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Molecular and Immunological assessment of anticancer activity of 1,2,4-triazolo[4,3-b] [1,2,4] triazole derivative in combination with Artemisia Judaica Extract in Murine Lung cancer model.

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Abstract

Lung cancer remains a leading cause of cancer-related mortality worldwide, emphasizing the urgent need for innovative therapeutic strategies. This study evaluates the anti-cancer impact of *Artemisia Judaica* (AJ) and 1,2,4-triazole derivatives (TRI), both individually and in combination, against lung cancer. Immunohistochemical (IHC) analysis revealed significant modulation in the expression of Caspase-3, Caspase-7, and tumor necrosis factor-alpha (TNF-α), indicating enhanced apoptosis and immune responses. Molecular investigations further demonstrated the compounds' regulatory effects on key oncogenic pathways, specifically EGFR and BRAF. Notably, the combined therapy displayed a synergistic effect, underscoring its potential as a novel therapeutic approach for lung cancer. These findings highlight AJ and 1,2,4-triazole derivatives as promising candidates for further clinical evaluation and development in lung cancer management.

Keywords: lung Cancer, Artemisia Judaica, 1,2,4-triazole derivatives, caspase-3, caspase-7, TNF-α.

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1. Introduction:

Lung cancer remains a predominant cause of morbidity and mortality associated with cancer on a global scale, accounting for a significant percentage of all cancer-related fatalities. The World Health Organization (WHO) indicates that lung cancer ranks as the second most frequently diagnosed malignancy worldwide, with its prevalence increasing, especially in low- and middle-income nations [1, 2]. In the Middle East, the burden of lung cancer has been steadily increasing, driven by factors such as high smoking rates, environmental pollution, and occupational hazards. This growing prevalence highlights the urgent need for effective therapeutic strategies that are tailored to the region's unique epidemiological and socioeconomic circumstances[3].

The main treatments for lung cancer today are surgery, chemotherapy, radiation therapy, and targeted therapies[4]. Although these methods have enhanced survival rates in many patients, they come with notable limitations. For example, chemotherapy and radiation therapy can cause serious side effects, including myelosuppression, organ toxicity, and drug resistance[5]. Additionally, the high costs and accessibility challenges associated with modern targeted therapies make them difficult to implement widely, particularly in developing regions[6].

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In light of these challenges, natural products derived from medicinal plants have gained attention as promising alternatives or adjuncts in cancer therapy. These natural compounds are recognized for their diverse bioactivities, which include antioxidant, anti-inflammatory, and anticancer properties, and they often have lower toxicity profiles compared to conventional treatments[7, 8]. Artemisia judaica, a medicinal plant native to arid regions, is noted for its potent therapeutic properties. The bioactive compounds found in abundance include flavonoids like apigenin, kaempferol, and rutin as well as phenolic acids like p-coumaric acid, ferulic acid, and caffeic acid [9].

Artemisia Judaica demonstrates anticancer effects against lung cancer by inducing apoptosis, arresting the cell cycle, and inhibiting key signaling pathways like EGFR and CDK-2. These actions result from a variety of bioactive compounds, including flavonoids and phenolic acids, which work together to target cancer cells while reducing systemic toxicity. Additionally, the plant exhibits anti-inflammatory and antioxidant activities, positioning it as a potential treatment for lung cancer with minimal side effects[9, 10].

Advancements in medicinal chemistry have led to the development of novel synthetic compounds that enhance therapeutic efficacy and selectivity, complementing natural products. One class of these compounds that has shown promise as a cancer treatment is 1,2,4-triazole derivatives. The biological activities of these heterocyclic compounds are diverse, encompassing anticancer, antifungal, and antibacterial effects, among others. Their versatility in chemical modification enables the creation of derivatives specifically designed to target distinct cancer pathways, which may improve treatment outcomes while minimizing systemic toxicity [11].

There is an ever-increasing need to discover new anticancer drugs for various types of cancer. There is significant evidence in the literature that hybrid 1,2,4-triazoles have a greater range of biological potential than their monocyclic analogues, and this includes strong anti-cancer effects[12].

The medicinal chemistry community is very interested in triazoles, and in particular 1,2,4-triazole derivatives, due to their potent biological activities and adaptability. These compounds are known for their chemical stability, straightforward synthesis, and capacity to interact with various biological targets, establishing them as essential components in drug discovery. Many studies in both animal and human settings have shown that triazoles have a variety of beneficial pharmacological effects, including those against cancer, bacteria, inflammation, and free radicals [11].

Several triazole-based drugs, including fluconazole and itraconazole, have been approved for clinical use, underscoring their therapeutic significance. Their capacity to inhibit key enzymes and modulate cellular pathways makes them promising candidates for the development of new treatments for difficult diseases such as cancer. Ongoing advancements in triazole synthesis and the optimization of their structure-activity relationships enhance their potential as effective and selective therapeutic agents, helping to overcome limitations in current treatments, such as drug resistance and toxicity [12].

This study investigates the synergistic potential of Artemisia judaica and 1,2,4-triazole derivatives in lung cancer therapy, emphasizing their ability to modulate essential molecular pathways. Specifically, it examines the extract's role in inducing apoptosis, halting the cell cycle, and inhibiting crucial signaling pathways such as the Epidermal Growth Factor Receptor (EGFR) and BRAF [13].

This research clarifies the mechanisms involved, contributing to the growing evidence for the therapeutic potential of natural compounds in oncology. It seeks to provide a comprehensive evaluation of their biochemical effects, molecular impacts, and therapeutic benefits [14, 15].

2. Material and methods

2.1. Preparation and Culture of B16/F10 Melanoma Cell Line

The B16/F10 melanoma cell line, which is derived from a gp100-positive spontaneous murine melanoma, was obtained from the cell culture department at NAWAH Scientific Center (Cairo, Egypt). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cultures were maintained at a temperature of 37°C within a humidified environment containing 5% carbon dioxide.

Cells were subcultured when they reached 70-80% confluency. Using a complete medium to neutralize the 0.25% trypsin-EDTA solution, the cells were detached. We used the trypan blue exclusion method to evaluate cell viability. After adding a 0.4% trypan blue solution to the cell suspension, the number of viable cells was measured using a hemocytometer while the sample was observed under a light microscope. Only cultures with over 95% viability were considered suitable for experimental use.

2.2. Chemicals and Reagents

Our suppliers in Alexandria, Egypt, El-Gomhoria Company for Chemicals and Medical Supplies, supplied the 96% ethyl alcohol, 10% neutral-buffered formalin, and sodium hydroxide pellets. The ethanolic extract of Artemisia Judaica was prepared at the Faculty of Science, Damanhour University, Egypt. Additionally, the 1,2,4-triazolo[4,3-b][1,2,4]triazole derivative was supplied by the chemistry department of the same faculty.

2.3. Plant material collection and extraction

Arish, North Sinai, Egypt was the site of the 2022 collection of fresh aerial parts of the Artemisia Judaica (AJ) plant. The ethanolic extract was made following the procedures outlined in [16, 17] The 1000g of plant material was mildly modified and then air-dried at room temperature. The herb was powdered from a somewhat coarse consistency using a mechanical mortar grinder; a finer powder was obtained by passing it through a sieve. Immersion in 96% ethyl alcohol at room temperature, with dilutions every three days, was performed on this dry powder for nine days. A rotary evaporator was used to concentrate the filtrate under vacuum after filtering the organic phase through filter paper. Exhaustion set in after several iterations of this process. Up until it was required, the dark-green sticky byproduct was refrigerated at 5°C. An analysis was conducted at Egypt's National Institute for Oceanography and Fisheries using gas chromatography-mass spectrometry (GC-MS) to identify the chemical composition of the aerial parts derived from an ethanolic extract of Artemisia judaica. After the compounds were discovered, their biological activities were assessed by reviewing the relevant literature.

2.4. Experimental Design and Animal Model

The experiment used 37 female C57BL/6 mice that were fully grown, with an average weight of 25 g, and were between 6 and 7 weeks old. The National Research Center's animal house in Dokki, Cairo, Egypt, was the source of the mice. The animals were provided with water at will and were fed mouse chow. In Egypt, the Institutional Animal Ethics Committee of Damanhour University's Faculty of Science gave their stamp of **approval to all research involving animals. with the following reference numbers: DMU-SCI-CSRE-24-9-03**, and all procedures followed the rules determined by the Committee for Control and Supervision of Experiments on Animals. Two primary groups of mice were established before the commencement of the experiment: A total of seven mice were used as a control group in the first study. In the second group, known as the tumor-bearing group, thirty mice were administered B16/F10 melanoma cells (1×10^1 cells/mL in 0.1 ml of phosphate-buffered saline [PBS]) through the tail vein through intravenous injection [18]. The tumor-bearing mice were monitored for 25 days to allow the development of lung tumor foci. After this period, two mice were

randomly dissected to confirm the presence of lung tumors. Following confirmation, the remaining 28 mice in Group 2 were further divided into four subgroups (Groups 2–5), each containing 7 mice, to receive specific treatments as outlined in the experimental protocol. Group 2: Served as lung cancer group without any treatment, Group 3: Lung cancer group treated with 1,2,4-triazolo[4,3-b] [1,2,4] triazole derivative (30 mg/kg.BW)[19]. Group 4: Lung cancer group treated with A. Judaica ethanolic extract (500 mg/kg.BW)[15, 20]. Group 5: Lung cancer group treated with a combination of 1,2,4-triazolo[4,3-b] [1,2,4] triazole derivative (30 mg/kg.BW) and A. Judaica ethanolic extract (500 mg/kg.BW), This experimental design allowed for the evaluation of the therapeutic effects of AJ extract, the 1,2,4-triazolo[4,3-b] [1,2,4] triazole derivative, and their combination on lung tumor progression in the B16/F10 melanoma model. All Treatment-induced peritoneal one dose, after 4 weeks from the last dose, the mice were deprived of food overnight. Following an intraperitoneal injection of 120 mg/kg of ketamine in a sterile saline solution, which anesthetized the animals, they were beheaded to ensure their sacrifice [21].

2.5. Blood Sample Collection

Blood samples were collected through cardiac puncture using sterile capillary tubes. A portion of each sample was mixed with dipotassium EDTA, an anticoagulant, for various hematological analyses.

2.6. Tissue Sample Collection and Preparation

Lung tissues were quickly excised and cut into small sections. These sections were then washed with ice-cold normal saline (0.9%) and preserved for later molecular and histological analyses.

2.7. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was used to evaluate the expression of Caspase-3, Caspase-7, and TNF-α proteins within the lung tumor microenvironment. Specific antibodies for these targets were employed to verify their associations. Tissue blocks of formalin-fixed, paraffin-embedded lung tissue were sectioned to a thickness of 4 µm. Antigen localization was conducted using 3,3'-diaminobenzidine (DAB) as a substrate, forming a brown precipitate upon antibody binding, followed by counterstaining with Mayer's Hematoxylin.

2.8. Gene Expression Analysis by qRT-PCR

Using quantitative real-time PCR (qRT-PCR), the levels of gene expression for EGFR, BRAF, and GAPDH (the internal control) were examined. The relative expression of the target genes was normalized to GAPDH, and the data were quantified using the comparative ($2^{-\Delta\Delta Ct}$) method.

2.8.1. RNA Isolation and Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was extracted from mice tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany; Catalog no. 74104) in the Physiology Laboratory at the Faculty of Science, Damanhour University, Egypt. The concentration and purity of the RNA were assessed with a NanoPhotometer® NP80 (IMCERT Lab, Damanhour University). RNA purity was evaluated based on the A260/A280 ratio, with values between 1.8 and 2.0 indicating high-quality RNA.

Complementary DNA (cDNA) was synthesized from the isolated RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Invitrogen, USA; Catalog No. 4368814). The reverse transcription took place in the IMCERT Lab, located in the Central Laboratories Building at Damanhour University. The resulting cDNA was then amplified with specific TaqMan primers using the Applied Biosystems StepOne™ Real-Time PCR system. Data analysis was conducted using StepOne™ software at the IMCERT Lab, Damanhour University.

Statistical analysis:

The means ± standard deviations are used to represent the group's data. For each pairwise comparison between the two groups, we used the Post Hoc Test (Tukey) and the One-way ANOVA test to establish statistical significance. In the 9th

edition of GraphPad Prism, a p-value of less than 0.05 was considered significant for graph generation. Results were classified as statistically significant. Conclusions indicated by * at P ≤0.05, ** at P ≤0.01, *** at P ≤0.001, and as well as by **** at P ≤ 0.0001. signify significance.

3. Results

3.1. The effect of lung cancer and treatments on complete blood count.

Table 1 shows that the analysis of hematological parameters, including red blood cell (RBC) count, hemoglobin (HGB) levels, platelet count, and white blood cell (WBC) count, revealed significant differences across the experimental groups ($P < 0.0001$ for all parameters), as indicated by the high F-values (ranging from 1032 to 367.6). The tumor control group exhibited a marked reduction in RBC count ($7.64 \pm 0.21 \times 10^6$/µl) and HGB level ($10.46 \pm 0.21$ g/dl) compared to the control group ($9.59 \pm 0.14 \times 10^6$/µl and 14.4 ± 0.31 g/dl, respectively). Conversely, platelet count ($1394 \pm 9.3 \times 10^3$/µl) and WBC levels (12.20 ± 0.16 K/µl) were significantly elevated in the tumor control group, indicating potential hematological disturbances associated with tumor progression.

Treatment with 1,2,4 Triazol derivative, Artemisia Judaica extract individually, or their combination led to varying degrees of hematological recovery. The 1,2,4 Triazol derivative and Artemisia judaica extract co-treated group showed the most significant improvement, with red blood cell count increasing to $8.92 \pm 0.15 \times 10^6$/µl and hemoglobin levels rising to 12.62 ± 0.16 g/dl. Additionally, there was a notable reduction in platelet count ($1209 \pm 7.8 \times 10^3$/µl) and white blood cell levels (9.1 ± 0.19 K/µl). According to these findings, the 1,2,4 Triazol derivative and Artemisia judaica extract work in concert to reduce tumor-induced hematological alterations and bring parameters closer to those observed in the control group. This highlights the potential therapeutic benefits of 1,2,4 Triazol derivative and Artemisia judaica extract for treating hematological dysregulation associated with tumour diseases, particularly when combined.

3.2. Effect of lung cancer and treatments on differential WBCs absolute count.

The differential white blood cell counts, including lymphocytes (LYMPH), neutrophils (NEUT), monocytes (MONO), basophils (BASO), and eosinophils (EOS), were evaluated across the experimental groups. The results showed significant variations in these hematological parameters, highlighting the therapeutic influence of Artemisia Judaica extract, 1,2,4-Triazole, and their combination, compared to the control tumor group (Table 2).

Table 2 shows that the analysis of white blood cell differentiation across the studied groups revealed significant variations ($p < 0.0001$ for all parameters), as indicated by the high F-values (ranging from 103.4 to 367.6). The tumor group exhibited the highest total WBC count (12.20 ± 0.16 K/µl), accompanied by elevated neutrophils (7.43 ± 0.14 K/µl), Monocytes (0.65 ± 0.03 K/µl), and eosinophils (0.43 ± 0.024 K/µl), alongside reduced lymphocytes (3.68 ± 0.13 K/µL), and basophils (0.013 ± 0.002 K/µl), suggesting a pronounced inflammatory response. In contrast, the control group demonstrated a balanced WBC profile with the highest lymphocyte count (6.26 ± 0.15 K/µl) and the lowest neutrophil count (1.62 ± 0.19 K/µl). The Artemisia Judaica extract and 1,2,4-Triazole derivative groups showed intermediate values, with 1,2,4-Triazole derivative and Artemisia Judaica extract co-treatment resulting in a WBC profile closest to the control, particularly in lymphocytes (6.5 ± 0.13 K/µl) and neutrophils (2.0 ± 0.27 K/µl). These findings highlight the potential of Artemisia Judaica extract and 1,2,4-Triazole derivative in modulating immune responses and restoring WBC homeostasis, providing valuable insights for therapeutic strategies in tumor-associated immune dysregulation.

Table (1): The effect of thymoquinone and 1,2,4-Triazole derivative treatment on complete blood count in all studied groups.

	RBC ($x10^6$/µl)	HGB (g/dl)	Platelet ($x10^3$/µl)	WBC ($x10^3$/µl)
Control	9.59 ± 0.14	14.4 ± 0.31	1068 ± 10.9	8.44 ± 0.11
Tumor	7.64^{a} ± 0.21	10.46^{a} ± 0.21	1394^{a} ± 9.3	12.20^{a} ± 0.16
TRI	8.2^{ab} ± 0.16	11.24^{ab} ± 0.23	1323^{ab} ± 8.4	10.24^{ab} ± 0.27
AJ	8.44^{abc} ± 0.11	11.65^{ab} ± 0.15	1288^{abc} ± 5.7	10^{ab} ± 0.13
TRI&AJ	8.92^{abcd}± 0.15	12.62^{abcd} ± 0.16	1209^{abcd} ± 7.8	9.1^{abcd} ± 0.19
F	213.6	216.6	1032	367.6
P	<0.0001	<0.0001	<0.0001	<0.0001

a: Significant with Control, b: Significant with Tumor, c: Significant with TRI, d: Significant with AJ
5 Replica for each group, Data was expressed using Mean ± SD., SD: Standard deviation

Table (2): The effect of thymoquinone and 1,2,4-Triazole derivative treatment on absolute differential leukocyte count in all studied groups.

	LYMPH (K/µl)	NEUT (K/µl)	MONO (K/µl)	BASO (K/µl)	EOS (K/µl)
Control	6.26 ± 0.15	1.62 ± 0.19	0.37 ± 0.02	0.038 ± 0.0019	0.15 ± 0.016
Tumor	3.68^{a} ± 0.13	7.43^{a} ± 0.14	0.65^{a} ± 0.03	0.013^{a} ± 0.002	0.43^{a} ± 0.024
TRI	5.28^{ab} ± 0.14	4.12^{ab} ± 0.4	0.53^{ab} ± 0.03	0.023^{ab} ± 0.0018	0.26^{ab} ± 0.025
AJ	5.6^{ab} ± 0.16	3.6^{abc} ± 0.10	0.43^{bc} ± 0.02	0.027^{ab} ± 0.0013	0.23^{ab} ± 0.024
TRI + AJ	6.5^{bcd} ± 0.13	2.0^{abcd} ± 0.27	0.39^{bc} ±0.016	0.033^{abcd} ± 0.002	0.2^{abc} ± 0.02
F	235.2	262.0	103.4	140.7	105.8
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

a: Significant with Control, b: Significant with Tumor, c: Significant with TRI, d: Significant with AJ. 5 Replica for each group, Data was expressed using Mean ± SD. SD: Standard deviation

3.3. Immunohistochemical Examination

3.3.1 Effect of lung cancer and treatments on the expression of Caspase-3, Caspase-7, and TNF-α (IHC Score)

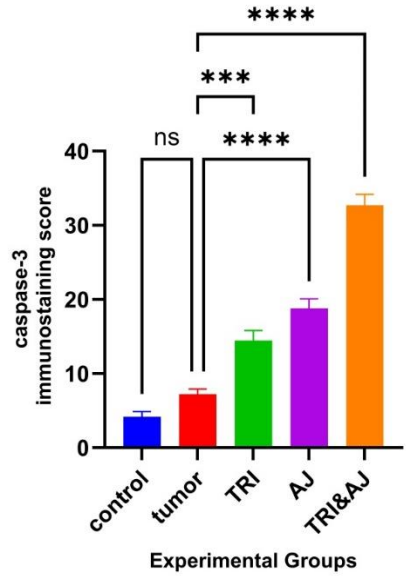

Figure 1. Comparison between the different studied groups according to Caspase-3 immunostaining positive area. (Mean±SD)

Figure 1 shows the immunostaining reactivity percentage of Caspase-3 in the tumor control group tissue (Fig.5), which did not significantly increase compared to the control group (Fig 4). This percentage value was significantly increased in the tumor with treatment groups TRI (Fig 7) and, AJ (Fig 6) individually or in combination (Fig 8) when compared with the tumor control group (Fig 5).

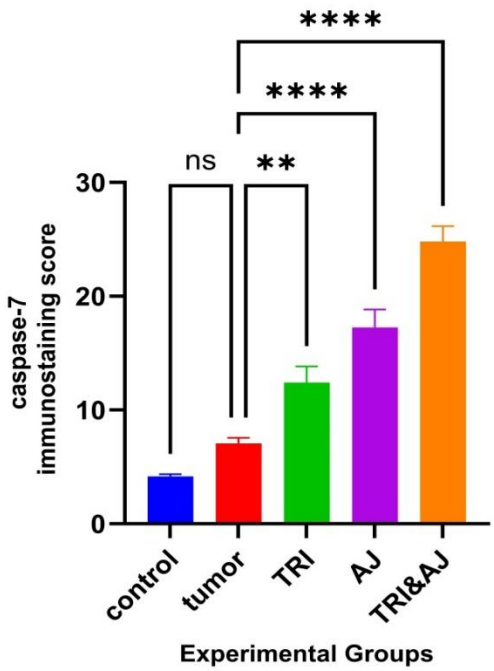

Figure 2. Comparison between the different studied groups according to Caspase-7 immunostaining positive area (Mean±SD).

Figure 2 demonstrates the immunostaining reactivity percentage of Caspase-7 in the tumor group tissue (Fig.10) which did not significantly increase compared to the control group (Fig 9). This percentage value was significantly increased in the tumor with treatment groups TRI (Fig 11) and, AJ (Fig 12) individually or in combination (Fig 13) when compared with the tumor group (Fig 10).

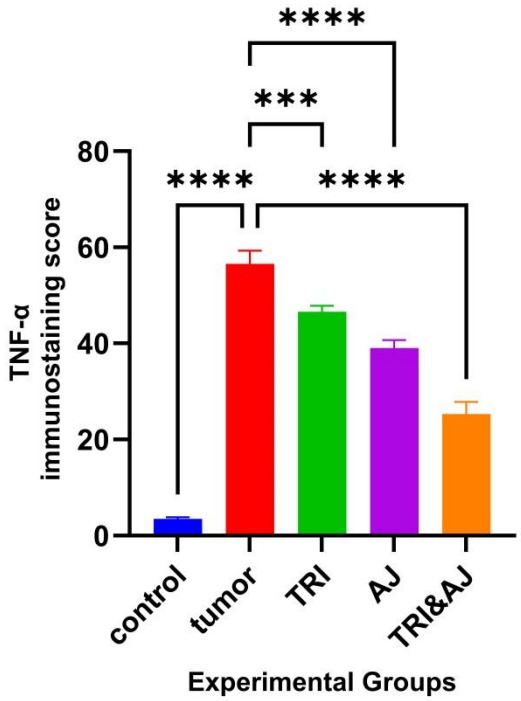

Figure 3. Comparison between the different studied groups according to TNF-α immunostaining positive area (Mean ±SD).

Figure 3 shows the immunostaining reactivity percentage of TNF-α in the tumor control group tissue (Fig.15) which was highly significantly increased compared to the normal control group (Fig 14). This percentage value was significantly decreased in the tumor with treatment groups TRI (Fig 16) and, AJ (Fig 17) individually or in combination (Fig 18) when compared with the tumor control group (Fig 15).

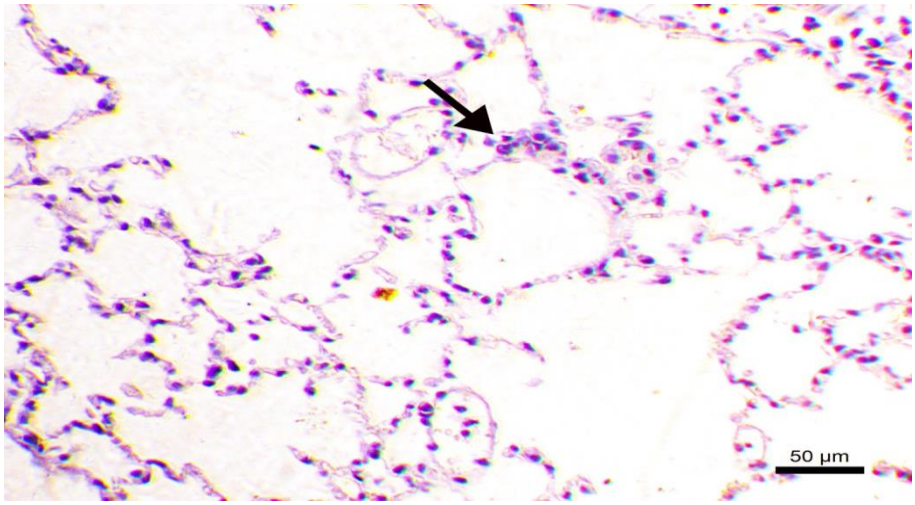

Figure 4. The lung tissue of the control animal shows a scanty expression of Caspase 3 antibody within the alveolar cells (arrow), Caspase 3 IHC, scale bar = 50 µm.

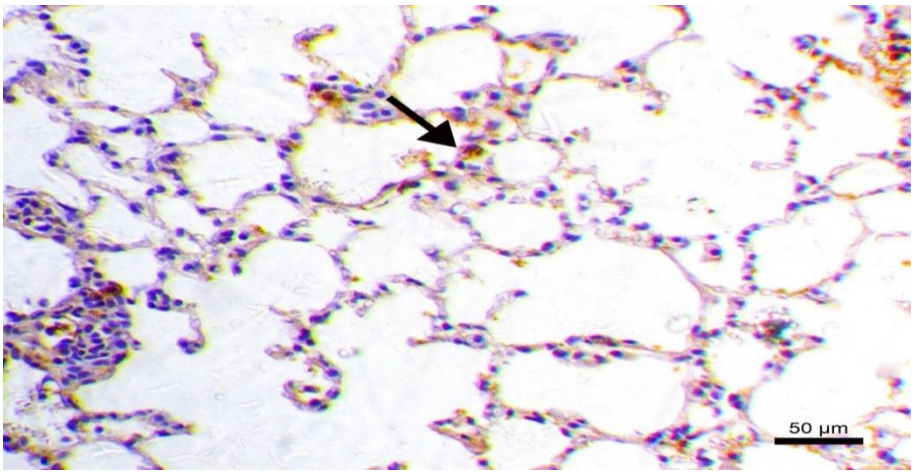

Figure 5. The lung tissue of the tumor control group shows minimal expression of Caspase 3 antibody within the alveolar cells (arrow), Caspase 3 IHC, scale bar = 50 µm.

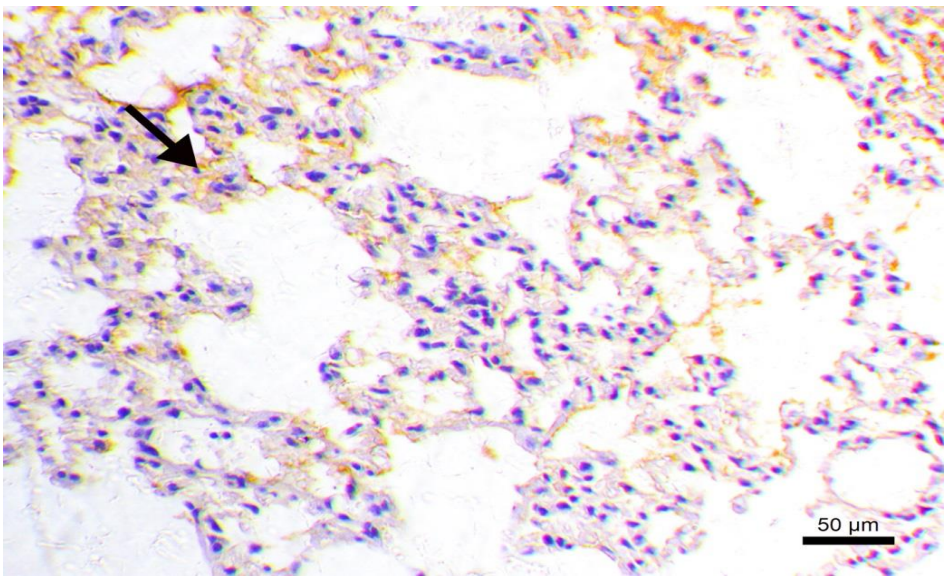

Figure 6. The lung tissue of the TRI-treated group shows an increase in the immunostaining of the Caspase 3 antibody in the alveolar cells (arrow), Caspase 3 IHC, scale bar = 50 µm.

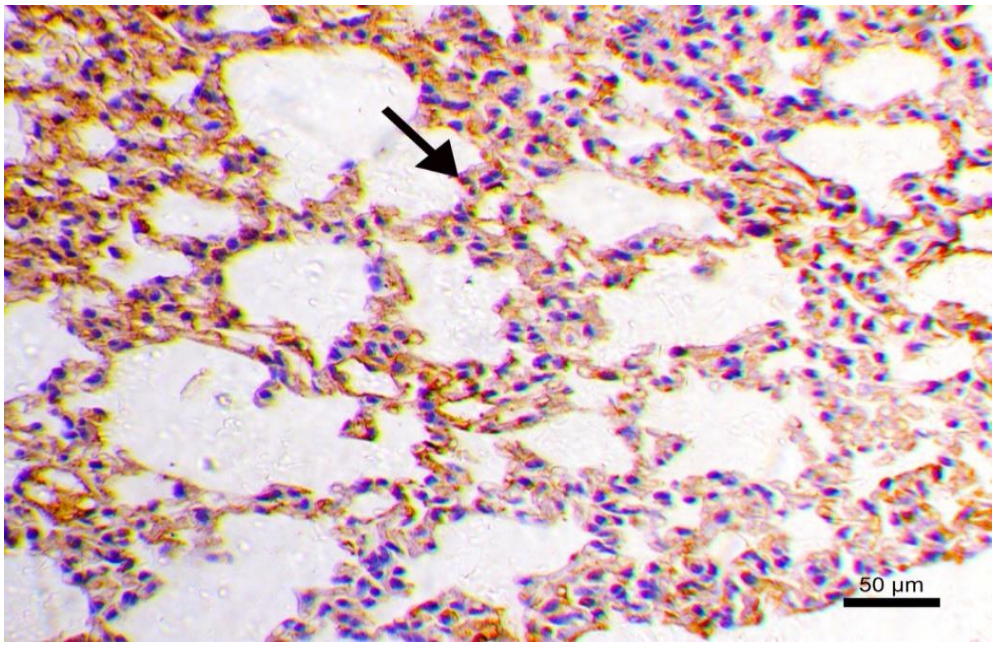

Figure 7. The lung tissue of the AJ-treated group shows an increase in the immunostaining of the Caspase 3 antibody in the alveolar cells (arrow), Caspase 3 IHC, scale bar = 50 µm.

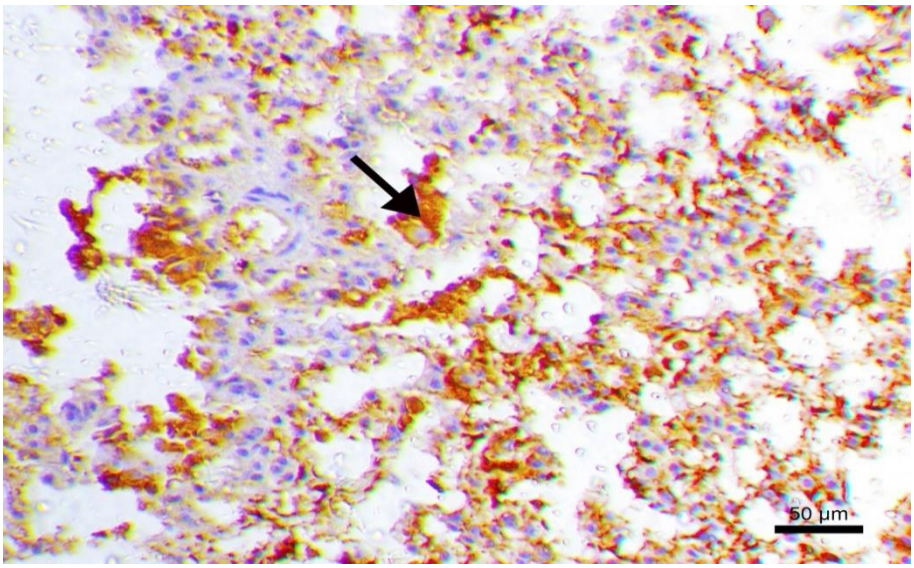

Figure 8. The lung tissue of the TRI and AJ treated group shows a marked increase in Caspase 3 immunostaining within the alveolar cells (arrow), Caspase 3 IHC, scale bar = 50 µm.

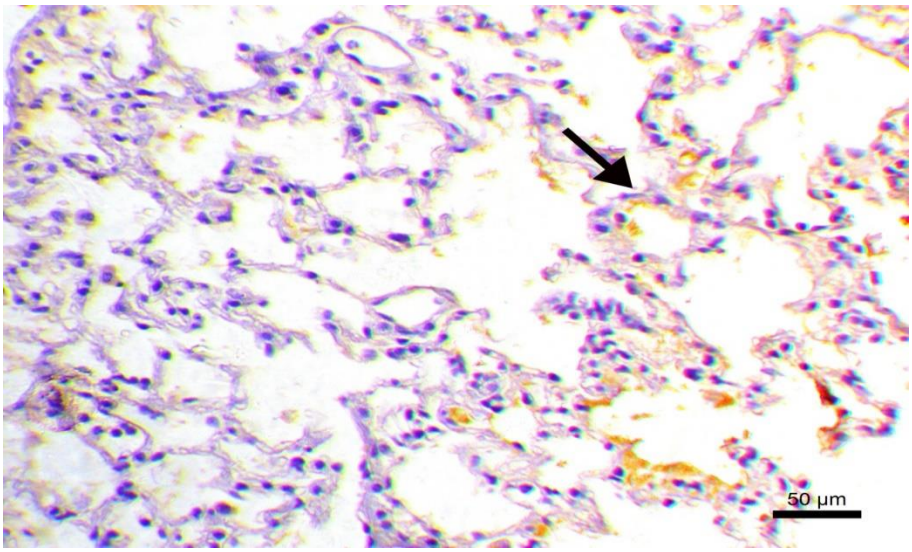

Figure 9. The lung tissue of the control animal shows a scanty expression of Caspase 7 antibody within the alveolar cells (arrow), Caspase 7 IHC, scale bar = 50 µm.

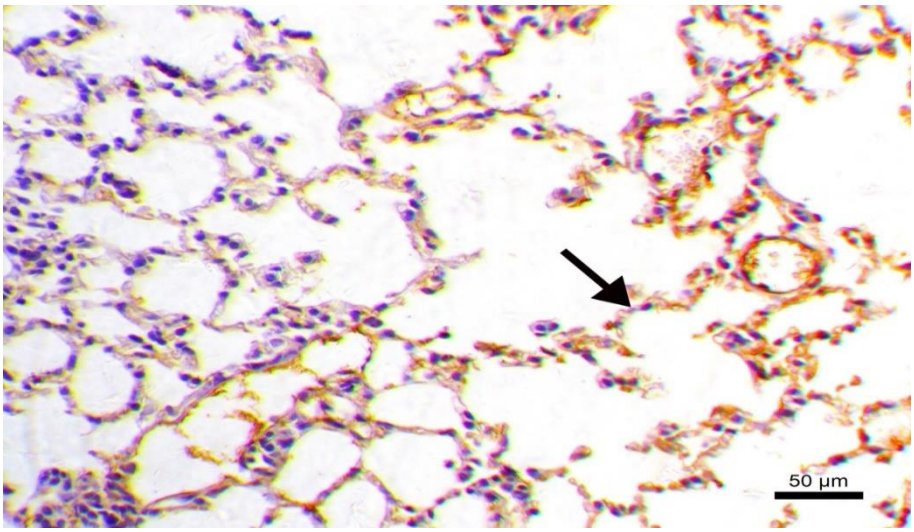

Figure 10. The tumor control group shows a scanty expression of Caspase 7 antibody within the alveolar cells (arrow), Caspase 7 IHC, scale bar = 50 µm.

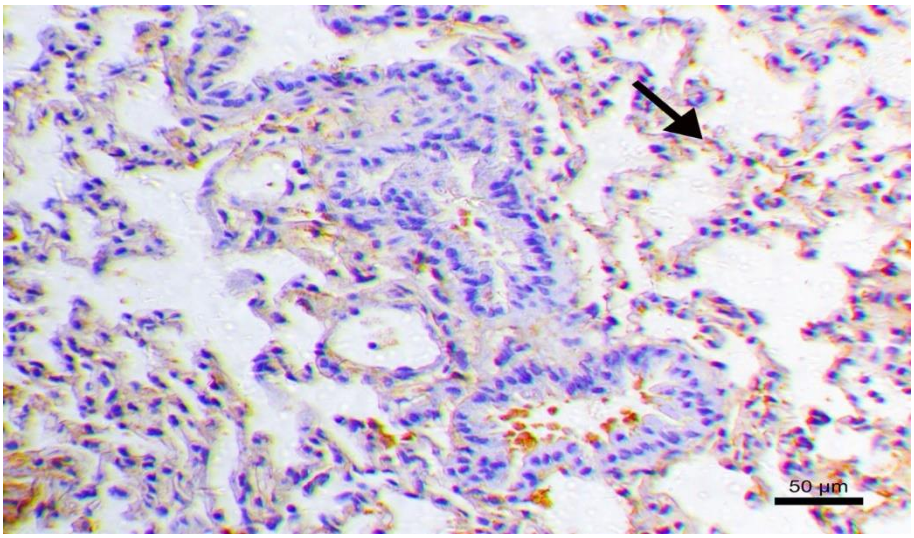

Figure 11. The TRI-treated group shows an increase in the expression of Caspase 7 antibody in the alveolar cells (arrow), Caspase 7 IHC, scale bar = 50 µm.

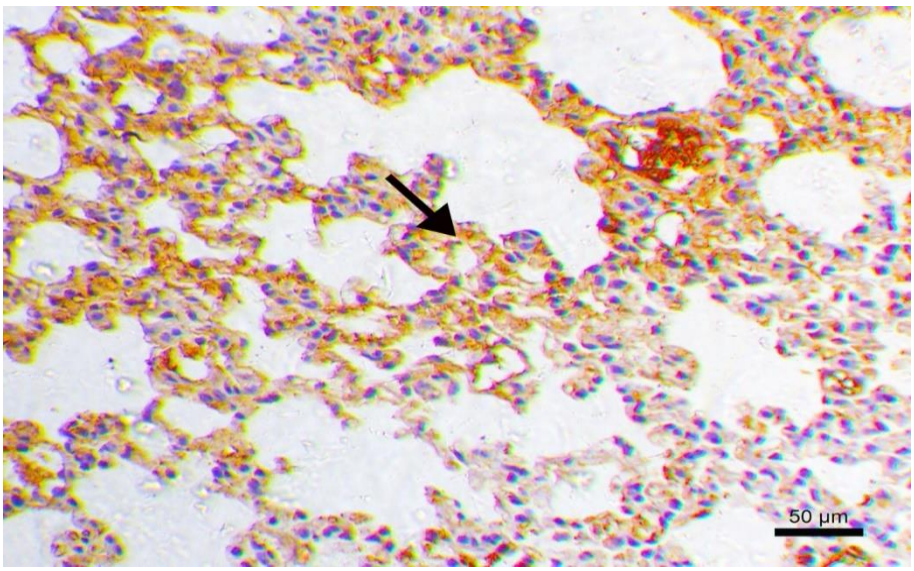

Figure 12. The AJ-treated group shows an increased Caspase 7 immunostaining within alveolar cells (arrow), Caspase 7 IHC, scale bar = 50 µm.

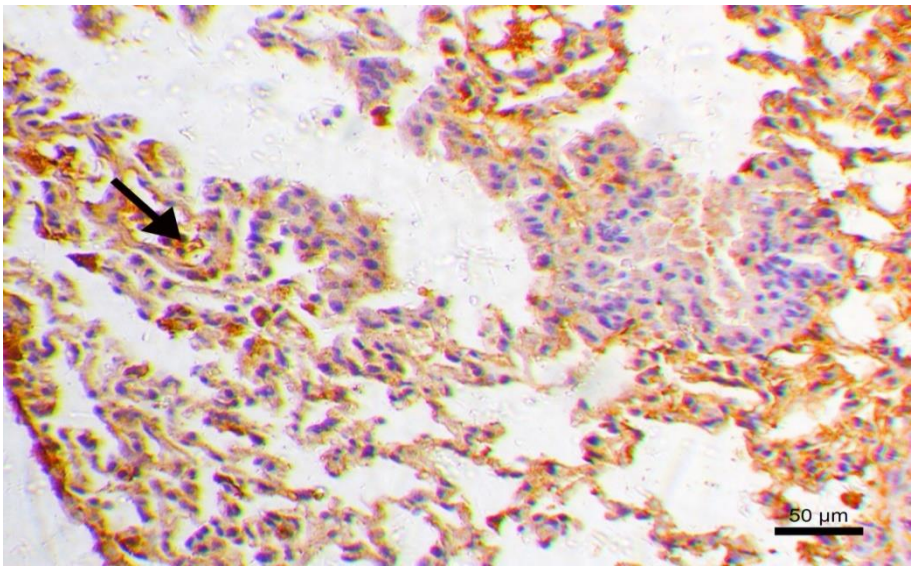

Figure 13. The TRI and AJ co-treated group shows a remarkable increase in Caspase 7 immunostaining within the alveolar cells (arrow), Caspase 7 IHC, scale bar = 50 µm.

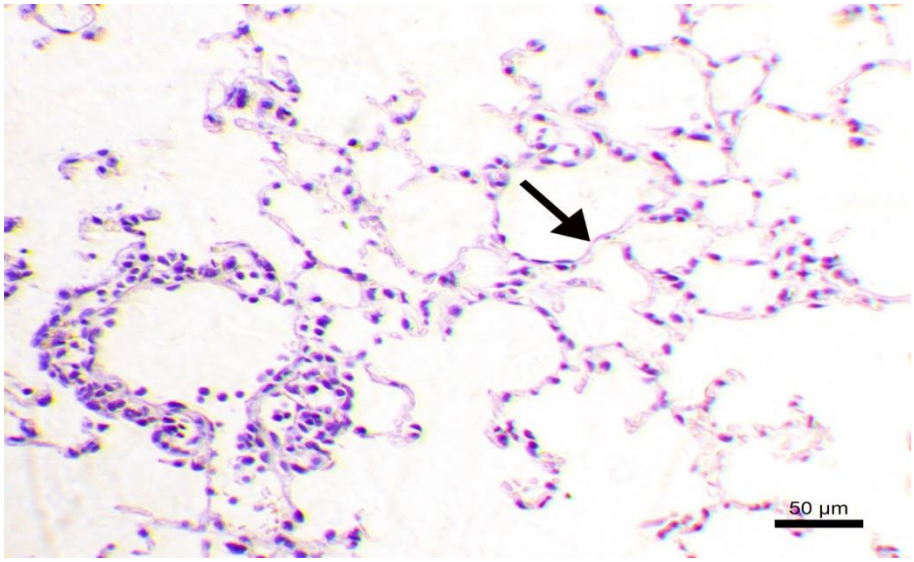

Figure 14. The lung of the control animal shows a scanty expression of TNF-α within the alveolar cells (arrow), TNF-α IHC, scale bar = 50 µm.

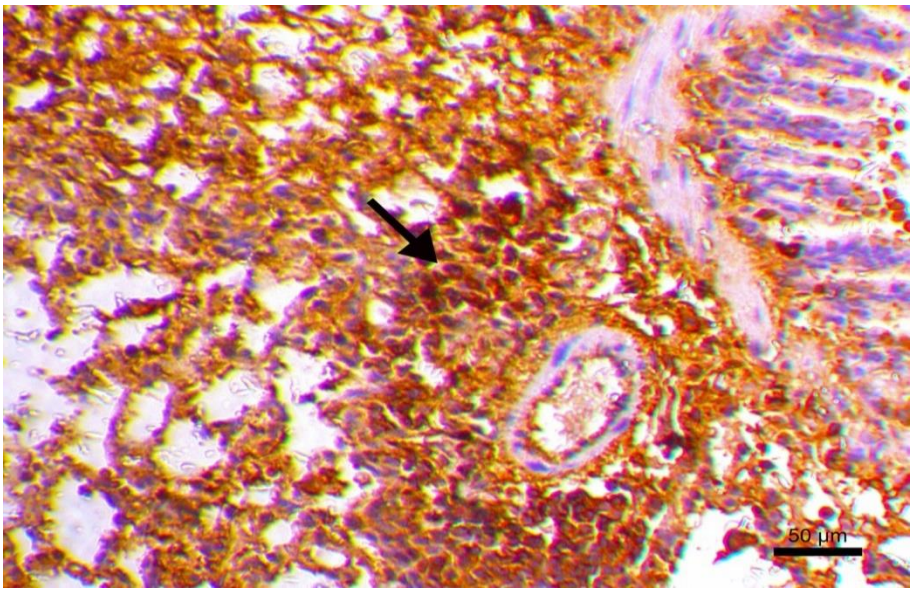

Figure 15. The lung of the tumor control group shows a marked expression of TNF-α within the alveolar cells (arrow), TNF-α IHC, scale bar = 50 µm.

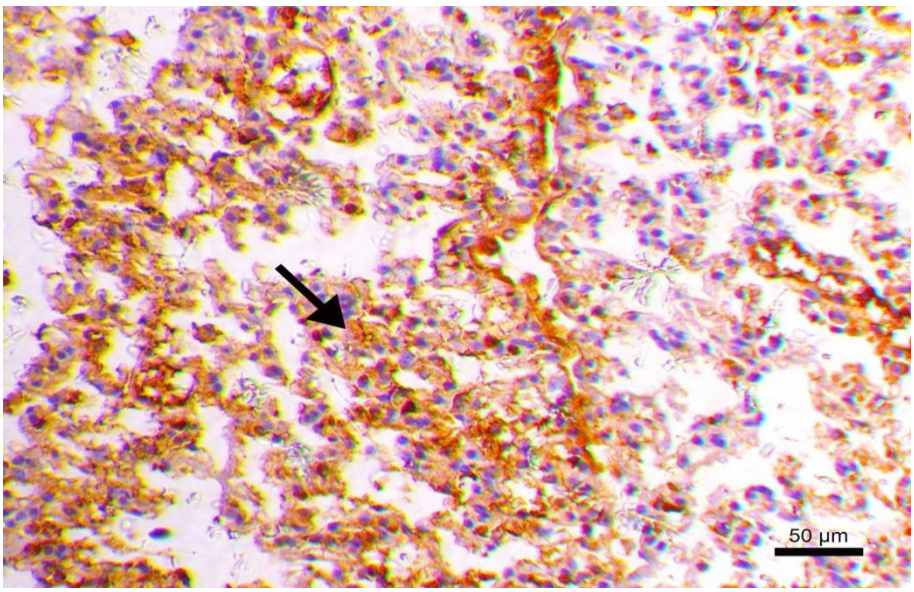

Figure 16. The TRI-treated group shows a decrease in the expression of TNF-α within the alveolar cells (arrow), TNF-α IHC, scale bar = 50 µm.

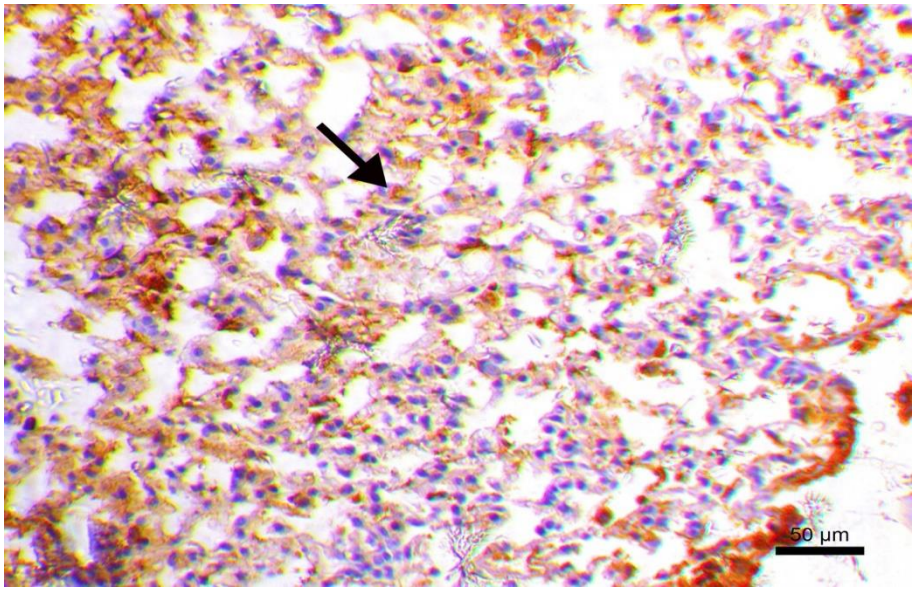

Figure 17. The AJ-treated group shows a decrease in the expression of TNF-α within the alveolar cells (arrow), TNF-α IHC, scale bar = 50 µm.

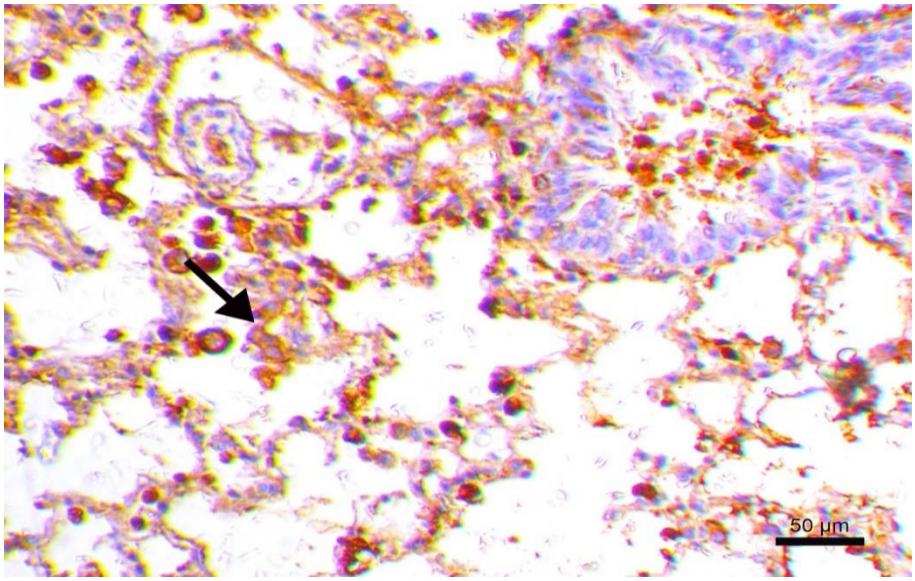

Figure 18. The TRI and AJ-treated group shows a marked decrease in the expression of TNF-α within the alveolar cells (arrow), TNF-α IHC, scale bar = 50 µm.

3.4. EGFR mRNA Expression in Cancer and Healthy Lung Tissues Assessed by RT-PCR

The expression levels of key genes involved in melanoma invasion and metastasis, EGFR, and BRAF were evaluated across the experimental groups. The results revealed significant variations in gene expression, reflecting the therapeutic impact of Artemisia judaica extract (AJ) and 1,2,4-Triazole derivative (TRI)

In the current study, the expression of the EGFR gene was detected in tumor control and the normal control lung tissues by real-time fluorescence quantitative PCR. The statistical analysis of EGFR gene expression revealed significant differences among the experimental groups (F = 306.0, P < 0.0001). The tumor group exhibited a significant increase in EGFR fold change (4.20 ± 0.16) compared to the control group (1.07 ± 0.14). Treatment with 1,2,4-triazole and Artemisia judaica extract independently reduced EGFR expression to 3.24 ± 0.13 and 3.10 ± 0.15, respectively. Notably, the combined treatment of TRI and AJ demonstrated the most pronounced effect, reducing EGFR expression to 2.30 ± 0.15, indicating a synergistic impact on downregulating this oncogene.

3.5. BRAF mRNA Expression in Cancer and Healthy Lung Tissues Assessed by RT-PCR

In this study, the expression of the BRAF gene was detected in the tumor control and normal control lung tissues by real-time fluorescence quantitative PCR. The statistical analysis of BRAF gene expression showed significant variations across the experimental groups (F = 525.1, P < 0.0001). The tumor control group displayed a marked increase in BRAF fold change (3.7 ± 0.08) compared to the normal control group (1.02 ± 0.1). Treatment with 1,2,4-triazole and Artemisia Judaica extract individually reduced BRAF expression to 2.92 ± 0.08 and 2.76 ± 0.1, respectively. The combined treatment exhibited the most significant reduction, bringing BRAF expression down to 2.1 ± 0.12, highlighting a synergistic effect in modulating this oncogenic pathway.

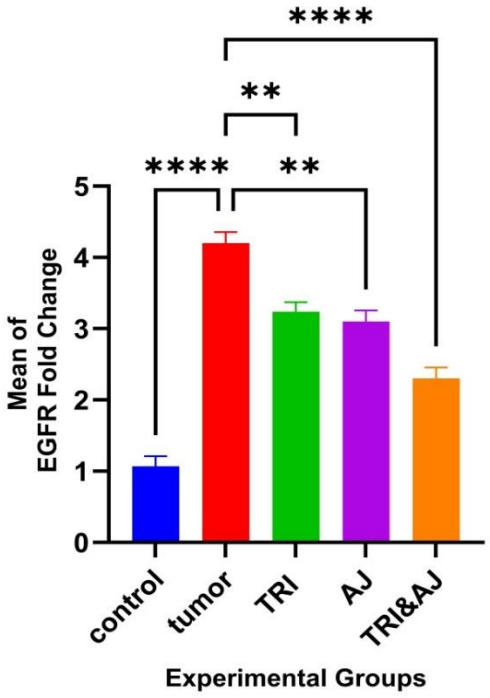

Figure 19. Comparison between the different studied groups according to EGFR fold change (Mean ±SD).

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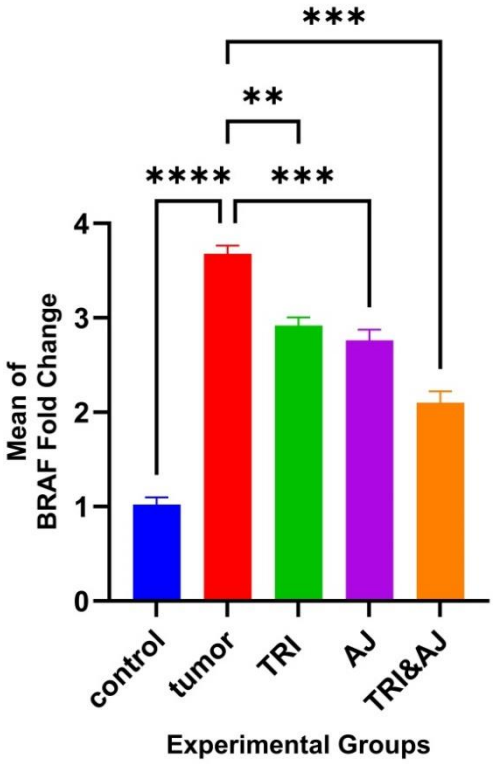

Figure 20: Comparison between the different studied groups according to fold change of BRAF (Mean±SD).

Discussion

The balance of cell division and cell death is maintained through apoptosis. The process of apoptosis is tightly regulated by a complex balance of factors that either promote cell death or prevent it. By manipulating these variables, cancer cells can evade the body's natural mechanisms that normally cause damaged cells to die [22]. New therapeutic approaches have been made possible by shedding light on basic biological processes through our understanding of the molecular mechanisms of apoptosis. Many diseases, including cancer, neurological disorders, and autoimmune diseases, are being studied as potential targets for apoptotic pathways [23]. In apoptosis, novel therapeutic approaches may lie in the regulation of molecules and pathways.

Tumor-bearing control animals(gp2) revealed alterations in apoptosis confirmed by the detection of immunostaining reactivity for caspase-3 and caspase-7, indicative of tumor-induced altered apoptosis inconsistent with [24]. These executioner caspases are activated through intrinsic pathways triggered by mitochondrial dysfunction and DNA damage caused by melanoma progression [25] Concurrently, TNF-α levels were significantly increased, reflecting tumor-associated inflammation driven by NF-κB activation and macrophage infiltration, which further promotes tumor survival and immune evasion [26-28].

One potential way that natural products can induce apoptosis in cancer cells is by upregulating anti-apoptotic genes and downregulating proapoptotic genes. Mitochondrial dysfunction is a molecular mechanism that many anticancer drugs use to induce cell death [29]. Caspases-9, -3, -6, and -7 are activated when cytochrome c is released from the

inner mitochondrial membrane into the cytosol via a cascade of events.

The current results revealed that the1,2,4-Triazole derivative-treated group (gp3) exhibited a significant increase in immunostaining reactivity for caspase-3 and caspase-7, likely due to 1,2,4-Triazole derivatives enhance caspase-3 and caspase-7 activity through similar mechanisms. They induce mitochondrial outer membrane permeabilization (MOMP) by modulating the balance between pro-apoptotic and anti-apoptotic proteins, leading to cytochrome c release and caspase-9 activation[30, 31]. These derivatives also increase ROS levels to trigger mitochondrial dysfunction and apoptosis while stabilizing mitochondrial membranes to prevent excessive damage[32, 33]. Additionally, they activate the extrinsic apoptotic pathway by upregulating death receptors and caspase-8, which directly or indirectly activates caspase-3 and caspase-7 [34]. Furthermore, they reduce TNF-α levels, via inhibiting NF-κB signaling by preventing IκBα degradation and NF-κB nuclear translocation, thereby reducing TNF-α transcription [35, 36].

When tested against cancer cell lines, some Artemisia species, like Artemisia Judaica, demonstrated encouraging anticancer effects [37]. The group treated with the Artemisia judiaca extract (gp 4) showed an upregulation of caspase-3 and caspase-7, as well as a downregulation of TNF-α, which was mainly caused by the activation of the intrinsic apoptotic pathway [38]. Additionally, it modulates ROS levels, initially increasing them to trigger mitochondrial damage and apoptosis, while at higher concentrations, it acts as an antioxidant to prevent excessive ROS-induced damage[39,40].

Artemisinin, a key component of the plant Artemisia, and its bioactive byproducts have shown promise in preventing the spread of cancer and the formation of new blood vessels in certain cell lines [41]. The majority of research has shown that apoptotic cell death is the primary mechanism by which artemisinin exerts its antitumor effects. Heme or free ferrous iron can reduce the recognized endoperoxide bridge pharmacophore, producing carbon-free radicals and reactive oxygen species (ROS) [42, 43]. We know that apoptotic cell death occurs when ROS production is excessive. Yet, artemisinin's anticancer effects are due in part to its ability to influence processes other than apoptosis, such as oncosis, autophagy, and ferroptosis [44-46].

This may be because Artemisia Judaica extract inhibits NF-κB signaling by reducing the transcription of TNF-α and other pro-inflammatory cytokines, as evidenced by the decreased immunohistochemical staining reactivity of TNF-α levels in the gp4 group [47, 48].

The co-treatment group (gp5) demonstrated the most significant increase in caspase-3 and caspase-7 immunohistochemical staining reactivity, and a significant decrease in TNF-α expression, suggesting a synergistic effect of the 1,2,4-Triazole derivative and Artemisia Judaica extract. This synergy likely arises from their complementary mechanisms. 1,2,4-Triazole targets ROS and mitochondrial stability, while Artemisia Judaica extract modulates NF-κB and cytokine production, collectively attenuating apoptosis and inflammation[49, 50].

EGFR and BRAF (B-Raf proto-oncogene) play significant roles in lung function, especially in the context of lung cancers. EGFR is a tyrosine kinase receptor that is frequently overexpressed or mutated in lung cancer, and its activation can affect both lung function and cancer progression[51]. Additionally, BRAF mutations can activate the MAPK signaling pathway, which may hinder the effectiveness of EGFR inhibitors in patients with EGFR-mutant lung cancer[52, 53]. This interaction complicates treatment responses and may negatively impact lung function.

BRAF and EGFR signaling frequently interact synergistically. When EGFR is activated, it can enhance BRAF signaling. Conversely, BRAF mutations can amplify pathways driven by EGFR,

resulting in a positive feedback loop that promotes tumor growth[54].

At the molecular level, qRT-PCR analysis demonstrated that metastatic tumor-bearing mic (gp2) showed a significantly increased expression of EGFR and BRAF. This finding aligns with the activation of the MAPK/ERK pathway driven by melanoma metastasis, which promotes cell proliferation and survival[55]. Both 1,2,4-Triazole derivative treatment (gp3) and Artemisia judaica extract treatment (gp4) individually downregulated EGFR and BRAF, likely by inhibiting upstream signaling pathways such as PI3K/Akt and RAS[9, 56]. Furthermore, some species of Artemisia extract inhibit the PI3K/Akt/mTOR pathway, a major downstream effector of EGFR signaling. This inhibition reduces cell proliferation, survival, and protein synthesis, thereby limiting tumor growth[57].The co-treated group (gp5) exhibited the most significant suppression of EGFR and BRAF[58], indicating that dual targeting of multiple signaling nodes enhances pathway inhibition.

Tumor-bearing control group (gp2) exhibited reduced hemoglobin concentration level, decreased RBCs count and leukocytosis with neutrophilia, characteristic of cancer-associated systemic inflammation and bone marrow suppression [59, 60]. The decreased RBCs count in the tumor control group (gp2) may be due to the elevated level of pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, that suppress erythropoiesis by inhibiting erythropoietin (EPO) production and impairing the responsiveness of erythroid progenitors to EPO [61-63].

1,2,4-Triazole derivative and Artemisia Judaica extract treatment (gp3 and gp4) ameliorated the hemoglobin level and restored RBCs count by reducing inflammation and oxidative stress, which are detrimental to erythropoiesis[64, 65]. The combination group (gp5) demonstrated the most significant normalization of hemoglobin level and RBCs count, further supporting the synergistic anti-inflammatory and antioxidant effects of 1,2,4-Triazole derivative and Artemisia Judaica extract.

Metastatic tumor-bearing mice (gp2) exhibited significant leukocytosis relative to the control group (gp1), attributed to the overproduction of pro-inflammatory cytokines, including IL-6, IL-8, and TNF-α, which enhance bone marrow production of white blood cells, resulting in leukocytosis [66]. Moreover, the tumor-associated macrophages (TAMs) and stromal cells in the TME activate NF-κB signaling, further amplifying cytokine production and systemic inflammation[67].

The significant neutrophilia in the metastatic tumor-bearing mice (gp2) may be due to TME components and the increased secretion of chemokines by melanoma cells such as CXCL1, CXCL2, and CCL2, which recruit neutrophils, monocytes, and other immune cells to the metastatic site[66]. Furthermore, melanoma cells produce Granulocyte Colony-Stimulating Factor (G-CSF), which promotes the proliferation and release of neutrophils from the bone marrow, contributing to neutrophilia[68, 69], that induce tumor progression by releasing ROS, proteases, and pro-angiogenic factors[70, 71], and form Neutrophil Extracellular Traps (NETs), which facilitate tumor cell adhesion and lung metastasis [72]. On the other hand, the pronounced lymphopenia in group 2 may be due to the promotion of T-cell apoptosis and the suppression of lymphocyte proliferation through PD-L1 expression and TGF-β secretion [73, 74].
The significantly increased monocyte count in group 2 as melanoma cells secrete chemokines that recruit monocytes from the bloodstream to the metastatic site in the lungs. The key chemokine involved is CCL2 (C-C Motif Chemokine Ligand 2), commonly referred to as monocyte chemoattractant protein-1 (MCP-1). Monocytes undergo differentiation into tumor-associated macrophages (TAMs), which facilitate tumor growth, angiogenesis, and immune suppression [67, 75]. Moreover, under hypoxic conditions, melanoma cells upregulate HIF-1α,

which increases the expression of CCL2 and other chemokines that recruit monocytes[76]. On the other hand, tumor-derived cytokines such as IL-6 and G-CSF stimulate the bone marrow to produce and release more monocytes into circulation[77].

The significant eosinophilia in tumor-bearing mice (gp2) may be due to tumor-derived cytokines such as IL-5, IL-33, and GM-CSF, which promote their production and recruitment to the tumor site. Eosinophils exert anti-tumor effects by releasing cytotoxic granules and promoting T-cell activation. Still, they can also contribute to tissue remodeling and tumor progression through the secretion of matrix metalloproteinases (MMPs) [78]. However, the pronounced decreased basophil count may be due to similar immune-suppressive mechanisms, including TGF-β and IL-10 signaling, which suppress basophil production and recruitment[79, 80]. Therapeutic strategies that target these cells, including cytokine modulation and immune checkpoint inhibitors, may help adjust their roles in melanoma progression and immune responses. Our results showed that treatment with Artemisia judaica extract and 1,2,4-Triazole derivative can modulate leukocytosis through the inhibition of NF-κB and cytokine signaling (e.g., IL-6, TNF-α), reducing systemic inflammation and bone marrow stimulation[36, 81, 82].

The current results revealed a highlighted modulation in the total and differential leukocyte count in both 1,2,4-Triazole derivative (gp3) and Artemisia judaica extract (gp4) treated mic individually or in combination (gp 5), this may be achieved by targeting inflammatory and immune pathways within the tumor microenvironment (TME). Artemisia judaica extract reduces neutrophilia by inhibiting the production of neutrophil-attracting chemokines such as CXCL1 and CXCL2, which are upregulated in metastatic tumor-bearing mice (gp2), while also suppressing NF-κB and MAPK/ERK signaling pathways that drive neutrophil recruitment and activation[82, 83]. Additionally, Artemisia judaica extract restores lymphocyte counts by downregulating immunosuppressive factors such as PD-L1 and TGF-β, thereby reversing T-cell exhaustion and promoting lymphocyte proliferation [84]. 1,2,4-Triazole derivative complements these effects by inhibiting PI3K/Akt/mTOR signaling, which reduces the expression of anti-apoptotic proteins and enhances T-cell activation[85, 86]. Both 1,2,4-Triazole derivative and Artemisia judaica extract decrease monocyte counts by suppressing CCL2 production, a key chemokine for monocyte recruitment, and by inhibiting their differentiation into tumor-associated macrophages (TAMs) through the modulation of cytokines like IL-10 and M-CSF[87, 88]. Furthermore, 1,2,4-Triazole derivative and Artemisia judaica extract can modulate eosinophil and basophil counts by balancing the cytokine milieu, reducing pro-inflammatory cytokines such as IL-5 and IL-33, and enhancing anti-tumor immune responses[89- 91].

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