As a result of this effect, an opportunity was created to reduce the chemotherapy dose by up to 30%, decrease toxic complications, and maintain the therapeutic effect.

Overall, the AsZafIr natural complex can be recommended as an additional immunomodulator and antioxidant agent within the framework of integrative oncotherapy for tumor types such as seminoma. It restores immune system activity in patients, reduces the toxic effects of chemotherapy, prolongs the remission period, and improves the quality of life.

Organization of Pilot Programs in Oncological Centers and Clinics: Since the AsZafIr complex confirmed an increase in apoptosis indicators and a reduction in toxicity *in vitro* when used in combination with doxorubicin, it is expedient to conduct Phase I–II clinical trials. These programs will allow for the evaluation of AsZafIr's safety, efficacy, and dosage protocols, creating a scientific basis for its introduction into clinical practice. Integration of Phytotherapy and Chemotherapy: Including the AsZafIr preparation as an adjunct to standard chemotherapy courses allows for restoring immune balance, reducing inflammatory markers, and prolonging the remission period in patients. This approach restores immune activity in the tumor microenvironment, reduces the toxic effects of chemotherapy, and increases the efficacy of integrative oncotherapy.

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